Diversität, antagonistisches Potential und Pathogenität von Mikroorganismen aus dem Boden und der Phyllosphäre von Apfelanlagen

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INHALTSVERZEICHNIS

7.2. PUBLIKATIONEN

7.2.1. Kucheryava, N., Fiss, M., Auling, G., and Kroppenstedt, R.M. 1999. Isolation and characterization of epiphytic bacteria from the phyllosphere of apple, antagonistic *in vitro* to *Venturia inaequalis*, the causal agent of apple scab. *System. Appl. Microbiol.* 22: 472-478.

7.2.2. Fiss, M., Kucheryava, N., Schönherr, J., Kollar, A., Arnold, G., and Auling, G. 2000. Isolation and characterization of epiphytic fungi from the phyllosphere of apple as potential biocontrol agents against apple scab (*Venturia inaequalis*). *J. Plant Dis. Protection* 107: 1-11.

7.2.3. Zherebilo, O.E., Kucheryava, N., Gvozdyak, R.I., Ziegler, D., Scheibner, M., and Auling, G. 2001. Diversity of polyamine patterns in soft rot pathogens and other plant associated members of the *Enterobacteriaceae*. *System. Appl. Microbiol.* 24: 54-62.

7.2.4. Hofemeister, J., Conrad, B., Adler, B., Hofemeister, B., Feesche, J., Kucheryava, N., Steinborn, G., Franke, P., Gramme, N., Zwintscher, A., Leenders, F., Hitzeroth, G., and Vater, J. 2004. Genetic analysis of the biosynthesis of non-ribosomal peptide- and polyketide-like antibiotics, iron uptake and biofilm formation by *Bacillus subtilis* A1/3. *Mol. Genet. Genomics* 272: 363-378.

7.2.5. Kucheryava, N., Bowen, J.K., Sutherland, P.W., Conolly, J.J., Mesarich, C.H., Rikkerink, E.H.A., Kemen, E., Plummer, K.M., Hahn, M., and Templeton, M.D. 2008. Two novel *Venturia inaequalis* genes induced upon morphogenetic differentiation during infection and *in vitro* growth on cellophane. *Fungal Genetetics and Biology*, 45: 1329-1339.

1. EINLEITUNG

Mikroorganismen dominieren auf der Erde nicht nur bezüglich ihrer Zellzahl (geschätzte 4-6 x 10^{30} Prokaryotenzellen), sondern machen Schätzungen zufolge auch mehr als die Hälfte der auf der Erde vorkommenden Biomasse aus (Whitman et al., 1998). Etwa die Hälfte des organischen Kohlenstoffs unseres Planeten ist in Mikroorganismen gebunden, bezogen auf Stickstoff und Phosphor sind es fast 90%. Mikroorganismen stellen außerdem ein großes genetisches Reservoir auf der Erde dar und besitzen aufgrund ihrer etwa 3,8 Milliarden Jahre dauernden Evolution eine außerordentlich hohe Diversität an Anpassungs- und Überlebungsstrategien sowie eine große Vielfalt der Habitate (Torsvik et al., 2002). Die Grenzen der Lebensmöglichkeiten auf der Erde werden durch Mikroorganismen festgelegt. So gibt es viele Prokaryoten, die unter extremen Lebensbedingungen vorkommen, wie beispielsweise in sehr salzhaltigen (bei etwa 25% Salzkonzentration), sauren (um pH 0) oder alkalischen (bis pH 13) Habitaten, unter hohem Druck (bis 1050 bar), in heißen Quellen (bis 113°C) oder unter dem dicken Eispanzer der Antarktis (bis zu –88°C) (Antranikian, 2005). Mikroorganismen kommen überall vor, in der Atmosphäre, im Meer, in den Meeressedimenten, im Boden und in der tiefen Erdkruste, wobei der größte Teil (90-95%) in Sedimenten gefunden wird (Whitman et al., 1998). Auch auf den Oberflächen (Rhizo-, Phyllosphäre bzw. Haut) und innerhalb von Pflanzen, Tieren und Menschen leben die verschiedensten mikrobiellen Gemeinschaften. So ist z.B. der gesunde Mensch mit 10-100-mal mehr Bakterienzellen besiedelt, als er eigene Zellen enthält (Schleifer and Horn, 2000).

Mikroorganismen sind die Grundlage der Biosphäre und des gesamten Stoffkreislaufs auf der Erde. Sie waren die ersten und lange auch die einzigen Lebewesen auf der Erde. Sie katalysieren essentielle Schritte der globalen Stoffkreisläufe (Methanbildung, Stickstofffixierung, Nitrifikation, Denitrifikation, Metallreduktionen) und produzieren auch wichtige Bestandteile der Erdatmosphäre (O_2, CO_2, N_2, N_2O, CH_4). Viele andere spezielle Syntheseleistungen wie sekundäre Metabolite (Antibiotika oder Toxine) sind nur bei den Mikroorganismen bekannt. Und es scheint keine natürlichen Stoffe zu geben, die nicht von Mikroorganismen abgebaut werden können.

Die überwiegende Mehrheit der in der Umwelt vorkommenden Mikroorganismen ist bis heute unerkannt. Man kennt heute nach aktuellen Schätzungen weit weniger als 1% der tatsächlich in der Natur vorkommenden mikrobiellen Arten (Torsvik et al., 1996, 1998; Dykhuizen, 1998; DeLong and Pace, 2001). Über 99% der in vielen Umwelthabitaten lebenden Mikroorganismen können mit heutigen Standardmethoden noch nicht isoliert und kultiviert werden (Amann et al., 1995; Torsvik et al., 2002). Diese Welt der bisher nicht kultivierten oder nicht kultivierbaren Prokaryoten wird aus Mangel an geeigneten Methoden erst seit wenigen Jahren sehr langsam erschlossen.

Die Gesamtzahl der verschiedenen mikrobiellen Arten auf der Erde wird auf etwa 100 Millionen, möglicherweise sogar bis zu einer Milliarde geschätzt (Wilson, 1992; Strickberger, 1996; Dykhuizen, 1998; Curtis et al., 2002). Ein Gramm Boden enthält zum Beispiel aufgrund von DNA-Reassoziationsstudien und mathematischen Datenanalysen bis zu 10 Milliarden Prokaryotenzellen, die vermutlich mehreren Tausenden bis zu eine Million verschiedenen Arten zugeordnet werden können (Torsvik et al., 1990; Rosello-Mora and Amann, 2001; Gans et al.; 2005; Curtis and Sloan, 2005). In einem Liter Seewasser sind mehr als 20.000 Arten mariner Mikroorganismen enthalten, Schätzungen zufolge leben 5 bis 10 Millionen Arten in den Ozeanen (Sogin et al., 2006). Bisher sind jedoch erst 7060 Prokaryotenarten (List of Prokaryotic Names with Standing in Nomenclature: http://www.bacterio.cict.fr, Stand Mai 2007) beschrieben. Die Artenzahl der bisher beschriebenen Prokaryoten erscheint sehr gering, zumal im Vergleich zu den Pilzen. Etwa 100.000 Pilzarten kennt die Wissenschaft bislang, ca. 80.000 sind in *Dictionary of the Fungi* beschrieben (Kirk et al., 2001; Hawksworth, 2004). Das sind lediglich auch nur 7% der von manchen Autoren geschätzten gesamten Artenzahl (1,5 Millionen) der Pilze auf der Erde (Hawksworth, 2001).

Die Beschreibung neuer Arten von Mikroorganismen beruht bisher fast ausschließlich auf Reinkulturen, die mit klassischen Kultivierungsverfahren isoliert wurden und nach wie vor die Voraussetzung für eine umfassende Charakterisierung und systematische Beschreibung sind (Brenner et al., 2001). Zudem sind die Artkonzepte in der Mikrobiologie sehr umstritten und eine Einheit „Species“ konnte für Prokaryoten bis heute nicht definiert werden. Es fehlt ein biologisch begründeter Artbegriff, da die übliche Definition über Fortpflanzungsgemeinschaften bei den sich klonal fortpflanzenden Prokaryoten nicht anwendbar ist (Rosello-Mora and Amann, 2001). Der phylo-phenetische Artbegriff wird für die Prokaryoten benutzt. Demnach wird eine Art anhand verschiedener genomischer Merkmale und phänotypischer Eigenschaften definiert (Stackebrandt et al., 2002).

Das am häufigsten genutzte Kriterium ist die ribosomale RNA bzw. die dafür kodierenden Gene (16S-rDNA-Sequenz). Als relevantes genomisches Kriterium wurde eine Ähnlichkeit der DNA-Sequenz (DNA-DNA-Hybridisierungs- oder DNA-Reassoziationswert) von mehr als 70% festgelegt (Stackebrandt and Goebel, 1994). Daher werden Prokaryoten, deren 16S-rDNA-Sequenzen zu mehr als 97% übereinstimmen, einer Art zugerechnet.

Es ist aber nicht möglich allein auf Basis einer rRNA-Analyse eine neue Art zu definieren. Die korrekte taxonomische Einordnung eines Mikroorganismus muss stets mit anderen Methoden bestätigt werden, d.h. basierend auf den polyphasischen Analysen (*polyphasic approach*) (Vandamme et al., 1996). Hierzu zählen nach wie vor auch morphologische, physiologische und chemotaxonomische Merkmale. Im Sinne einer polyphasischen molekularen Strategie kommen weitere Markergene (sogar vergleichende Genomanalyse) ergänzend zum Einsatz.

Die rRNA-Moleküle sind als molekularphylogenetischer Marker besonders geeignet, da sie ubiquitär verbreitet sind und außerdem Spezies-spezifisch hochkonservierte wie auch variable Bereiche enthalten und somit eine in vielen Fällen eindeutige Identifizierung einzelner Spezies bzw. 16S-rRNA-Typen (phylogenetischer Typen, Phylotypen) erlauben.

Mit der Etablierung der 16S-rRNA als phylogenetischem Marker führte Carl Woese die moderne Systematik der Prokaryoten ein und teilte die damals bekannten Bakterien nach der 16S-rRNA-Genanalyse in 11 Phyla (Hauptabstammungslinien) ein (Woese, 1987). Archaea wurden in 2 Phyla eingeteilt und bilden demnach eine eigene Domäne (Woese und Olsen, 1986; Woese et al., 1985). Die Einteilung aller Organismen in die drei Domänen *Bacteria, Archaea* und *Eucarya* wurde durch Sequenzvergleiche der 16S- der Prokaryoten bzw. 18S-rRNA-Gene der Eukaryoten festgelegt und daraus der phylogenetische Stammbaum der Lebewesen rekonstruiert (Woese et al., 1990).

Dieses auf rRNA-Analyse basierte phylogenetische Konzept wurde zur kultivierungs-unabhängigen Analyse mikrobieller Lebensgemeinschaften in der Umwelt fortentwickelt (Pace et al., 1986) und ermöglichte somit nach deren Detektion auch phylogenetische Einordnung unkultivierter Mikroorganismen.

Die Anwendung von kultivierungsunabhängigen Techniken hat in den letzten Jahren unsere Kenntnisse über die mikrobielle Diversität revolutioniert und neu definiert. Eine Vielzahl an molekularen Untersuchungen unterschiedlicher Habitate auf der Basis von direkten Analysen der 16S-rRNA aus Umweltproben hat zur Detektion von vielen bisher nicht kultivierten Prokaryotenarten und zur Identifizierung neuer 16S-rRNA-Typen geführt. 41 neue bakterielle Abstammungslinien im Range eines Phylums haben den Stammbaum, der nur 11 Phyla in der Domäne der *Bacteria* umfasste (Woese et al., 1985), bereits 20 Jahre später auf 53 bakterielle Phyla erweitert (Rappé und Giovannoni, 2003; Keller und Zengler, 2004; Abbildung 1). Die Hälfte davon (27 Phyla) sind durch beschriebene (kultivierbare) Vertreter repräsentiert, wobei die meisten Phyla nur wenige Isolate und einige nur eine beschriebene Art umfassen. Die andere Hälfte, 26 so genannte Kandidaten-Phyla (Hugenholtz et al., 1998), enthalten keine kultivierten Mikroorganismen und sind nur durch aus Umweltproben isolierte 16S-rRNA-Sequenzen von bisher nicht kultivierbaren Bakterien repräsentiert, die weniger als 75% Ähnlichkeiten zu bisher charakterisierten Phyla aufweisen.

Auch unter den Archaea konnten durch 16S-rRNA Analysen von Umweltproben zwei neuen Phyla *Korarchaeota* und *Nanoarchaeota* (Abbildung 2) sowie neue phylogenetische Gruppen von unkultivierten Archaea (*AAG - ancient archaeal group*) innerhalb und sogar außerhalb aller bisher bekannten Phyla identifiziert werden (Schleper et al., 2005). Das neue Phylum *Nanoarchaeota* ist nur von einem Vertreter *Nanoarchaeum equitans* repräsentiert, der durch Kultivierung in einer Co-Kultur entdeckt wurde (Huber et al., 2002). Bislang wurden die kultivierten Vertreter der bereits bekannten archaealen Phyla (*Crenarchaeota* und

Euryarchaeota) (Woese et al., 1990) als ausschließlich in extremen oder anaeroben Habitaten vorkommenden Mikroorganismen beschrieben. Überraschend wurde das ubiquitäre Vorkommen von rDNA-Sequenzen von bisher nicht kultivierten Archaea auch in aeroben und mesophilen Habitaten entdeckt, z.B. in marinem mikrobiellen Plankton, in Seesedimenten und Böden, im Gewebe mariner Schwämme und auf den Pflanzenwurzeln (Fuhrman et al., 1992; Buckley et al., 1998; Schleper et al., 1998; Simon et al., 2000).

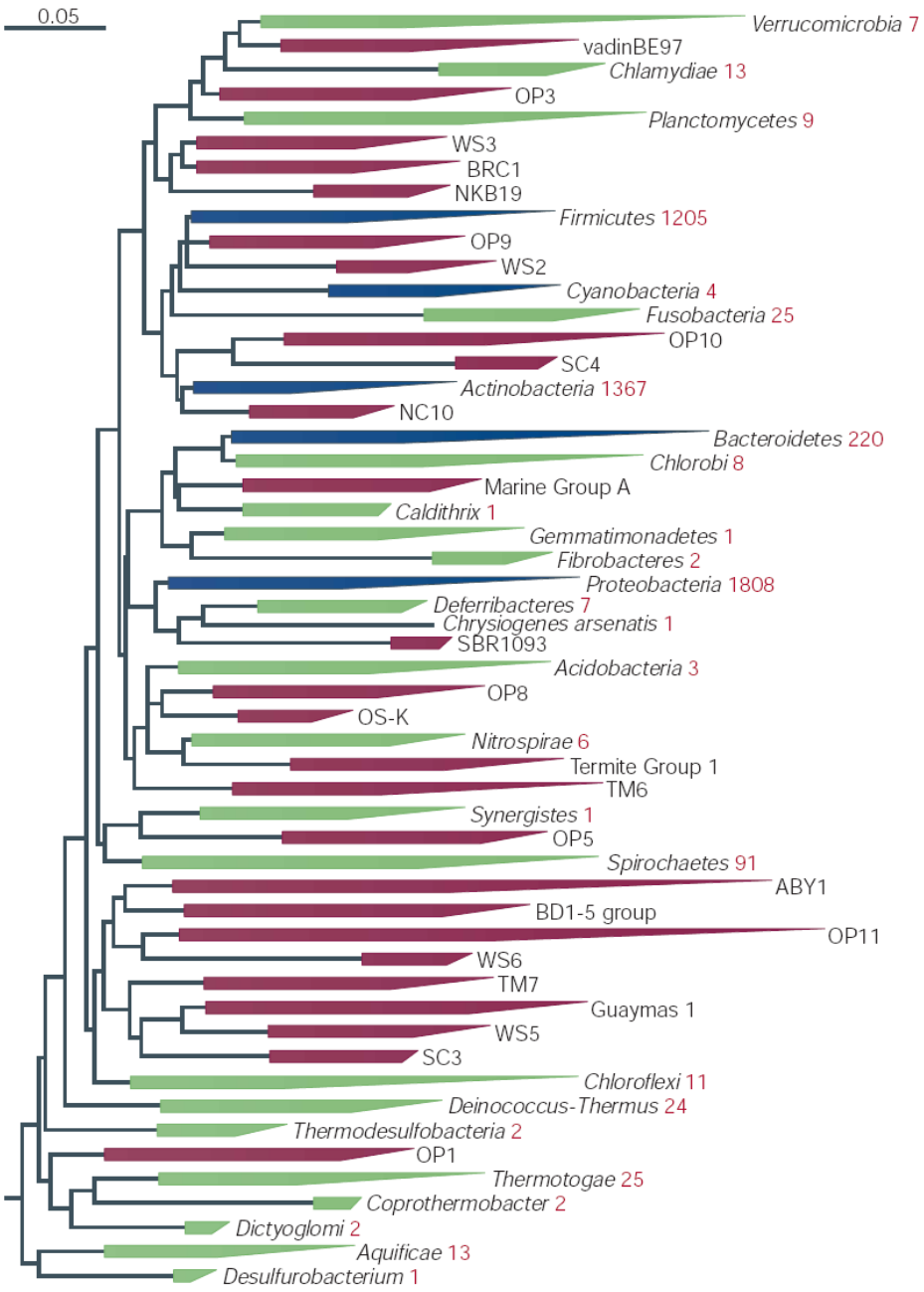

Abbildung 1. Phylogenetischer Stammbaum der bakteriellen Phyla auf Basis der 16S-rRNA-Sequenzen (aus Keller und Zengler, 2004). Bereits identifizierten Phyla (grün und blau dargestellt) mit der Zahl von beschriebenen Arten (Stand Juli 2003) und Kandidaten-Phyla (rot dargestellt), die keine kultivierten Bakterien, sondern nur 16S-rRNA-Sequenzen von Umweltproben repräsentieren.

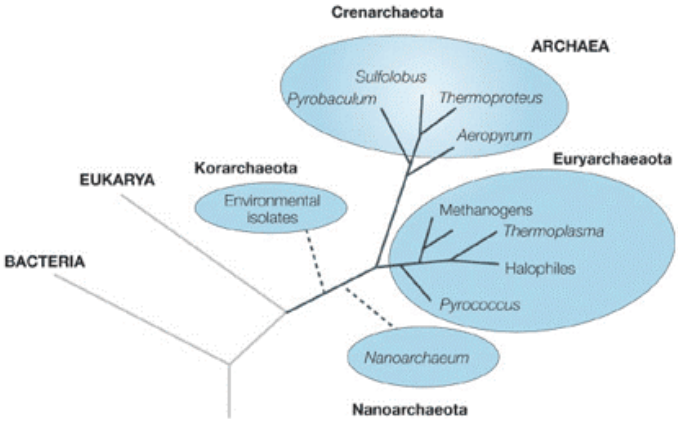

Abbildung 2. Phylogenetischer Stammbaum der Domäne der Archaea auf Basis der 16S-rRNA-Sequenzen (aus Ettema et al., 2005).

Aufgrund der 18S-rRNA-Analysen wurde auch der Stammbaum der Pilze rekonstruiert. Die arbuskulären Mykorrhiza-Pilze, die zuvor zu den *Zygomycota* gerechnet waren, wurden in ein eigenes Pilz-Phylum, die *Glomeromycota*, gestellt (Schüßler et al., 2001; Walker and Schüßler, 2004). Auf Basis von 18S-, 28S- und ITS- (*internal transcribed spacer*) -rRNA-Sequenzen wurden auch neue phylogenetische Linien von bisher unbekannten, unkultivierten Pilzen im Boden und in den Pflanzenwurzeln identifiziert (Vandenkoornhuyse et al., 2002; Schadt et al., 2003; O'Brien et al., 2005). Aufgrund der Sequenzanalyse von sechs verschiedenen Genen aus 200 Pilzarten wurde der Stammbaum der Pilze revidiert und verfeinert (James et al., 2006). Zu den traditionellen Phyla (*Ascomycota*, *Basidiomycota*, *Zygomycota, Glomeromycota* und *Chitridiomycota*) wurden nun die *Microsporidia* als tiefabzweigende Abstammungslinie in das Reich der Pilze gestellt (Abbildung 3). Basierend nur auf 18S-rRNA wurden sie zuvor als tiefste phylogenetische Abzweigung in der Domäne *Eucarya* fern von Pilzen positioniert.

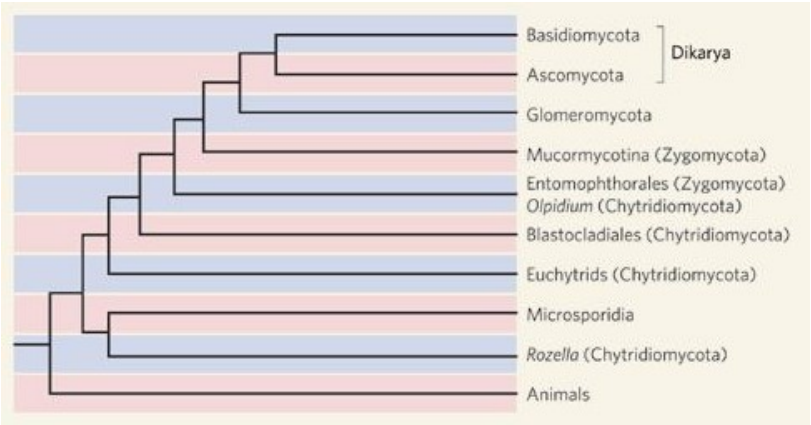

Abbildung 3. Phylogenetischer Stammbaum des Königreichs der Pilze auf Basis der Sequenzanalyse von sechs Genen (aus Bruns, 2006).

Die direkte Analyse von ribosomaler RNA aus Umweltproben ermöglichte es, die Zusammensetzung mikrobieller Gemeinschaften in ihrem Habitat kultivierungsunabhängig zu ermitteln und mit Hilfe von Oligonukleotidsonden die Mikroorganismen direkt in der Umwelt zu identifizieren (Pace et al., 1985; Amann et al., 1995). Die neuen molekulargenetischen Techniken wurden auf ökologische Fragestellungen angewandt und für diese weiterentwickelt. Das ist besonders wichtig für die Umweltforschung, da die Mikroorganismen in ihren natürlichen Habitaten erforscht werden können. Mit Hilfe der Fluoreszenz-*in-situ*-Hybridisierung (FISH) (Lee et al., 1999; Amann et al., 1995; DeLong et al., 1999) ist es möglich die Mikroorganismen auch ohne vorherige Kultivierung *in situ* (in der Probe) nachzuweisen und zu identifizieren. Die quantitative *Real-time*-PCR (Takai und Horikoshi, 2000) ermöglicht es auch kleinste Subpopulationen (<1% der Gesamtpopulation) auf der Basis der 16S rRNA-Gene zu quantifizieren.

Allerdings geben die rRNA-Genanalysen allein keine Hinweise auf metabolische oder physiologische Eigenschaften der Mikroorganismen und auf deren Funktion im Habitat. Eine zusätzliche Möglichkeit, mehr über Aktivität und physiologische Eigenschaften von Bakterien ohne vorherige Kultivierung zu erfahren, ist die Kombination aus FISH und Mikroautoradiographie (Lee et al., 1999; Ouverney und Fuhrman, 1999) oder stabile Isotopenbeprobung (SIP) (Radajewski et al., 2000). Hierbei werden radioaktiv markierte Substrate zu einer Probe gegeben. Anschließend kann analysiert werden, welche Organismen die markierten Substrate aufgenommen und möglicherweise umgesetzt haben. Diese Methoden etablierten sich auf dem Gebiet der mikrobiellen Ökologie und erlauben es, die Mikroorganismen in der Umweltprobe nicht nur zu visualisieren und zu quantifizieren, sondern auch deren genetische Ausstattung und damit verbundenes metabolisches Potential abzuschätzen.

Um Informationen über die metabolischen Eigenschaften der mikrobiellen Gemeinschaften in ihren natürlichen Habitaten zu gewinnen, werden auch PCR-basierte Methoden angewendet. Dabei können auch die proteinkodierenden Gene mit spezifischen Markersequenzen (konservierten Genregionen) direkt aus Umweltproben amplifiziert werden. So konnten viele Gene, die für ökologisch wichtige Schlüsselenzyme (z.B. Polyketidsynthetasen, Proteo-rhodopsine oder Nitritreduktasen) kodieren, auch aus nicht kultivierten Mikroorganismen nachgewiesen werden (Hallin and Lindgren, 1999; Sheu et al., 2000; Eschenfeldt et al., 2001; de la Torre et al., 2003). Allerdings hat diese Methode den Nachteil, dass nur bekannte oder diesen ähnliche Gene gefunden werden können, da die verwendeten Primer von bereits bekannten Gensequenzen abgeleitet wurden. Ein alternativer PCR-Ansatz, bei dem degenerierte Oligonukleotide von konservierten Genregionen oder flankierenden Elementen abgeleitet werden, ermöglichte das Auffinden neuartiger Gene und ganzer Genkassetten (Seow et al., 1997; Stokes et al., 2001).

Eine neue Strategie und alternative kultivierungsunabhängige Technik, die so genannte Metagenomik, wurde in den letzten Jahren entwickelt. Es handelt sich um die genomische Analyse aller Mikroorganismen eines Habitats, eine Kombination aus Metaanalyse und Genomik. Dabei wird die gesamte genomische DNA einer natürlich vorkommenden Mikrobengemeinschaft, d.h. die Gesamtheit der Genome aller Mikroorganismen in einem Habitat - als Metagenom bezeichnet (Handelsman et al., 1998) - direkt aus Umwelthabitaten isoliert, in geeigneten Vektoren (Plasmiden, Cosmiden, BACs) kloniert und als rekombinante Metagenombank in einem gut kultivierbaren heterologen Wirt (meist *E. coli*) archiviert und anschließend exprimiert und analysiert (Abbildung 4).

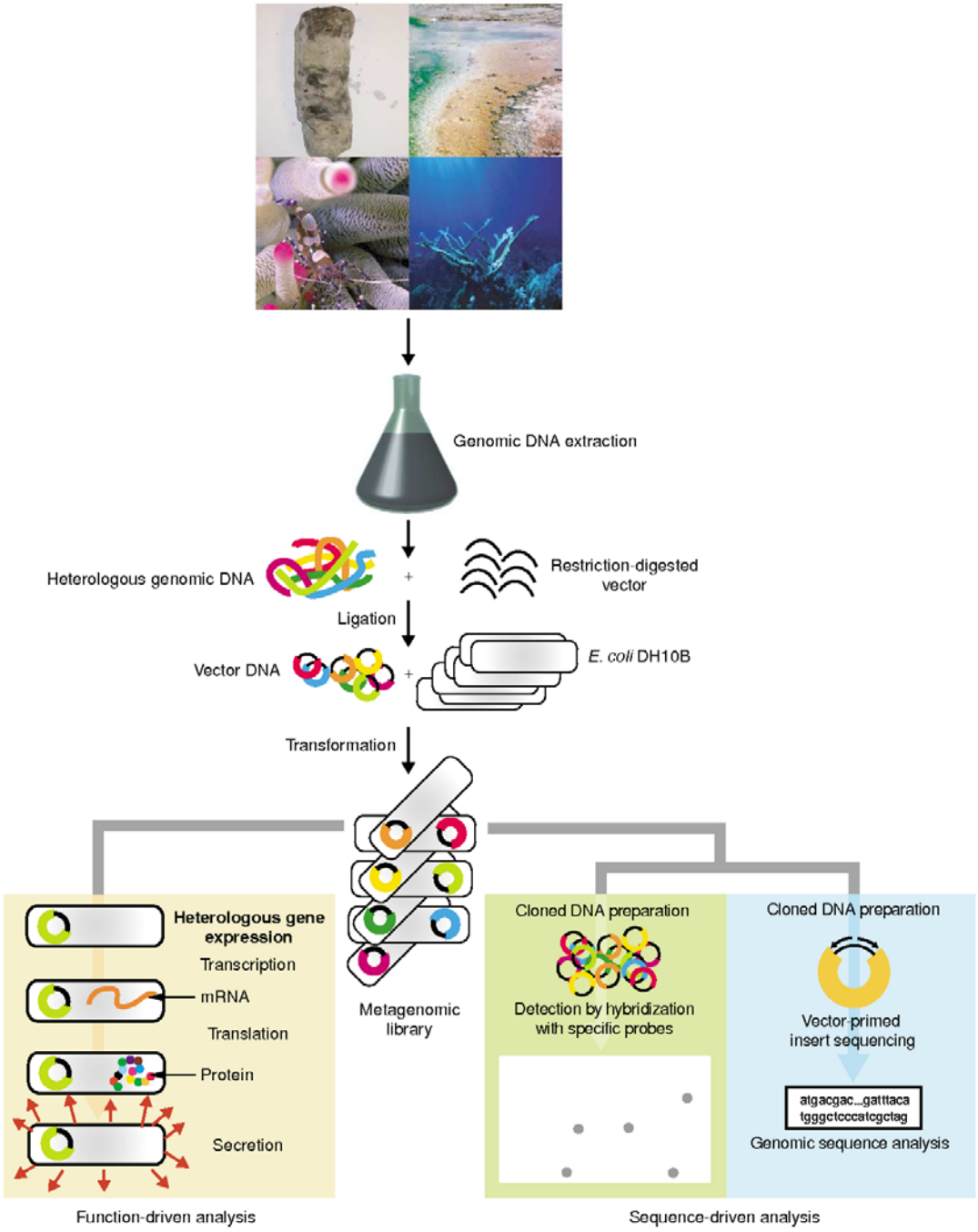

Abbildung 4. Schematische Darstellung der Metagenomik-Strategie zur Analyse mikrobieller Gemeinschaften in den Umwelthabitaten (aus Schloss und Handelsman, 2003).

Eine solche Metagenombank enthält den Hauptanteil der gesamten genomischen Information einer mikrobiellen Gemeinschaft. Auf Basis der genetischen Ausstattung lassen sich metabolische und biochemisch relevante Eigenschaften ableiten. Somit wird die in der Metagenombank enthaltene genetische und metabolische Diversität für die Forschung verfügbar. Auf der Basis von Sequenzähnlichkeiten kann eine Metagenombank durch Hybridisierung oder PCR-Amplifikation mit entsprechenden Sonden durchmustert oder durch ein aktivitätsbasiertes, sequenzunabhängiges Screening, oder durch *shotgun* Sequenzierung (Schrotschusssequenzierung) untersucht werden.

Die Metagenombanken dienen sowohl als Grundlage für umfangreiche Analysen im Bereich der Umweltgenomik und der mikrobiellen Ökologie als auch für die Entdeckung neuer Enzyme, Biokatalysatoren und Wirkstoffe. Die bisher aus den Metagenombanken isolierten Gene für Enzyme (Henne et al., 1999, 2000; Rondon et al., 2000; Knietsch et al., 2003; Voget et al., 2003), Antibiotika-Biosynthesewege (Wang et al., 2000; Brady et al., 2001, 2002, 2004; MacNeil et al., 2001; Gillespie et al., 2002), Sekundärstoffbiosynthesen (Entcheva et al., 2001; Knietsch et al., 2003) und Membranproteine (Majernik et al., 2001) waren oft neuartig oder deutlich verschieden zu denen, die in kultivierten und charakterisierten Mikroorganismen identifiziert wurden.

Bei der *shotgun* Sequenzierung des Metagenoms planktonischer Mikroorganismen aus der Sargassosee (Venter et al., 2004) entstand das Bild einer sehr viel größeren mikrobiellen Diversität als sie in Stammsammlungen und Sequenzdatenbanken repräsentiert wurde. Dabei stammen die neuen Sequenzen von marinen Mikroorganismen nach bioinformatischer Schätzung von wenigstens 1800 Spezies, darunter 148 bislang unbekannte Phylotypen. Der riesigen Flut von in Sargassosee gewonnenen DNA-Sequenzen (über 2 Millionen) standen erstaunlich wenige Daten in öffentlichen Genbanken gegenüber. Über 1,2 Millionen potenzielle Gene wurden identifiziert, mehr als doppelt so viele wie in allen Genbanken verfügbaren mikrobiellen Genomen (ca. 500.000 Gene) enthalten waren. Etwa 70.000 (ca. 65%) der identifizierten Gene wurden als neuartig (*hypotheticals* bzw. *unknows*) annotiert.

In einer ähnlichen Sequenzierstudie des Metagenoms eines natürlichen acidophilen Biofilms, der FISH- und 16S-rDNA-Sequenzanalysen zufolge aus sechs prokaryotischen Spezies bestand, gelang sogar die fast komplette Rekonstruktion der Genome von dominierenden Mikroorganismen, *Leptospirillum* sp. und *Ferroplasma* sp. (Tyson et al., 2004).

Die Sequenzierung großer unmittelbar aus der Umwelt gewonnener DNA-Abschnitte und Entschlüsseln ganzer Genome von nicht kultivierbaren Prokaryoten erweitern erheblich unsere Kenntnisse über deren Funktion und physiologischen Fähigkeiten. Auf diese Weise wurden bisher unbekannte neuartige Stoffwechselwege entdeckt, wie z.B. die Proteorhodopsin-abhängige Phototrophie bei marinen unkultivierbaren *γ-Proteobacteria* (SAR86-Gruppe) (Béjà et al., 2001) oder anaerobe Ammonium-Oxidation bei unkultivierten

Planctomycetes (Kuypers et al., 2003) oder anaerobe Methan-Oxidation (*reversed methanogenesis*) bei der archaealen Methanotrophen der ANME1-Gruppe (Hallam et al. 2004).

Die Fortschritte in der Bioinformatik ermöglichen auch den genomischen Inhalt verschiedener Metagenombanken miteinander und mit einzelnen ausgewählten Genomen kultivierter Organismen zu vergleichen, somit einen Überblick über das metabolische Potential der unterschiedlichen Habitate zu gewinnen. Habitatspezifische funktionelle Fingerprints werden durch die Analyse des Vorhandenseins von habitatspezifischen Markergenen und überwiegenden Genfamilien, dem funktionellen Inventar, erstellt. Eine solche Vergleichsstudie aus vier verschiedenen Ökosystemen (Walskeletten am Meeresgrund, Ackerboden, Oberflächenwasser des Sargassosee und acidophiler Biofilm) ermöglichte habitatspezifische Genprofile zu erstellen (Tringe et al., 2005). Denn in jedem Ökosystem waren jeweils bestimmte Genklassen angereichert – im Oberflächenwasser etwa Gene für Photorezeptoren, im Boden Gene für Enzyme, die pflanzliches Material zersetzen, oder Gene für Spezieskommunikation und Antibiotikabiosynthesen.

Ziel der Erforschung mikrobieller Diversität ist einerseits ein besseres Verständnis der Struktur und Funktion der natürlichen mikrobiellen Gemeinschaften und andererseits die biotechnologische Nutzung der vorhandenen und bisher noch unerschlossenen metabolischen Diversität. Die meisten der heute verwendeten Antibiotika (75%) oder Krebstherapeutika (50%) basieren auf Naturstoffen und wurden durch Kultivierung von Bakterien, Pilzen oder aus Pflanzen gewonnen (Hentschel et al., 2002; Newman et al., 2003). Dabei ist manchmal nicht die Pflanze selbst für biologische Aktivität verantwortlich, sondern die von Epi- oder Endophyten produzierte Sekundärstoffe.

Der Sekundärmetabolismus ist bislang nur in wenigen bakteriellen und pilzlichen Taxa studiert worden. Nur 5 bakterielle Taxa (*Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes* und *Proteobacteria*) (Abbildung 1, blau dargestellt) enthalten Arten, die bioaktive Substanzen produzieren (Keller und Zengler, 2004). Bei den Pilzen ungefähr die Hälfte aller bekannten Ascomyceten und Basidiomyceten produzieren Wirkstoffe. Die neuen Aktivitäten der Genomsequenzierung haben gezeigt, dass fast alle bisher sequenzierten Bakterien und Pilze zumindest einige für die Naturstoffproduktion typische Biosynthesegene zeigen (Bode und Müller, 2005). Auch viele bislang nicht als Naturstoffproduzenten bekannte Organismen enthalten die typischen Gene und oft große Biosynthese-Gencluster für die Produktion hypothetischer oder noch nicht erkannter Naturstoffe. Im Genom von *Streptomyces coelicolor* wurden 23 für Sekundärstoffe kodierende Gencluster gefunden, obwohl zuvor nur sechs der Substanzen bekannt waren (Ikeda et al., 2003). Bekanntgabe der Genomsequenz von *Streptomyces coelicolor* hat bereits zur Isolierung von zwei Substanzen geführt, deren

Existenz anhand der Sequenzdaten postuliert wurde (Barona-Gomez et al., 2004; Lautru et al., 2005).

Extrapoliert man von bekannten Leistungen der kultivierten Organismen auf die metabolischen Fähigkeiten der unkultivierten Mikroorganismen, so kann man deren natürliche Habitate aufgrund ihrer dominierenden Abundanz und enormen Diversität als die nahezu unerschöpfliche Quelle für neuartige biologisch aktive Substanzen und Biosynthesewege betrachten (Bull et al., 1992, 2000).

Natürlich werden die klassischen Methoden weiterhin angewendet, um neue Naturstoffe, Enzyme und Stoffwechselwege zu entdecken. Die Entdeckungsrate von neuen Naturstoffen mittels traditioneller Kultivierungsverfahren ist mittlerweile stark gesunken, und die Reisolierungsrate von bekannten Substanzen beträgt bereits >99% (Zähner und Fiedler, 1995; Strohl, 2000). Klassische Methoden zur Gewinnung neuer Naturstoffe aus unbekannten Mikroorganismen, die auf deren Kultivierbarkeit beruhen, lassen daher das beträchtliche Potential der unkultivierbaren Organismen völlig ungenutzt. Tatsächlich sind viele Mikroorganismen, die in der Umwelt in verschiedenen komplexen Habitaten wie Boden, Biofilmen, Schwämmen oder Pflanzen, insbesondere in symbiotischen und pathogenen Interaktionen leben, bisher nicht kultivierbar. Die ökologischen Nischen solcher Habitate wie Boden und Pflanze (Rhizo- und Phyllosphäre) beherbergen eine große mikrobielle Diversität. Diese Habitate weisen ein immenses Potential zur Wirkstoffsynthese auf. Die antimikrobiell wirksamen Substanzen werden von Mikroorganismen offenbar bei hoher mikrobieller Dichte produziert und spielen eine Rolle entweder bei der Verteidigung gegen Konkurrenten um dasselbe Substrat oder für die erfolgreiche Besiedlung des Habitats.

Naturstoffe werden in der Regel in Mikroorganismen von verschiedenen Enzymen über mehrstufige Biosynthesewege synthetisiert, wobei in Bakterien die dafür benötigten Gene fast immer dicht gruppiert in Genclustern auf dem Genom angeordnet sind. In einigen Bakterien und insbesondere oft bei Pilzen können diese Gene aber auch im ganzen Genom verstreut liegen. Somit bleibt die Isolierung der entsprechenden Mikroorganismen in Reinkultur und eine anschließende genaue genetische, physiologische und biochemische Charakterisierung die beste Lösung, um alle an dem Naturstoff-Biosyntheseweg beteiligten Gene zu identifizieren.

Jedoch sind die etablierten Kultivierungs- und Isolierungsmethoden für die meisten Prokaryoten unzureichend und meist nicht spezifisch genug, um neue Mikroorganismen aus der Umwelt zu isolieren. Die konventionellen Medien enthalten für den Großteil der Mikroorganismen zu hohe Nährstoff- und Salzkonzentrationen (Torsvik et al., 1990; Connon and Giovannoni, 2002). Für die meisten chemoorganotrophen Mikroorganismen, die in oligotrophen Standorten vorkommen, spiegeln die üblichen im Labor Kultivierungsverfahren keine natürlichen Bedingungen wider (Oren, 2004). In der Natur sind nährstoffarme

Bedingungen die Regel und in vielen Habitaten stehen sogar zeitweise keine Nährstoffe zur Verfügung. Für jede neu zu kultivierende mikrobielle Art muss ein spezifisches Set von Kultivierungsparametern ermittelt und eingestellt werden, wie beispielsweise der pH-Wert, die Temperatur und die Verfügbarkeit des Sauerstoffs.

Neue Kultivierungsverfahren und Strategien wurden bereits entwickelt, welche die natürlichen Umweltbedingungen simulieren, z.B. durch lange Inkubationszeiten und durch Verwendung von Habitat nachahmenden Medien mit relativ niedrigen Konzentrationen von Nährstoffen, mit der Zugabe von bestimmten Signalmolekülen oder von Inhibitoren (Kaeberlein et al., 2002; Connon and Giovannoni, 2002; Janssen et al., 2002; Zengler et al., 2002; Joseph et al., 2003; Stevenson et al., 2004). Die neuen Kultivierungsmethoden sind technisch sehr aufwendig und zeitintensiv. Dennoch erscheint deren Ansatz sehr erfolgversprechend. So ist es z.B. gelungen, durch die Kokultivierung oder Hochdurchsatz-Kultivierung von in Mikrokapseln eingeschlossenen Mikroorganismen, Zellsortierung mit Durchflusszytometrie oder Anwendung der so genannten optischen Pinzette einige neuartige Organismen aus den Phyla der *Proteobacteria*, *Bacteroides*, *Planktomycetes*, *Acidobacteria* und *Verrucomicrobia* zu kultivieren (Zengler et al., 2002; Kaeberlein et al., 2002; Stevenson et al., 2004).

Durch die Kokultivierung an der Oberfläche eines Archaeums *Ignicoccus hospitalis* konnte *Nanoarchaeum equitans*, der erste Vertreter des neuen Phylum *Nanoarchaeota,* im Labor gezüchtet werden (Huber et al., 2002). Durch die Analyse seines Genoms, eines kleinsten Genoms aller Prokaryoten (0,5 Mb), konnten keine Stoffwechsel- oder Biosynthesegene nachgewiesen werden, was seine Abhängigkeit von symbiotischer oder parasitischer Beziehung mit dem bekannten archaeellen Wirt unterstreicht. Dabei wurden auch die einzigartigen 16S-rRNA-Sequenzen identifiziert, die das neue Phylum *Nanoarchaeota* repräsentieren.

Connon und Giovannoni (2000) entwickelten eine *High-Throughput*-(Hochdurchsatz)-Methode. Dabei werden die Mikrotiterplatten und Zellarrays eingesetzt, die mikroskopisch mit Hilfe spezifischer Sonden (FISH) auf neue Mikroorganismen gescreent werden (Connon und Giovannoni, 2002). Mit dieser Technik wurde ein in den Weltmeeren abundantes α-Proteobakterium *Pelagibacter* sp. (früher als unkultivierbar geltender SAR11-Phylotyp) isoliert, welches nur sehr geringe Zelldichten erreicht (Rappe et al., 2002). Durch die 16S-rRNA-Analysen wurde gezeigt, dass die SAR11-Bakterien als zahlreichste, dominierende marine Mikroorganismen (30-50% der mikrobiellen Gemeinschaft) und als erfolgreichste (ubiquitär verbreitete) auf der Erde gelten (Morriss et al., 2002). Aber die ökologische Rolle dieser Bakterien, die aufgrund ihrer Abundanz wahrscheinlich eine wesentliche Bedeutung für die biogeochemischen Kreisläufe unseres Planeten haben, bleibt unaufgeklärt.

Neulich ist die erfolgreiche Kultivierung noch eines Archeons *Nitrosopumilus maritimus* gelungen (Könneke et al., 2005), das wegen geringer Größe (0,5-0,9 µm) im Vergleich zu den meisten anderen Bakterien nur schwer unter dem Lichtmikroskop sichtbar ist und zur Gruppe von marinen *Crenarchaeota*, den häufigsten Mikroorganismen in den Ozeanen zählt. Sie repräsentieren mehr als ein Drittel der mikrobiellen Planktongemeinschaft und galten bislang als nicht kultivierbar. Durch die erfolgreiche Kultivierung konnte ihre chemolithoautotrophe, Ammonium-oxidierende Lebensweise und ökologische Rolle aufgeklärt werden.

Solange eine Kultivierung von Mikroorganismen der neuen Kandidaten-Phyla nicht gelingt, bietet nur die Isolierung, Klonierung und Charakterisierung von deren genomischen DNA den direkten Zugang zur Mehrzahl nicht kultivierter Prokaryonten. Damit werden detaillierte Einblicke in deren Biologie, Ökologie und Evolution unabhängig von der Kultivierung möglich.

Aus diesen Gründen wird heutzutage eine umfassende polyphasische Strategie unter Anwendung einer Kombination verschiedener kultivierungsabhängiger und -unabhängiger Methoden (*polyphasic studies*) bevorzugt, um mikrobielle Diversität auf allen Ebenen zu erfassen und verschiedene Aspekte zu integrieren (Torsvik and Øvreås, 2002). Auf diese Weise wird unser Wissen über die tatsächliche Vielfalt der Mikroorganismen in der Natur über die Grenzen der bisher beschriebenen (kultivierbaren) Arten hinaus ständig erweitert.

Das Ziel dieser Arbeit war es, die genetische und metabolische Diversität der komplexen mikrobiellen Gemeinschaft von kultivierbaren und nicht kultivierbaren Mikroorganismen in ausgewählten Umwelthabitaten wie des Bodens und der Phyllosphäre von Apfelanlagen zu ermitteln. Dabei sollten Mikroorganismen mit antagonistischem oder pathogenem Potential charakterisiert bzw. bisher unbekannte Gene identifiziert werden.

Die hier beschriebenen Verfahren basieren daher sowohl auf klassischen kultivierungsabhängigen mikrobiologischen und chemotaxonomischen Methoden als auch auf molekularbiologischen, kultivierungsunabhängigen Techniken. Die aus den Umwelthabitaten gewonnenen Isolate oder aus den Umweltproben isolierten DNAs wurden mit Hilfe von unterschiedlichen Techniken auf biologische Aktivitäten oder auf die Existenz von Genen für biologisch aktive Substanzen oder Pathogenitätsfaktoren untersucht. Als Grundlage für die genomischen Analysen dienten die angelegten komplexen Metagenombanken von natürlich vorkommenden mikrobiellen Gemeinschaften oder Genbanken von einzelnen Bakterien- bzw. Pilz-Stämmen. Die vorliegende Arbeit zeigt Möglichkeiten und Methoden auf, die zum Einsatz gebracht werden können, um die Diversität und das genetische Potential von Umweltmikroorganismen zu erforschen, ihre enormen metabolischen Fähigkeiten zu ermitteln und nutzbar zu machen.

2. ZUSAMMENFASSUNG DER ERGEBNISSE

Die Arbeit beinhaltet die Anwendung unterschiedlicher Strategien und Techniken zur Darstellung der mikrobiellen Diversität in ausgewählten Umwelthabitaten, einer ökologisch geführten und einer naturbelassenen Apfelanlage, in verschiedenen Habitatnischen - in der Phyllosphäre und im Boden. Die genetische und metabolische Diversität der natürlichen Gemeinschaften von Mikroorganismen wurde bezüglich des antagonistischen bzw. pathogenen Potentials ermittelt.

Die hier beschriebenen Studien tragen zur Charakterisierung von in der Umwelt vorkommenden Mikroorganismen bei und evaluieren die Effizienz von molekular- und mikrobiologischen sowie chemotaxonomischen und bioinformatischen Methoden bei deren Charakterisierung. Die Anwendung eines Methodenspektrums auf verschiedenen Ebenen und Fragestellungen ergibt den kumulativen Charakter dieser Arbeit, die in wissenschaftlichen Publikationen und Manuskripten zu folgenden voneinander abgegrenzt dargestellten Themen vorgestellt ist.

2.1. Mikrobielle Diversität in der Phyllosphäre einer ökologisch geführten Apfelanlage (Kucheryava et al., 1999, Anhang 7.2.1; Fiss et al., 2000, Anhang 7.2.2)

Die Diversität einer Gemeinschaft von Mikroorganismen in der Phyllosphäre von Apfelbäumen wurde durch traditionelle Kultivierung von ca. 280 Einzelisolaten phänotypisch untersucht. Dazu stand im Institut für Mikrobiologie der Universität Hannover eine Samlung von epiphytischen Bakterien- und Pilzisolaten aus der Apfelphyllosphäre von einem ökologisch geführten Apfelanbau des Alten Landes bei Hamburg zur Verfügung. 150 Isolate von Bakterien und 107 filamentösen Pilzen dieser Samlung sowie 23 zusätzlich isolierte eigene Isolate von Hefen wurden morphologisch untersucht und in 15 bzw. 13 und 5 Morphotypen eingeordnet.

Alle Umweltisolate wurden auf ihre antagonistischen Eigenschaften gegenüber den Apfelschorferreger *Venturia inaequalis* getestet. Die antagonistische Wirkung wurde im Labor bzgl. der Hemmung der Keimung und des Myzelwachstums des Apfelschorferregers *V. inaequalis* untersucht. Die Abundanzen und die Diversität der antagonistischen Isolate in der Apfelphyllosphäre wurden ermittelt. Der Anteil der Antagonisten in der Bakterien-population lag bei 18%, der Anteil von Pilzantagonisten bei 23% der untersuchten Isolate.

Insgesamt 27 Bakterien und 23 Pilze erwiesen sich als Antagonisten aufgrund der Hemmung der Konidienkeimung und des Myzelwachstums von *V. inaequalis*. Diese 50 antagonistischen Isolate aus der Apfelphyllosphäre wurden mit verschiedenen Methoden charakterisiert. Es erfolgte eine umfassende phänotypische und taxonomische

Charakterisierung der antagonistischen Bakterienisolate mit unterschiedlichen mikrobiologischen und chemotaxonomischen Methoden (Gramfärbung, Endosporenbildung, Substratnutzungsanalyse, Nachweis fluoreszierender Pigmente, Bestimmung von Polyaminen). Sie zeigte, dass die 27 bakteriellen Antagonisten vier Gattungen (*Pseudomonas, Pantoea, Bacillus, Curtobacterium*) angehörten. Die Identifizierung der antagonistischen Bakterienisolate erfolgte aufgrund einer Fettsäure-Analyse mit Hilfe des *Microbial Identifications System* (MIS) in der Arbeitsgruppe von Prof. Dr. Kroppenstedt (DSMZ, Braunschweig). Die 27 bakteriellen Antagonisten wurden von 7 Arten repräsentiert, der größte Teil davon *P. fluorescens, P. syringae* und *B*. *mycoides*. Die Identifizierung der Pilzisolate wurde von Dr. M. Fiss und Dr. G. Arnold (Pilzkulturensammlung der Universität Jena) durchgeführt (Fiss et al., 2000; Fiss, 2001). Demzufolge waren die 23 antagonistischen Pilze aus der Phyllosphäre fünf Gattungen (*Cladosporium, Aureobasidium, Epicoccum, Botrytis, Fusarium*) und sechs Arten zuzuordnen. Vor allem sind Spezies *C. herbarum* und *E. nigrum* vertreten.

Die antagonistische Wirkung dieser epiphytischen Mikroorganismen sollte auf der Pflanze gegenüber dem Apfelschorferreger *V. inaequalis* geprüft werden. Der zum Test der Myzelwachstumshemmung verwendete Referenzstamm *V. inaequalis* DSM 1002 hatte die Fähigkeit zur Konidienproduktion und seine pathogenen Eigenschaften verloren und war daher für die Infektionsversuche auf Apfelsämlingen nicht geeignet. In der verfügbaren Sammlung von Mikroorganismen, die aus den mit Apfelschorf befallenen Blättern isoliert wurden, war kein *V. inaequalis*-Isolat vorhanden, da diese mit der Blattabdruck-Isolierungsmethode schwer zu isolieren sind. Um virulente Apfelschorferreger zu isolieren, wurden daher selektive Isolierungs- und Kultivierungsparameter sowie lange Kultivierungszeit eingestellt, die gezielt für die Auskeimung von *V. inaequalis*-Konidien und die Eliminierung schnell wachsender Mikroorganismen geeignet sind. So wurden aus den Schorfläsionen von Blättern und Früchten verschiedener Apfelsorten über 30 neue Einzelsporenisolate von *V. inaequalis* erhalten, die sich phänotypisch (nach Kolonie-morphologie) unterschieden. Alle Isolate sporulierten *in vitro*, waren pathogen und konnten daher für Infektionsversuche verwendet werden. Da die Antagonisten gegen verschiedene Einzelsporenisolate von *V. inaequalis* unterschiedlich wirksam waren, wurde eine definierte dafür hergestellte infektiöse Population (Mischung von 18 bzw. 30 frisch isolierten monokonidialen Isolaten) als repräsentative natürliche Pathogenpopulation für die Infektionsversuche und die Tests auf antagonistische Wirkung von epiphytischen Mikroorganismen auf Apfelsämlingen sowie *in vitro* verwendet. Dabei zeigten im Labor 37% der bakteriellen Antagonisten keine antagonistische Wirkung gegenüber der hergestellten Pathogenpopulation. Bei den Infektionsversuchen mit *V. inaequalis* auf Apfelsämlingen bewirkten nur noch ein Bakterien- und drei Hefe-Isolate eine vollständige Hemmung der

Schorfinfektion. Die hier gefundenen bakteriellen Antagonisten wurden auf mögliche Wirkmechanismen (Antibiose und Bildung von Siderophoren) sowie auf ihre Pathogenität an Tabak (als hypersensitiv reagierende Pflanze) und Apfelsämlingen (als Wirtpflanze) untersucht. Alle untersuchten Isolate waren zur Bildung von Siderophoren unter Eisenmangelbedingungen und ein Drittel zur Produktion von antimykotischen Wirkstoffen befähigt. Alle *P. syringae*-Isolate zeigten sich als Phytopathogene, die eine hypersensitive Reaktion (HR) an Tabakpflanzen und Nekrose an Apfelsämlingen hervorrufen.

2.2. Polyamine als chemotaxonomischer Marker für die Analyse der Diversität von pflanzenpathogenen und pflanzenassoziierten Enterobakterien (Zherebilo et al., 2001, Anhang 7.2.3)

Die Polyamine, niedermolekularen für den Metabolismus von Mikroorganismen essenziellen Zellinhaltsstoffe, wurden als chemotaxonomischer Marker für die Analyse der Diversität und taxonomische Zuordnung von pflanzenassoziierten Enterobakterien verwendet. Für 91 *Erwinia*-Stämme aus 30 Taxa von pflanzenpathogenen und pflanzenassoziierten Mitgliedern der Familie *Enterobacteriaceae*, die in der Sammlung von phytopathogenen Bakterien im Institut für Mikrobiologie und Virologie der Nationalakademie der Wissenschaften der Ukraine in Kiew zur Verfügung standen, wurden die Polyamin-Profile erstellt. Die erhaltenen Polyaminmuster deuten auf eine große chemotaxonomische Diversität innerhalb der untersuchten pflanzenpathogenen Stämme hin und lassen sich zu deren Unterscheidung verwenden. So wurden charakteristische Polyaminmuster für *Pectobacterium chrysanthemi* und *P. carotovorum* (basonym *E. chrysanthemi* bzw. *E. carotovora*) erhalten, welche eine Unterscheidung von anderen Arten der Gattung *Erwinia* ermöglichen. Damit unterstützt dieser chemotaxonomische Ansatz die durch 16S rRNA-Sequenzierung nachgewiesene Abgrenzung der Gattungen *Pectobacterium, Brenneria, Panthoea* und *Erwinia* (Kwon et al., 1997; Hauben et al., 1998; Spröer et al., 1999). Aufgrund der Polyaminmuster konnten auch innerhalb der Gattung *Pectobacterium* die Arten *P. chrysanthemi*, *P. carotovorum* und *P. cypripedii* sicher voneinander abgegrenzt werden.

2.3. Genetische und metabolische Diversität der mikrobiellen Gemeinschaft im Boden einer naturbelassenen Apfelanlage (Kucheryava et al., Anhang 7.1.1)

In einem weiteren Projekt sollte die komplexe Gemeinschaft von Bodenmikroorganismen einer unbehandelten und naturbelassenen Apfelanlage in Gatersleben untersucht werden. Um dabei auch unkultivierbare Arten erfassen zu können, wurden Mikroorganismen direkt in Bodenproben, ohne vorherige Isolierung und Kultivierung, lysiert. Um möglichst viele Arten

von Mikroorganismen, auch diejenigen, die fest an Bodenpartikel gebunden sind, zu lysieren und deren DNA dann zu extrahieren, wurden verschiedene Methoden der direkten Lyse parallel angewendet (Zhou et al., 1996; Porteous et al., 1997; Kuske et al., 1998; Edgcomb et al., 1999; kommerziell erhältlicher FastDNA® SPIN Kit for Soil (Bio 101). Sie beruhen auf einer Kombination der verschiedenen Lyseverfahren, wie physikalisches Aufbrechen der Zellen (Gefrier-Tau-Zyklen, Ultraschall oder Mörsern in flüssigem Stickstoff), chemische Lyse mit Verwendung von Detergenzien wie SDS, Lösungsmitteln wie Phenol, Chloroform, Chelatbildner wie EDTA oder hohen Salzkonzentrationen oder enzymatische Lyse (Lysozym, Proteinase K). Anschließend wurde die gesamte genomische DNA (das Metagenom) aus Bodenproben extrahiert.

Bei der Extraktion der klonierbaren DNA aus Bodenhabitaten stellte die Eliminierung von Humin- und Fulvinsäuren ein wesentliches Problem dar. Diese hochmolekularen heterozyklischen Polyphenole komplexieren mit der extrahierten DNA und inhibieren die folgenden, zur Klonierung notwendigen, enzymatischen Reaktionen. Da dieses Problem bei den Versuchen zur Reinigung der DNAs tatsächlich auftrat, wurde die CsCl-Gradientenzentrifugation angewendet. Diese Methode ist sehr zeitaufwendig. Um den Zeitaufwand zu kompensieren, wurden mehrere DNA-Proben parallel gereinigt. Die so erhaltenen gereinigten hochmolekularen Boden-DNAs waren mit verschiedenen Restriktions-enzymen (*Eco*RI, *Sau*3A oder *Bam*HI) verdaubar und konnten daher für die Klonierung oder in PCR-Reaktionen eingesetzt werden.

Auf diese Weise erhaltene metagenomische DNA der mikrobiellen Bodengemeinschaft wurde durch PCR-Amplifizierung der 16S-rRNA mit gruppenspezifischen Primern auf charakteristische Bodenbakterien untersucht. Bakterien verschiedener phylogenetischen Gruppen wurden identifiziert. Dabei wurden neben bekannten *Bacillus* spp. überwiegend unkultivierbare Bakterien detektiert, deren 16S-rRNA-Sequenzen hohe Übereinstimmungen mit Umweltsequenzen von nicht identifizierten Mikroorganismen aufwiesen. Dies deutet darauf hin, dass die aus Bodenproben extrahierte metagenomische DNA die tatsächliche Diversität der Prokaryoten in Bodenhabitaten widerspiegelt.

Neben den 16S rRNA-Genen wurden auch spezifische Protein kodierende Gene mittels PCR aus der metagenomischen DNA amplifiziert. Auf diese Weise wurden mit spezifischen, aus dem Genom von *B. subtillis* Isolat A1/3 abgeleiteten Oligonukleotiden, zwei Genfragmente (*orf1-eriS* bzw. *bmyD*) aus dem Gencluster für die Synthese von antibakteriellem Ericin- bzw. antifungalem Bacillomycin-L-Wirkstoff (Hofemeister et al., 2004, Anhang 7.1.4) in zwei getesteten Bodenproben amplifiziert. Die Sequenzierung von amplifizierten Fragmenten zeigte, dass beide Genfragmente hohe Übereinstimmungen (97-98% bzw. 100%) mit entsprechenden Genen (*orf1-eriS* bzw. *bmyD*) von *B. subtillis* A1/3

Stamm aufwiesen. Damit war die Abundanz von *B. subtillis* A1/3 im beprobten Bodenhabitat nachgewiesen.

Zur Identifizierung von neuartigen Genen wurden aus verschiedenen Bodenproben acht Metagenom-Expressionsbanken im Phagen-Expressionsvektor Lambda Zap Express (Stratagene) angelegt. Insgesamt wurden 1,6 x 10^6 rekombinante Bakteriophagen-Klone mit durchschnittlichen Insert-Größen von 4,8 kb (zwischen 2,0 und 9,5 kb) erhalten. Das entsprach ca. 7,7 Gb klonierter metagenomischen DNA von Bodenorganismen. Unter der Annahme, dass ein durchschnittliches prokaryotisches Genom aus ca. 4 Mb besteht, entspricht dies ca. 1900 prokaryotischen Genomäquivalenten. Für die Expression einzelner Gene wie beispielsweise Hydrolasen mit einer Länge von maximal 1 - 2 kb (Lorenz et al., 2002) ist eine Klonierung von Fragmenten dieser Größe ausreichend, es kann sich dabei um bis zu 7 Millionen klonierter Gene handeln. Zusammengefasst zeigt es, dass die Kombination der verschiedenen Extraktionsmethoden und Restriktionsenzymen mit der Verwendung des Phagenexpressionsvektors eine beliebige Erweiterung der Genbanken sowie Identifizierung einzelner Gene als auch kleiner Gencluster möglich macht.

Zwei von den erhaltenen Genbanken wurden durch aktivitätsbasierte Screeningverfahren untersucht, die in Form von einfachen Plattentests mit verschiedenen Substraten und Indikatormikroorganismen durch Nachweis exprimierter Genaktivitäten hinsichtlich des Auftretens hydrolytischer Enzyme oder antimikrobieller Wirkstoffe durchgeführt wurden. Auf diese Weise wurden insgesamt 11 positive Phagenklone isoliert, die glukanolytische oder proteolytische Phänotypen exprimierten. Aus den positiven rekombinanten Phagenklonen wurden die klonierten Inserts in *E. coli* subkloniert, um Inserts im Plasmidsystem besser charakterisieren zu können. So wurden zwei Gene in *E. coli* identifiziert, die einen stabilen glukanolytischen bzw. proteolytischen Phänotyp vermittelten. Die gefundenen Gene wiesen eine deutliche Übereinstimmung (96-98%) zu bekannten Enzymen, einer endo-ß-1,4-Glukanase (Cellulase, EC 3.2.1.4) und einer intrazellulären Serinprotease (EC 3.4.21), beide aus *Bacillus* sp. auf. Acht weitere Plasmidinserts von rekombinanten *E. coli*-Subklonen, die keine detektierbare Enzymaktivität beim Screening von *E. coli*-Kolonien zeigten, wurden ebenfalls sequenziert. Die Sequenzanalyse ergab nur schwache Ähnlichkeiten zu bekannten Proteinsequenzen vorwiegend bakterieller Herkunft, die aber keine signifikanten Übereinstimmungen mit bekannten Nukleotidsequenzen in Sequenzdatenbanken zeigten.

Diese Ergebnisse zeigen, dass das untersuchte Habitat Boden einer naturbelassenen Apfelanlage eine große Vielfalt von Mikroorganismen, einschließlich bisher unbekannten, möglicherweise nicht kultivierbarer Arten beinhaltet. Die angelegten Metagenombanken beinhalten eine große Vielfalt von Genen der Bodenorganismen und sind demnach gut

geeignet, um die große Diversität der mikrobiellen Gemeinschaft im Boden auf genomischer Ebene zu erfassen und sowohl bekannte als auch neuartige Gene zu identifizieren.

2.4. Konstruktion von einem in *E. coli* und *B. subtilis* mobilisierbaren Shuttle-Cosmidvektor pSB 681 zur Erstellung der Genbank von einem antagonistischen Umweltisolat *B. subtilis* A1/3 (Kucheryava et al., Anhang 7.1.2; Hofemeister et al., 2004, Anhang 7.2.4)

Das antagonistische Wirkstoff produzierende Unweltisolat *B. subtilis* A1/3 wurde aus der Rhizosphäre von Tomaten isoliert (Griesbach und Lattauschke, 1991) und kommt nachweislich im untersuchten Bodenhabitat vor (Kucheryava et al., Anhang 7.1.1). Ein breites Spektrum an antagonistischer Aktivität gegenüber Bakterien, Pilzen und Phytoviren wurde beobachtet (Huber et al. 1991) und die Synthese von 8 verschiedenen Wirkstoffen und einer Siderophore bereits identifiziert (Hofemeister et al., 2004, Anhang 7.2.4).

Um die metabolischen und genetischen Kapazitäten von *B. subtilis* A1/3 zu ermitteln und mit bekannten Genomsequenzen des Modellorganismus *B. subtilis* 168 speziell hinsichtlich dessen Gene für die Synthese von Sekundärmetaboliten zu vergleichen, wurde eine Genbank von *B. subtilis* A1/3 in einem neuartigen mobilisierbaren Cosmidvektor pSB 681 angelegt (Kucheryava et al., Anhang 7.1.2). Hierzu wurde ein Shuttle-Cosmidvektor konstruiert, der sowohl in *E. coli* als auch in *B. subtilis* vermehrt werden kann. Vorteil dieses Shuttle-Vektors bei der Erstellung von Genbank ist, dass die rekombinanten Cosmide mit hinreichend großen Inserts aus *E. coli*- in *B. subtilis*-Wirt für die funktionelle Analyse der klonierten Gene mobilisiert werden können. Dafür wurden in dem Cosmidvektor Supercos1 (Stratagene) plasmidale Replikationsgene für *Bacillus* und ein zweiter Antibiotikaresistenzmarker (Erythromycin) zur Selektion einrekombiniert. Nach der Transformation in *E. coli* wurde der rekombinante Shuttle-Cosmidvektor pSB 681 isoliert und in *B. subtilis* transformiert. Nach der Reisolierung aus beiden Wirtsorganismen zeigten alle untersuchten Cosmide identische Restriktionsmuster, womit gezeigt werden konnte, dass der konstruierte Shuttle-Cosmidvektor pSB 681 in beiden Organismen stabil vermehrt wird und somit auch eine Genbank in *B. subtilis* transferiert und funktionell untersucht werden kann. Außerdem kann der konstruierte Shuttle-Cosmidvektor die Analyse der Biosynthese von Wirkstoffen sowohl in heterologen *E. coli*- als auch in verschiedenen *Bacillus*-Wirtsorganismen ermöglichen, die aufgrund ihrer natürlichen biosynthetischen Kompetenz als Expressionswirte bevorzugt werden.

Im neu konstruierten Shuttle-Cosmidvektor pSB 681 wurde eine Genbank von *B. subtilis* A1/3 angelegt, die etwa 5000 Klone mit durchschnittlichen Insertgrößen von 34 kb umfasste. Dies entspricht insgesamt ca. 170 Mb klonierter genomischen DNA und einer 40-facher

Abdeckung des Genoms von *B. subtilis* (ca. 4 Mb). Etwa 4600 Klone der amplifizierten Genbank wurden mit Hilfe eines Roboters in 384-Loch-Mikrotiterplatten vereinzelt und auf Nylonfiltern in dichten Arrays für Hochdurchsatzscreening mittels Koloniehybridisierungen aufgetragen.

Die antifungale Wirkung von *B. subtilis* A1/3 wurde gegenüber verschiedenen pflanzenpathogenen Oomyzeten und Pilzen (einschließlich *V. inaequalis*) untersucht (Kucheryava et al., Anhang 7.1.2). Zellen und zellfreie Überstände von *B. subtilis* A1/3 verursachten eine starke Hemmung des Myzelwachstums aller getesteten Phytopathogenen sowie eine starke Hemmung der Konidienkeimung von *V. inaequalis*, wobei morphologische Veränderungen, wie z.B. Hyphenschwellung von *V. inaequalis*-Keimlingen festgestellt wurde.

Um das gesamte Gencluster für die Biosynthese eines stofflich vorher mittels MALDI-TOF-MS nachgewiesenen antifungalen Wirkstoffes Bacillomycin L zu ermitteln, der zu den nicht ribosomal synthetisierten zyklischen Lipopeptiden der Iturin-Gruppe gehört (Hofemeister et al., 2004, Anhang 7.2.4), wurde die im Cosmidvektor pSB 681 angelegte Genbank von *B. subtilis* A1/3 mittels Koloniehybridisierung mit entsprechenden *bmyD* Gensonden durchmustert. Insgesamt wurden 11 positive Klone detektiert, darunter ein Cosmid mit dem kompletten Gencluster für die Biosynthese von Bacillomycin L isoliert und analysiert (Hofemeister et al., 2004, Anhang 7.2.4). Dieses Ergebnis zeigt, dass die angelegte Genbank einen Zugang zu einem repräsentativen Spektrum der Gene von *B. subtilis* A1/3 bietet und als Grundlage für die Identifizierung einzelner Gene sowie großer Biosynthese-Gencluster eingesetzt werden kann.

2.5. Erstellung der cDNA-Genbank von *V. inaequalis* zur Ermittlung der Genexpression bei der biotrophen pathogenen morphogenetischen Differenzierung *in vitro* und während der Infektion in der Pflanze (Kucheryava et al., 2008, Anhang 7.2.5)

Bei der Infektion entwickeln die meisten Pilze, die als biotrophe oder hemibiotrophe Pathogene auf lebende Wirtszellen angewiesen sind, hochspezialisierte Strukturen, die Voraussetzung für die Infektion sind (*infection-related morphogenesis*). So werden bei der Invasion in das Pflanzengewebe verschiedene morphologisch spezialisierten Hyphen, so genannte Infektionsstrukturen gebildet, die für die Anpassung an das Habitat Pflanze und für Etablierung und Aufrechterhaltung der Pathogenese erforderlich sind. Das können die Appressorien, Penetrationhyphen, Infektionshyphen und Haustorien sein.

Obwohl der hemibiotrophe Erreger des Apfelschorfes, *V. inaequalis,* seinen Lebenszyklus *in vitro* außerhalb der Pflanze vervollständigen kann, entwickelt er unter Standard-kultivierungsbedingungen im Labor keine spezialisierten Infektionsstrukturen, die für die

Infektion essenziell sind. Die morphogenetische Differenzierung des Pathogens *V. inaequalis* während der Infektion *in planta* konnte *in vitro* mit Hilfe eines dafür hergestellten Infektionsmodells nachgebildet werden. Das hier eingesetzte Infektionsmodell besteht aus einer Zellophanmembran, die auf Agarmedium aufgebracht und mit einer Konidiensuspension von *V. inaequalis* inokuliert wird. Dabei kann die infektionsrelevante Differenzierung des Pathogens von der Appressorienbildung bis zur Sporulation vollständig beobachtet werden. Auch die späteren Stadien der *V. inaequalis*-Infektion, nämlich die Differenzierung von Infektionshyphen und das subkutikuläre Wachstum nach der Penetration, konnten *in vitro* mit der Zellophantechnik induziert werden.

Nach dem Eindringen der Hyphen in die Zellophanmembran differenzierte der Pilz Infektionshyphen- und Stroma-ähnliche Strukturen und zeigte ein starkes Wachstum, das nach 2-3 Wochen zu einer fast vollständigen Besiedlung des Zellophans und dessen teilweiser Auflösung führte. Dieses Infektionsmodell wurde genutzt, um molekulare Mechanismen der infektionsrelevanten morphogenetischen Differenzierung aufzuklären. Die Gene sollten identifiziert werden, die spezifisch bei der Entwicklung in dem Modellhabitat Zellophan exprimiert werden, welche vermutlich auch während einer biotrophen Infektion in der Pflanze exprimiert werden.

Die RNA aus im Zellophan wachsenden Myzel von *V. inaequalis* wurde isoliert, die poly(A)-mRNA wurde danach aus den RNA-Extrakten mittels kovalent an Magnetpartikel gebundenes oligo(dT) angereichert, in cDNA umgeschrieben und mit Hilfe des Expressionsvektors Lambda ZAP-Express in *E. coli* kloniert. Die so erstellte cDNA-Bank von *V. inaequalis* ermöglichte mit Hilfe der differenziellen Hybridisierung die Identifizierung von Pathogenese-relevanten Genen von *V. inaequalis*.

Insgesamt vier Gene wurden identifiziert, die im Zellophan spezifisch exprimiert wurden. Zwei identifizierte Gene werden auch spezifisch während einer Kolonisierung der Pflanze exprimiert. Die Expression von beiden Pilzgenen wurde mittels quantitativer RT-PCR bei der Infektion von Apfelsämlingen nachgewiesen. Diese Ergebnisse bestätigten die Hypothese, dass im Zellophan gewachsene *V. inaequalis* den Infektionsprozess nachahmt.

Zwei der identifizierten Gene, *cin1* und *cin3* (***c***ellophane-***in***duced) genannt, die in der infizierten Pflanze sehr stark induziert werden, kodieren hypothetische Proteine mit einer neuartigen Repeat-Sequenzstruktur. Das *cin1*-Gen kodiert für ein sekretiertes Protein mit 7-8 Repeats von 60 Aminosäuren, mit vier konservierten Cystein-Aminosäuren pro Repeat, welches wahrscheinlich in der Zellwand lokalisiert ist. Die Immunofluoreszenzmarkierung mit anti-CIN1p-Antikörpern bestätigte die Lokalisation des Cin1-Proteins in der Zellwand. Das *cin3*-Gen kodiert für ein sekretiertes Protein mit 4-5 Repeats von 32 Aminosäuren ohne Cystein. Die Funktion(en) der genannten Gene sind bisher unbekannt, da die beiden

Sequenzen keine Ähnlichkeiten zu Proteinen in Datenbanken aufwiesen und können bisher nur auf Basis der Sequenz postuliert werden. Somit ist es erstmalig gelungen die infektionsrelevanten Gene von *V. inaequalis* zu identifizieren, die spezifisch während der Infektion exprimiert werden und für die Besiedlung in der Pflanze und biotrophe Pathogenese möglicherweise essentiell sind. Diese potentiellen für Pathogenese relevanten Gene sind von besonderem Interesse, da sie für die Steuerung der Differenzierungsprozesse und für die Aufrechterhaltung des speziellen Metabolismus von *V. inaequalis* während der biotrophen pathogenen Interaktion von Bedeutung sein könnten.

3. ZUSAMMENFASSENDE DISKUSSION

Wie der Boden, stellt auch die Pflanze für Mikroorganismen einen Lebensbereich mit sehr komplexen Nischenhabitaten im Inneren wie auch auf den Oberflächen, in der Rhizo- und Phyllosphäre von Pflanzen dar. Hier können sich Gemeinschaften von Mikroorganismen mit außerordentlicher Diversität entwickeln, die in komplexer Weise miteinander als auch mit der Pflanze kooperieren und interagieren. Dabei kann die Beziehung zwischen Pflanze und Mikroorganismus von symbiotisch über saprophytisch bis hin zu pathogen reichen. In all diesen Habitaten sind viele mikrobielle Prozesse noch unvollständig verstanden. Außerdem sind viele der daran beteiligten Mikroorganismen, u.a. auch solche mit einem hohen Potenzial als Pathogene, Antagonisten und Wirkstoffproduzenten noch unbekannt. Der Anteil kultivierbarer Spezies bleibt weiterhin gering, weil viele Mikroorganismen aufgrund ihrer speziellen Wechselwirkungen mit der Umwelt und mit anderen Organismen nicht in Reinkultur kultivierbar sind.

3.1. Mikrobielle Diversität in der Phyllosphäre eines ökologisch geführten Apfelanbaus

In den vorliegenden Arbeiten (Kucheryava et al., 1999, Anhang 7.2.1; Fiss et al., 2000, Anhang 7.2.2) konnte mit Hilfe kultivierungsabhängiger Methoden die strukturelle Diversität und das antagonistische Potential von Mikroorganismen in der Phyllosphäre von Apfelbäumen gezeigt werden. Die Phyllosphäre stellt einen sehr komplexen Lebensraum dar, in dem verschiedene mikrobielle Arten mit der geschätzten Populationsdichte von 10^4-10^6 cfu/cm^2 vorkommen (Whitman et al., 1998). Dennoch es stellte sich heraus, dass eine Sammlung von ca. 280 epiphytischen Isolaten aus der Phyllosphäre von gesunden und mit Apfelschorf befallenen Apfelblättern nur über eine begrenzte Diversität von Bakterien, Hefen und Pilzen verfügte, die in 15, 5 bzw. 13 Morphotypen eingeordnet werden konnten. Deren antagonistisches Potential wurde bezüglich einer Wirkung gegen den Apfelschorferreger *V. inaequalis* untersucht. Dadurch konnten die Abundanzen und die Diversität der antagonistischen Isolate in der Apfelphyllosphäre ermittelt werden. Diese Ergebnisse zeigen, dass die Phyllosphäre-assoziierten Mikroorganismen eine reiche Quelle von natürlichen Antagonisten darstellen.

Bei dem antagonistischen Unweltisolat *B. subtilis* A1/3 wurde die Synthese von acht verschiedenen Wirkstoffen und einer Siderophore bereits identifiziert (Hofemeister et al., 2004, Anhang 7.2.4).

Nicht alle Mikroorganismen in der Phyllosphäre sind aber ausschließlich assoziative Epiphyten, sondern auch solche, wie *P. syringae* in dieser Studie, die eindeutig phytopathogene Eigenschaften haben. Der gefundene hohe Anteil von *Pseudomonas* und

Bacillus spp. unter den Bakterien, oder von *Cladosporium* und *Epicoccum* spp. unter den Pilzen, dürfte weniger auf deren hohe Abundanz in der Apfelphyllosphäre, sondern hauptsächlich auf deren relativ leichte Kultivierbarkeit zurückzuführen sein. Da jedoch nur ein geringer Anteil der tatsächlich vorkommenden Mikroorganismen unter Laborbedingungen kultivierbar ist, bleibt bei dieser Untersuchung das vermutlich beträchtliche Potential von unkultivierbaren Mikroorganismen unbekannt. Viele pflanzenassoziierten Mikroorganismen sind aufgrund ihrer speziellen Wechselwirkungen mit der Pflanze und mit anderen Mikroorganismen in Reinkultur nicht oder nur schwer kultivierbar. Da die Standardmethoden schnell wachsende Stämme bevorzugen und meist nicht spezifisch genug sind, um bestimmte bzw. langsam wachsende, schwer kultivierbare Mikroorganismen gezielt zu isolieren, muss ein spezifisches Set von Kultivierungsparametern ermittelt werden und dadurch auch das Wachstum unerwünschter schnell wachsenden Organismen zu unterdrücken.

Außerdem tritt häufig das Phänomen der Stammredundanz auf (Demirjian et al., 1999). Sie kann dazu führen, dass ein neu isolierter Stamm nach seiner phänotypischen und phylogenetischen Charakterisierung als vermeintliches Duplikat eines bereits vorhandenen Stammes eliminiert wird. Ein weiteres Problem stellt die Refermentation der mikrobiellen Kulturen dar, die in über 50% der Fälle bei traditionellem Naturstoffscreening auftritt (Osburne et al., 2000). Insbesondere die aus Umweltproben angereicherten mikrobiellen Kulturen zeigen bei der ersten Anzucht bestimmte Eigenschaften bzw. biologische Aktivität, die sie aber bei weiteren Anzuchten verlieren oder ihr Wachstum überhaupt einstellen. Auch im Rahmen dieser Arbeit wurde dieses Phänomen beobachtet. Bei der Isolierung aus der Apfelphyllosphäre gingen ein Zehntel der Bakterienisolate (vermutlich aus der Kategorie der Endophyten) und später noch 6 weitere Isolate aus der Sammlung verloren, weil sie nicht weiterkultiviert werden konnten. Der Referenzstamm *V. inaequalis* DSM 1002, der als Apfelschorferreger zum Test der Hemmung von Myzelwachstum verwendet wurde, hat beispielsweise durch die anhaltende Kultivierung auf künstlichen Nährmedien die Fähigkeit zur Konidienproduktion und seine pathogenen Eigenschaften verloren und war daher für die Infektionsversuche auf Apfelsämlingen nicht geeignet. Um virulente Apfelschorferreger zu isolieren, wurden selektive Isolierungs- und Kultivierungsparameter eingestellt, die für die Auskeimung von *V. inaequalis*-Konidien und die Eliminierung schnell wachsender Mikroorganismen geeignet sind. Auf diese Weise wurden aus den Schorfläsionen über 30 virulente Einzelsporenisolate von *V. inaequalis* isoliert. Zusätzlich wurden dabei 23 Isolate von epiphytischen Hefen isoliert, die das Auskeimen von *V. inaequalis*-Konidien aus den Schorfläsionen *in vitro* deutlich hemmten.

3.2. Polyamine als chemotaxonomischer Marker bei der Analyse der Diversität und taxonomischen Zuordnung von pflanzenpathogenen und pflanzenassoziierten Enterobakterien

In der Umweltmikrobiologie werden unterschiedliche Methoden zur Charakterisierung und taxonomische Einordnung von Mikroorganismen eingesetzt. So ist die Chemotaxonomie ein Verfahren, mit dem weitere phänotypische Merkmale von Mikroorganismen erfasst werden können, und wird in der bakteriellen Systematik erfolgreich herangezogen. Durch den Nachweis spezieller Zellinhaltsstoffe wie Chinone, Polyamine, Fettsäuren oder Phospholipide, die nach Extraktion aus einer Kultur chromatographisch aufgetrennt und somit bzgl. ihrer Zusammensetzung analysiert werden, können die Mikroorganismen charakterisiert und identifiziert werden.

Polyamine sind niedermolekulare, positiv geladene Verbindungen, die bei fast allen Lebewesen vorkommen. Sie sind im Organismus unter anderem an der Biosynthese von DNA, RNA und Proteinen beteiligt (Tabor und Tabor, 1985) und für das Zellwachstum, die Stabilisierung von DNA und von Membranstrukturen notwendig (Walters, 2000). Die Polyaminbiosynthese ist daher auch ein effizientes Target zur Therapie humanpathogener Protozoa-Infektionen und einiger Krebskrankheiten (Giffin et al., 1986; Kaur et al., 1986; Bitonti et al., 1987). Es ist auch ein interessantes Target für die Entwicklung von neuen antifungalen Wirkstoffen geworden (Guevara-Olvera et al., 1997; Bailey et al., 2000).

Als chemotaxonomischer Marker dienen Polyamine für bestimmte Bakteriengruppen, vor allem für gramnegative Bakterien. Grampositive Bakterien enthalten Polyamine nur in geringen Mengen, sind jedoch für bestimmte Bakterien charakteristische Polyaminmuster bekannt (Hamana et al., 1989; Busse et al., 1996). Die Eignung von Polyaminen als Biomarker wurde für Proteobakterien und für deren Subklassen nachgewiesen, wobei sich die charakteristischen Polyaminmuster der α-, β- und γ-*Proteobacteria* leicht zuordnen lässt (Busse und Auling, 1988; Hamana und Matsuzaki, 1992, 1993). Dabei dienen Polyamine als gut geeigneter Marker für die Abgrenzung phytopathogener und pflanzenassoziierter *Proteobacteria* (Auling et al., 1991; Auling, 1992).

Polyamine als chemotaxonomischer Marker wurden in der vorliegenden Arbeit (Zherebilo et al., 2001, Anhang 7.2.3) für die Analyse der Diversität und taxonomische Zuordnung von pflanzenpathogenen oder pflanzenassoziierten Mitgliedern der Familie *Enterobacteriaceae* verwendet. Die Polyamin-Profile wurden für 91 *Erwinia*-Stämme aus 30 Taxa der Familie *Enterobacteriaceae* erstellt, die in einer Sammlung von phytopathogenen Bakterien (Institut für Mikrobiologie und Virologie, Kiew) zur Verfügung standen. Die erhaltenen Polyaminmuster deuteten auf eine große chemotaxonomische Diversität der untersuchten Stämme innerhalb der Gattung *Erwinia* hin und lassen sich zu deren Unterscheidung

verwenden. So wurden die charakteristischen Polyaminmuster für *Pectobacterium chrysanthemi* und *P. carotovorum* (basonym *E. chrysanthemi* bzw. *E. carotovora*) erhalten, welche eine Unterscheidung von anderen *Erwinia*-Arten und Gattungen der Familie *Enterobacteriaceae* ermöglichen. Damit unterstützt die Polyaminanalyse die durch 16S rRNA-Sequenzierung nachgewiesene Abgrenzung der Gattungen *Pectobacterium, Brenneria* und *Erwinia* (Kwon et al., 1997; Hauben et al., 1998; Spröer et al., 1999).

Aufgrund der erhaltenen Polyaminmuster konnten auch innerhalb der Gattung *Pectobacterium* die Arten *P. chrysanthemi*, *P. carotovorum* und *P. cypripedii* sicher voneinander abgegrenzt werden.

Mit Hilfe der Analyse von Polyaminen wurde im Rahmen dieser Arbeit auch die taxonomische Diversität von antagonistischen Bakterienisolaten aus der Apfelphyllosphäre aufgeklärt (Kucheryava et al., 1999, Anhang 7.2.1). Die untersuchten Umweltisolate konnten aufgrund der erhaltenen Polyaminmuster, ergänzt durch morphologische und physiologische Charakterisierung, zu vier Gruppen zusammengefasst werden. Aufgrund der charakteristischen Polyaminmuster konnten drei Gattungen (*Pseudomonas*, *Pantoea* und *Bacillus*) identifiziert werden. Diese Ergebnisse zeigen, dass die Analyse von Polyaminen - in Ergänzung zu molekulargenetischen Verfahren - eine geeignete Methode zur Charakterisierung und taxonomischen Einordnung von unbekannten Umweltisolaten bieten. Da mit der Polyaminanalyse eine große Anzahl von Isolaten gleichzeitig, relativ schnell und kostengünstig untersucht werden kann, können auch Aussagen über die Zusammensetzung der Bakterienpopulation eines bestimmten Habitats getroffen werden. Dabei dienen Polyamine als chemotaxonomischer Biomarker für Nachweisen von bestimmten Bakteriengruppen im Habitat, so dass ein Profil dieser Mikroorganismen in den komplexen Populationen erfassbar ist.

3.3. Metabolische und genetische Diversität der mikrobiellen Gemeinschaft im Boden einer naturbelassenen Apfelanlage

Ein weiterer Schwerpunkt in der vorliegenden Arbeit (Kucheryava et al., Anhang 7.1.1.) war es, die genetische und metabolische Diversität sowie das antagonistische Potenzial der im Boden einer unbehandelten und naturbelassenen Apfelanlage mittels einer metagenomischen Strategie zu erfassen. Die Anwendung von molekular-biologischen kultivierungsunabhängigen Methoden wie Isolierung der metagenomischen DNA von mikrobiellen Gemeinschaften, PCR, Klonierung und Sequenzierung ermöglichte die Detektion und Identifizierung von Mikroorganismen ohne vorherige Anreicherung und Kultivierung.

In allen bisher untersuchten Bodenproben wurden fast ausschließlich unbekannte Mikroorganismen gefunden (Buckley und Schmidt, 2002). Die extrem hohe mikrobielle Diversität in Böden ist durch die Vielzahl von Mikrohabitaten zu erklären, die den Mikroorganismen mit unterschiedlichsten Ansprüchen die Koexistenz erlauben. Aufgrund der extrem hohen Diversität von Bodengemeinschaften kann nur eine Stichprobe mittels Sequenzierung klonierter PCR-Produkte untersucht werden. Denn zehntausende von 16S rDNA-Klone müssten sequenziert werden, um an die geschätzte Diversität mit 10^4 Arten (Torsvik et al., 1990) heranzukommen. Die Ergebnisse zur Diversität in den Bodenproben beziehen sich daher lediglich auf die PCR-amplifizierbaren Gene ausgewählter Stichproben.

Die in dieser Arbeit isolierte aus Bodenproben metagenomische DNA, die das Metagenom der mikrobiellen Gemeinschaft im ausgewählten Habitat darstellt, wurde zuerst mittels 16S rDNA-Sequezierung hinsichtlich ihrer phylogenetischen Diversität untersucht. Auf diese Weise wurden Bakterien der unterschiedlichen phylogenetischen Gruppen, einschließlich überwiegend unidentifizierter Mikroorganismen detektiert. Dies weist darauf hin, dass die isolierte metagenomische DNA für die tatsächlich vorhandene Diversität der Prokaryoten im Boden repräsentativ ist.

Die Analyse von rRNA-Genen ist jedoch nicht ausreichend, um die Rückschlüsse auf die physiologische Rolle von einzelnen Mikroorganismen in der Population zu ziehen (Rodríguez-Valera, 2002). Außerdem können Mikroorganismen mit identischen 16S rDNA-Sequenzen über unterschiedliche Genome, d.h. auch unterschiedliche metabolische und physiologische Eigenschaften verfügen (Jaspers and Overmann, 2004; Hahn and Pöckl, 2005). Dagegen kann die Beprobung der Mikroorganismengemeinschaft auf bestimmte metabolische oder biodegradierende Aktivitäten durch genspezifische PCR-Amplifikation erreicht werden (McDonald et al., 1995; Watanabe et al., 1998; Hallin and Lindgren, 1999; Sheu et al., 2000).

Im Rahmen dieser Arbeit wurde diese Strategie angewendet, um die erhaltene metagenomische DNA auf bestimmte antimikrobielle Aktivitäten zu untersuchen. So wurden aus jeweils zwei getesteten Bodenproben zwei Genfragmente für Biosynthese eines antibakteriellen (Ericin) und eines antifungalen (Bacillomycin L) Wirkstoffes mit genspezifischen Oligonukleotiden amplifiziert, die aus einem Wirkstoffproduzenten *B. subtillis* A1/3 abgeleitet wurden.

B. subtillis A1/3 ist ein Umweltisolat, der aus der Rhizosphäre von Tomaten in der Region Gatersleben isoliert wurde (Griesbach and Lattauschke, 1991), aus der auch die untersuchten Bodenproben stammen. Dieses Umweltisolat unterscheidet sich von Referenzmodellorganismen und anderen bekannten *B. subtillis*-Stämmen durch ein breites Spektrum antibiotischer Wirkungen gegen phytopathogene Viren, Bakterien und Pilze

(Griesbach and Lattauschke, 1991; Huber et al., 1991). Bisher wurden aus diesem Stamm neun verschiedene Wirkstoffe identifiziert (Steller et al., 1998; Steinborn and Hofemeister, 1998, 2000; Stein et al., 2002; Hofemeister et al., 2004).

Die Lantibiotika Ericin S und Ericin A sind Stamm-spezifisch, obwohl sich Ericin S von Subtilin (*B. subtillis* ATCC 6633) nur durch vier Aminosäuren unterscheidet (Stein, 2005). Das Ericin Gencluster von *B. subtilis* A1/3 enthält zwei strukturelle Gene, *eriA* und *eriS*, getrennt durch ein *orf1* mit unbekannter Funktion. Diese spezifischen Genfragmente wurden mittels genspezifischer PCR aus zwei verschiedenen Bodenproben amplifiziert und wiesen hohe Übereinstimmungen (97-100%) mit entsprechenden Genen von *B. subtillis* Stamm A1/3 auf (Stein et al., 2002; Hofemeister et al., 2004).
Das zweite amplifizierte *bmyD*-Genfragment aus dem Gencluster für Biosynthese von antifungalen Wirkstoff der Iturin-Gruppe wies Identität mit der Malonyl-CoA-Transacylase von Bacillomycin L von *B. subtillis* A1/3 auf. Dabei zeigte diese Gensequenz auch annähernd 100%-ige Übereinstimmung zu den Biosynthesegenen anderer Lipopeptidantibiotika der Iturin-Gruppe, wie Bacillomycin D von *B. amyloliquefaciens* (99%) und *B. subtilis* (98%), sowie auch zu Iturin A (98%) und Mycosubtilin (81%) von *B. subtilis*. Dies beweist, dass Malonyl-CoA-Transacylase ein gutes Zielgen für die PCR-basierte Detektion von Biosynthesegenen der Lipopeptidantibiotika der Iturin-Gruppe in Umweltproben darstellen könnte.
Durch die PCR-Amplifizierung dieser zwei Gene konnte auch die Abundanz von *B. subtillis* A1/3 im beprobten Bodenhabitat nachgewiesen werden.

Durch die Anwendung von Oligonukleotiden, die von konservierten Genregionen oder Gen-flankierenden Elementen abgeleitet werden, konnten neuartige Gene und ganze Gen-Kassetten amplifiziert werden (Stokes et al., 2001; Eschenfeldt et al., 2001). So wurden Gene für Polyketidsynthasen, die an der Synthese von Polyketidantibiotika beteiligt sind, mittels PCR aus dem Boden kloniert (Seow et al., 1997). Allerdings ist die PCR-Amplifizierung von großen Genclustern noch nicht möglich. Deshalb konnten viele Gene und Gencluster nur unvollständig kloniert werden. Das gesamte Genprodukt kann dann durch weitere Amplifizierungen (*primer walking*) ermittelt werden. So konnten bereits neue Lipasen und Nitrilhydratasen identifiziert werden (Bellet al., 2002; Liebeton and Eck, 2004). Dabei bleiben allerdings ihre Herkunft (produzierende Mikroorganismen), ihre Relevanz und Funktion oder die Bedeutung im Ökosystem ungeklärt.

Die direkte Klonierung der aus Umweltproben isolierten DNA in Metagenombanken ermöglicht den Zugang zu unbekannten, neuartigen Genen. Verschiedene Screening-strategien ermöglichen die Überprüfung einer sehr großen Anzahl von Klonen aus Metagenombanken mit relativ einfachen Methoden. Nach der Sequenzierung dieser

Metagenome kann die funktionelle *in silico* Annotation einzelner Gene oder komplette genetische Information einzelner Organismen durch etablierte Methoden der Bioinformatik und computerbasierte Algorithmen wieder zusammengesetzt werden. Auf sequenz-unabhängige Weise, mittels aktivitätsbasiertem Screening auf die Produktion der gewünschten biologisch aktiven Substanzen oder Enzyme, ist die Entdeckung von neuartigen Proteinen möglich.

Mit dieser Technik wurden in dieser Arbeit aus extrahierten metagenomischen DNAs insgesamt acht Metagenombanken in dem Phagen-Expressionsvektor angelegt. Die heterologe Expression in *E. coli* ist in diesem System sowohl von den nativen Promotoren als auch von dem vektoreigenen Promotor abhängig. Im Falle eines Expressionsscreenings kann die Transkription der Gene dabei auch vom vektoreigenen Promotor aus stattfinden, sodass die Erkennung fremder Promotoren durch die wirtseigene RNA-Polymerase nicht erforderlich ist und somit die Expression des Proteins und Detektion der exprimierten Aktivität von den nativen Promotoren unabhängig möglich ist.

Die Durchmusterung der Metagenombank auf exprimierbare Aktivität (z.B. enzymatische) hat gegenüber sequenzbasierten Screeningmethoden den Vorteil, dass auch Gene bisher unbekannter Mikroorganismen gefunden werden können, die keine Homologie zu bereits bekannten Sequenzen aufweisen. Daher wurden zwei von den angelegten Metagenom-banken mit Hilfe von aktivitätsbasierten Screeningverfahren zur Identifikation von neuartigen Genen für Hydrolasen sowie für antibakteriellen und antifungalen Wirkstoffen durchgeführt. Es wurden insgesamt 11 positive Phagenklone isoliert, die glukanolytische oder proteolytische Phänotypen exprimierten. Nur zwei identifizierte Gene exprimierten stabil einen glukanolytischen bzw. proteolytischen Phänotyp in *E. coli-S*ubklonen und wiesen eine deutliche Übereinstimmung (96-98%) zu bekannten Enzymen, endo-ß-1,4-Glukanase (Cellulase, EC 3.2.1.4) und Serinprotease (EC 3.4.21), aus *Bacillus* spp. auf. Die Sequenzanalyse von anderen *E. coli*-Subklonen, die keine detektierbare Enzymaktivität beim Rescreening der *E. coli*-Kolonien zeigten, ergab eine Reihe von abgeleiteten Proteinen, die nur geringe Ähnlichkeiten zu bekannten Proteinsequenzen vorwiegend bakterieller Herkunft und gar keine Übereinstimmungen zu Nukleotidsequenzen in den Datenbanken aufwiesen.

Es ist somit anzunehmen, dass die hier angelegten Metagenombanken auch Gene von noch nicht identifizierten oder kultivierbaren Mikroorganismen beinhalten und die Expression dieser Gene in *E. coli* erschwert oder nicht möglich war. Denn unabhängig von einer erfolgreichen Expression in Bakteriophagen, ist Enzymaktivität in *E. coli-S*ubklonen (*E. coli*-Kolonien) nur nachweisbar, wenn das Substrat in die Zellen gelingt oder das Enzym nach Zelllyse freigesetzt wird und mit dem Substrat in Kontakt kommen kann. Die Bildung eines Hofes (Substrathydrolyse) bei einem Screeningverfahren unter Verwendung von ganzen

Zellen (Kolonien) kann üblicherweise nur erfolgen, wenn die Bakterien zumindest teilweise lysieren und die Enzyme in den Agar diffundieren. Ist diese Voraussetzung nicht gegeben, muss mit Zellextrakten gearbeitet werden. Durch Zelllyse könnte dann eine hydrolytische Aktivität detektiert werden. Daher könnte die relativ niedrige Anzahl exprimierter Aktivitäten durch Verwendung von *E. coli* als Wirt erklärt werden. Tatsächlich konnte die Expression von Genen für Cellulase und Serinprotease aus dem grampositiven *Bacillus* mit dem hier gewählten Screening in *E. coli* gezeigt werden. Es wurde geschätzt, dass unter Standardbedingungen nur etwa 40% der metagenomischen Gene in *E. coli* aktiv exprimiert werden können (Gabor et al., 2004). Nach bioinformatischer Schätzung ist die Genexpression von *Firmicutes*, zu denen *Bacillus* gehört, im Vergleich zu anderen taxonomischen Gruppen am ehesten (73%) in *E. coli* möglich (Gabor et al., 2004).

Ein weiterer zentraler Punkt ist die Entwicklung einer zuverlässigen Screeningmethode. Die hier gewählten Anzucht- und Analysebedingungen waren möglicherweise nicht optimal für die heterologe Genexpression bzw. Aktivität von Enzymen und biologisch wirksamen Substanzen. Durch die Optimierung von Screeningverfahren und durch weitere Erhöhung der Anzahl rekombinanter Klone in jeweiligen Genbanken (Klonierungseffizienz) könnte die Identifizierung weiterer Gene und Genprodukte (wahrscheinlich auch mit antibiotischem Phänotyp) möglich sein. So wurden in anderen Studien die Antibiotika-produzierende Klone mit der Frequenz von 0,01% beobachtet (Brady and Clardy, 2000; Brady et al., 2001; Gillespie et al., 2002).

Aufgrund der hohen mikrobiellen Diversität im Boden sind die acht Metagenombanken zusammen dennoch nicht abdeckend und liefern eher einen „Schnappschuss“ des entsprechenden Genpools. Daher müsste eine sehr große Anzahl von rekombinanten Klonen auf die gewünschte Aktivität untersucht werden. Insgesamt wurden 1,6 Millionen rekombinante Bakteriophagen-Klone mit durchschnittlichen Insertgrößen zwischen 2,0 und 9,5 kb erhalten. Das entspricht 7,7 Gb klonierter DNA von anonymen Bodenorganismen. Nach einer Schätzung sollte eine Metagenombank mit durchschnittlichen Insertsgrößen von 10 kb etwa 20 Million Klone enthalten, um eine achtfache Abdeckung des Metagenoms von mikrobiellen Bodengemeinschaft zu erreichen (Handelsman, 2005). Metagenombanken dieser Ausmaße konnten aber bisher noch nicht erstellt werden. Nach einer Schätzung enthält ein durchschnittlicher Boden-DNA-Extrakt etwa 500 bakterielle 5 Mb-Genome und 100 pilzliche 20 Mb-Genome, insgesamt etwa 5 Gb von DNA-Sequenzen, das sind mehr als ein 3 Gb-Menschengenom (Cowan et al., 2004). Um Gene von seltenen Vertretern der Gemeinschaft ($<$1%) zu erfassen, soll die Metagenombank etwa 100 bis 1000-fache Abdeckung der Metagenome aufweisen. Für die Klonierung von Bodenmetagenomen entspricht dies etwa 10.000 Gb (Riesenfeld et al., 2004).

Die Auswahl eines geeigneten Vektorsystems für die Herstellung von Metagenombanken ist im Wesentlichen abhängig von der anschließend vorgesehenen Anwendung, wie z.B. Auffinden von einzelnen Genen oder Isolierung großer Biosynthese-Gencluster. So werden einzelne Wirkstoffe in Bakterien oftmals mit Hilfe einer Vielzahl von Enzymen synthetisiert. Deren Gene liegen in Produzenten in der Regel als Operons oder in komplexen Genclustern vor. Die Größe derartiger Gencluster schwankt in Abhängigkeit von der Komplexität des zu synthetisierenden Wirkstoffes zwischen 10 und über 100 kb. Ein Nachteil von verwendetem Zap Express-Vektor ist, dass nur relativ kleine (bis zu 12 kb) DNA-Fragmente kloniert werden können. Die ermittelten durchschnittlichen Insertgrößen lagen zwischen 2,0 und 9,5 kb. Dies bedeutet, dass für die Isolierung einzelner Gene, wie beispielsweise Hydrolasen mit einer Länge von maximal 1-2 kb (Lorenz et al., 2002), eine Klonierung von Fragmenten dieser Größe ausreichend ist, nicht aber für die Klonierung von größeren Genclustern. Bei der Suche nach kompletten Genclustern, die ganze Stoffwechselwege umfassen, wie beispielsweise Gencluster der Polyketidsynthasen, die oft größer als 100 kb sind, wären dagegen Fragmentgrößen von mehreren Dutzend Kilobasen erforderlich (Lorenz et al., 2002). Es wurden bereits Cosmid-Genbanken aus Umweltproben mit DNA-Fragmentgrößen von ca. 30 kb angelegt. Dadurch gelang es, neuartige Biotinbiosynthese-Operons und Antibiotika (Violocein, Long-Chain N-acyltyrosin) in Cosmid-Genbanken zu identifizieren (Entcheva et al., 2001). Die Nutzung von Fosmid- (F-Faktor-basierenden Cosmiden) oder BAC-Vektoren (*bacterial artificial chromosomes*) erlaubte die Klonierung von Umwelt-DNA-Fragmenten von mehr als 40 kb und hat zur Identifizierung bisher unbekannter neuartigen Stoffwechselwege und Antibiotika (z.B. Turbomycin) geführt (Stein et al., 1996; Beja et al., 2000; Rondon et al., 2000).

Durch Verwendung verschiedener Wirtsstämme für die Expression könnten Gene gefunden werden, die zu anderen phylogenetischen Gruppen gehören. In den meisten publizierten Arbeiten auf dem Gebiet der Metagenomik wurde *E. coli* als Wirtsorganismus eingesetzt. Ein erstes erfolgreiches Screening von Metagenombanken mittels Expression antibakterieller Aktivitäten, das zur Isolierung eines neuen Antibiotikums, wie z.B. Turbomycin A und B führte (Brady et al., 2001; MacNeil et al., 2001; Gillespie et al., 2002) zeigte, dass *E. coli* zur heterologen Expression zumindest kleinerer Biosynthese-Gencluster grundsätzlich geeignet ist. Im Vergleich zu Wirtsorganismen wie *Bacillus* oder *Streptomyces* bietet *E. coli* den Vorteil, dass weniger stringente Bedingungen bezüglich der Promotorerkennung und der Translationsinitiation vorliegen (Lorenz et al., 2002). Das Potenzial des funktionellen Screenings wurde in einer Arbeit mit einer genomischen BAC-Genbank von *B. cereus* in *E. coli* analysiert. Es wurde gezeigt, dass nur 6 von 10 untersuchten Aktivitäten in *E. coli* detektierbar waren (Rondon et al., 1999). Zum Beispiel war Cereolysin von *B. cereus* sehr schwach in *E. coli* exprimiert. Es zeigte aber in *B. subtilis* nach Subklonieren in einen

bifunktionellen Plasmid-Shuttlevektor die gleiche hämolytische Aktivität wie des *B. cereus*-Donorstammes (Kreft et al., 1983).

Allerdings ist es mit *E. coli* als Expressionswirt nicht möglich, die Gesamtheit der physiologischen Diversität einer Mikrobengemeinschaft darzustellen. Nicht alle Proteine werden exprimiert, einige sind in *E. coli* nicht aktiv oder sogar toxisch. Genexpression und Proteinsynthese im heterologen Wirt werden neben Erkennung von Genregulationssignalen, Transkription und Translation auch von korrekter Proteinfaltung, posttranslationalen Modifizierungen und Prozessierungen oder Proteinsekretion und proteolytischer Aktivierung beeinflusst (Makrides, 1996; Georgiou and Segatori, 2005; Lorenz and Eck, 2005; Terpe, 2006). Darum ist es sinnvoll neben *E. coli* andere Wirtsorganismen zur Expression und zum Screening von Metagenombanken zu nutzen. Die rekombinante Biosynthese von Sekundärstoffen in bekannten hochaktiven Wirkstoffproduzenten (*Streptomyces*, *Bacillus, Pseudomonas*) ist erfolgversprechender, weil aufgrund des stark ausgeprägten Sekundärmetabolismus die Prozessierung von Enzymen, die Bereitstellung von Grundbausteinen für die Naturstoff-synthese sowie geeignete Export- oder Resistenzmechanismen zum normalen Stoffwechsel-repertoire dieser Wirtsstämme gehören (Li et al., 2004). Die Verwendung verschiedener Wirte beim Screening erweitert das Expressionspotential. Beispielsweise wurde das neue Antibiotikum Terragin durch Expression von Metagenombanken in S. *lividans* isoliert (Wang et al., 2000).

Der Einsatz unterschiedlicher Expressionswirte wird durch die Verwendung von Shuttle-Vektoren ermöglicht. Hier wird auf Vektorsysteme gesetzt, die einerseits die effiziente Herstellung komplexer Metagenombanken in *E. coli* erlauben, aber andererseits die Expression dieser Banken in verschiedenen Expressionswirten wie z.B. *Streptomyces, Mycobacterium, Pseudomonas, Rhizobium* oder in einem grampositiven Organismus wie *Bacillus* oder sogar in Archea wie *Sulfolobus solfataricus* ermöglichen (Jonuscheit et al., 2003). Für die Expression von Biokatalysatoren und niedermolekularen Substanzen wie Antibiotika gelten auch die Pilze (z.B. *Penicillium, Aspergilus, Ashbya gossipii*) als Industriestandard (Stahmann et al., 2000). Mit Hilfe solcher Ansätze kann die Expression neuartiger Naturstoffbiosynthese-Gencluster optimiert bzw. die Wahrscheinlichkeit der Entdeckung neuer Aktivitäten oder Substanzen erhöht werden (Li et al., 2005).

Ein möglicher Ansatz wäre z.B. das Anlegen von Metagenombanken in einem Shuttle-Cosmidvektor wie das in der vorliegenden Arbeit neu konstruierte Shuttle-Cosmid pSB 681 (Kucheryava et al., Anhang 7.1.2). Dieser Vektor wurde entwickelt, um große DNA-Fragmente von 30-40 kb zu klonieren und sowohl in *E. coli* als auch in *Bacillus* Wirtsstämmen exprimieren zu können. Mit Hilfe dieses Shuttle-Vektors wurde eine Cosmid-Genbank von dem wirkstoffproduzierenden Umweltisolat *B. subtillis* A1/3 mit durchschnittlichen Insertgrößen von ca. 34 kb angelegt. Die angelegte Genbank bietet

Zugang zu den ganzen Genclustern für Wirkstoffbiosynthese von *B. subtilis* A1/3 und kann sowohl in *E. coli* als auch in *B. subtilis* vermehrt werden kann. Beispielsweise wurde das komplette Gencluster für die Synthese des antifungalen Lipopeptids Bacilomycin L als Cosmidklon identifiziert (Hofemeister et al., 2004, Anhang 7.2.4). Der hier konstruierte Shuttle-Cosmidvektor kann auch für Anlegen von Metagenombanken aus Umweltproben eingesetzt werden. Auf diese Weise konstruierte Metagenombanken können in geeignete, gut etablierte *Bacillus*-Wirte transferiert werden, die dann auf die Bildung und Expression der gewünschten biologisch aktiven Substanzen und Enzyme hin getestet werden können.

3.4. Auffinden und Expression von eukaryotischen Genen

Bei der Klonierung von Metagenomen natürlicher Habitate werden Genprodukte aus eukaryotischen Organismen wie Hefen und Pilze normalerweise nicht erfasst. Selbst wenn Metagenombanken die klonierte DNA aus Eukaryoten enthalten, werden sie mit den aktivitätsbasierten Screeningverfahren in bakteriellen Wirtsorganismen nicht detektiert. Die aus Bodenproben isolierten DNA-Extrakte können aber bis zu 60% eukaryotische DNA enthalten (Gabor et al., 2003).

Die überwiegende Mehrheit der eukaryotischen Gene beinhaltet neben den Protein-kodierenden Sequenzen (Exons) ein oder mehrere nicht kodierende Introns, die von Prokaryoten nicht prozessiert werden können. Für die erfolgreiche Expression eukaryotischer DNA wäre daher die Isolierung von bereits prozessierter mRNA und anschließende Klonierung von cDNA oder die Verwendung von Shuttle-Vektoren und Expressionssystemen vom Typ der YAC-Vektoren (*yeast artificial chromosomes*) in Hefen (*Saccharomyces*) und Pilzen (*Penicillium, Aspergilus, Ashbya gossipii*) als eukaryotischen Wirtsorganismen sinnvoller. Obwohl solche Ansätze erfolgreich bei der Untersuchung von eukaryotischer Genexpression angewendet werden, wird die Analyse von mRNA aus mikrobiellen Gemeinschaften bisher noch kaum genutzt (Lamar et al., 1995; Wilson et al., 1999).

Allerdings wurden die bisher entwickelten Methoden zur RNA-Isolierung aus den Umwelt-proben insbesondere bei phylogenetischen Untersuchungen angewendet (Frischer et al., 2000; Griffiths et al., 2000). So wurde bereits die Verwendung von 16S rRNA zur Untersuchung von mikrobiellen Populationen eingesetzt (Yakimov et al., 2001), um die Diversität von metabolisch aktiven Mikroorganismen in einem Habitat zu charakterisieren. Auch nach Extraktion von mRNA konnte die Expression bestimmter Gene in unterschiedlichen Bodenhabitaten nachgewiesen werden (Chandler et al., 1997; Rogers et al., 2002). Die Isolierung von eukaryotischer mRNA aus Umwelthabitaten und Erstellung von cDNA-Umweltgenbanken ist eine gute Möglichkeit der Identifizierung von eukaryotischen

Genen, um das metabolische Potential von eukaryotischen Organismen nutzen zu können. Entscheidend dafür ist insbesondere die Isolierung reiner und intakter RNA nach effektiver Lyse der eukaryotischen Zellen in den Umweltproben. Überhaupt sind die verwendeten Methoden zur Zelllyse in Umweltproben überwiegend auf prokaryotische Mikroorganismen ausgerichtet. Bei allen großen Metagenomik-Sequenzierungsprojekten wurde DNA aus Umweltproben durch chemische Extraktionsmethoden gewonnen, die doch nur ein Teil der Organismen erfassen. Die Lyseeffizienz in Bezug auf Pilze wird insbesondere durch den Einsatz von Glasperlen und Schüttelhomogenisatoren (*bead beating*) verbessert. So ist z.B. das in der vorliegenden Arbeit (Kucheryava et al., 2008, Anhang 7.2.5) verwendete FastPrep® Instrument vielverprechend.

3.5. Genexpression von Apfelschorferreger während der morphogenetischen Differenzierung in einem *in vitro* Infektionsmodell und in der mit *V. inaequalis* infizierten Pflanze

Im Rahmen dieser Arbeit wurde der Ansatz von FastPrep® FP120 Instrument (Bio101 Savant Instruments Inc., Holbrook, NY, USA) verwendet, um mRNA aus gesunden und infizierten Apfelblättern oder aus im Zellophan wachsenden Myzel des Pilzes *V. inaequalis* zu isolieren. Dieser Ansatz hat sich als sehr effizient erwiesen, um Myzel und Pilzsporen sowohl im Pflanzengewebe als auch im Zellophan zu lysieren und deren mRNA dann zu extrahieren und cDNA im Expressionsvektor Lambda ZAP-Express zu klonieren. Die hergestellte cDNA-Bank wurde genutzt, um molekulare Pathogenitätsmechanismen bei *V. inaequalis* aufzuklären und neue Gene zu isolieren, die bei einer biotrophen Infektion in der Pflanze exprimiert werden.

Die Analyse der differenziellen Genexpression von *V. inaequalis* ermöglichte die Identifizierung von für Pathogenese relevanten Genen, die spezifisch während der Infektion exprimiert werden. Zwei der identifizierten Gene, *cin1* und *cin3*, werden in der infizierten Pflanze sehr stark (mehr als tausendfach) induziert. Sie kodieren für hypothetische Proteine, deren Funktionen bisher unbekannt sind. Somit ist es erstmalig gelungen, bestimmte Gene von *V. inaequalis* zu identifizieren, die für die Besiedlung, Ausbreitung und Infektion in der Pflanze von Bedeutung sein könnten.

Mit Hilfe der Genomanalyse und Analyse der Genexpression von pathogenen Mikroorganismen ist es möglich, neue Virulenz-assoziirte Gene zu identifizieren, Mechanismen der Pathogenität zu entdecken und charakteristische Sequenzen aufzuspüren, die eine Bedeutung für die Diagnostik erlangen können. Pathogene Mikroorganismen produzieren bestimmte Genprodukte, sogenannte Virulenz- oder Pathogenitätsfaktoren, mit deren Hilfe sie Wirtsstrukturen schädigen oder ihre Wirte töten können. Viele dieser

Pathogenitätsfaktoren wie Adhäsine, Toxine, Eisenaufnahmesysteme, Kapseln oder Moduline sind inzwischen analysiert worden, wobei immer wieder neue interessante Strukturen gefunden werden (Hacker and Heesemann, 2000; Hacker and Dobrindt, 2006). Weiterhin ist bekannt, dass pathogene Pilze, Protozoen, Bakterien und Viren durchaus gemeinsame Pathogenitätsstrategien und Stoffwechsel-Funktionen entwickelt haben, so dass sie optimal an die Lebensbedingungen in den Wirtsorganismen angepaßt sind und die Wirtsabwehr umgehen oder -abwehrfaktoren ausschalten können. Die Genomanalyse und Analyse der Genexpression pathogener Mikroorganismen bringt unser Veständnis auf die molekularen Grundlagen der Infektionsprozesse erheblich weiter voran.

Die vorliegende Arbeit zeigt Möglichkeiten auf, die genetische und metabolische Diversität von in der Umwelt vorkommenden prokaryotischen als auch eukaryotischen Mikroorganismen zu erforschen und deren metabolisches, antagonistisches oder pathogenes Potential zugänglich zu machen. Die in dieser Arbeit verwendeten Strategien und Techniken zur Darstellung der mikrobiellen Diversität in ausgewählten Umwelthabitaten ermöglichen einen ersten Zugang für die Erforschung eines vermutlich äußerst diversen Bereiches der Mikroorganismenwelt, die derzeit mit sehr großer Intensität und immer effizienteren experimentellen und bioinformatischen Methoden erschlossen wird. Das rasant wachsende Wissen um die Diversität der Mikroorganismen in der Umwelt, neue Kultivierungsmethoden und kultivierungsunabhängige molekularbiologische Metagenomikansätze, die Fortschritte der heterologen Expression, Sequenzierungstechnologien und Bioinformatik lassen die Zukunft der Umweltforschung verheißungsvoll erscheinen.

4. ZUSAMMENFASSUNG

Die große Mehrheit der in der Umwelt vorkommenden Mikroorganismen lässt sich nicht isolieren und unter Laborbedingungen kultivieren, daher bleibt der beträchtliche Anteil der unkultivierbaren mikrobiellen Populationen unerschlossen. Durch die Entwicklung von kultivierungsunabhängigen Methoden wird unser Wissen über die tatsächliche Vielfalt der Mikroorganismen ständig erweitert. In dieser Arbeit wurden verschiedenen Methoden etabliert, um die Diversität von mikrobiellen Gemeinschaften aus Umwelthabitaten wie Boden und Phyllosphäre von Apfelanlagen zu ermitteln und deren metabolisches, antagonistisches bzw. pathogenes Potential zugänglich zu machen.

Eine Sammlung von 280 epiphytischen Bakterien-, Hefe- und Pilzisolaten aus der Apfel-phyllosphäre wurde auf phänotypische Diversität untersucht. Das antagonistische Potential einiger Mikroorganismen wurde gegenüber dem Apfelschorferreger *Venturia inaequalis* ermittelt. Diversität und taxonomische Einordnung der antagonistischen Bakterienisolate wurde aufgrund phänotypischer Charakteristika und chemotaxonomisch, durch Polyamin- und Fettsäurenanalyse, aufgeklärt. Polyamine wurden auch als Biomarker für die Analyse der taxonomischen Diversität von 91 Stämmen aus 30 Taxa von pflanzenpathogenen und pflanzenassoziierten Mitglieder der *Enterobacteriaceae* verwendet.

Die mikrobielle Diversität im Boden einer naturbelassenen Apfelanlage wurde durch Anwendung der Metagenomik-Strategie untersucht. Die PCR-Analyse und Expressions-klonierung der aus Bodenproben extrahierten metagenomischen DNA ermöglichte die Identifizierung von Genen und den Nachweis von Mikroorganismen ohne deren vorherige Kultivierung. Die phylogenetische Diversität der mikrobiellen Gemeinschaft wurde durch Analyse der 16S-rRNA-Gensequenzen in der metagenomischen Boden-DNA untersucht. Bakterien verschiedener phylogenetischer Taxa, einschließlich bekannte *Bacillus* spp. und überwiegend unkultivierbare Mikroorganismen, wurden detektiert. Insgesamt acht Metagenombanken mit durchschnittlichen Insertgrößen von 4,8 kb (zwischen 2,0 und 9,5 kb) wurden angelegt. Sie entsprechen insgesamt etwa 7,7 Gb klonierter metagenomischer DNA. Die Biosynthese von Hydrolasen und antimikrobiellen Wirkstoffen durch *Bacillus* spp. wurde durch genspezifische PCR-Amplifizierung sowie direkte Klonierung und Expression der Metagenombanken nachgewiesen. Mit Hilfe der angewandten Methoden gelang auch die Identifizierung neuartiger Gene, deren Sequenzen nur geringe Ähnlichkeit zu bereits bekannten Proteinen aufwiesen.

Für die Identifizierung der Gencluster für Wirkstoffsynthesen von einem besonders aktiven antagonistischen Umweltisolat *B. subtilis* A1/3 wurde genomische Genbank in einem neu konstruierten *E. coli-Bacillus*-Shuttle-Cosmidvektor hergestellt. Diese Genbank wurde für Auffinden der Gene für die Synthese eines antifungischen Wirkstoffes der Iturin-Gruppe genutzt und ermöglichte die Identifizierung des kompletten Genclusters (16,5 kb) für die Biosynthese von Bacillomycin L.

Für die Identifizierung und Expressionsanalyse von eukaryotischen Pathogenese-relevanten Genen wurde eine cDNA-Genbank aus einem *in vitro* etablierten Infektionsmodell oder aus mit *V. inaequalis* infizierten Pflanzen angelegt. Zwei bisher unbekannte potentiell für die Pathogenese relevante Gene, *cin1* und *cin3*, wurden identifiziert, die für neuartige Proteine mit bisher unbekannter Funktion kodieren und im Pathogen spezifisch während der biotrophen Infektion in der Pflanze exprimiert werden.

5. ABSTRACT

The vast majority of microorganisms existing in nature cannot be isolated and cultivated by standard techniques, hence the tremendous microbial variety in nature remains largely undetected. Development of molecular techniques for culture-independent approaches will circumvent the limitations of cultivation and extend our view of real microbial diversity. Various methods have been applied in this study to explore the entire diversity of microbial populations in the given habitats, soil and phyllosphere of apple trees, and to access to their metabolic, antagonistic or pathogenic potential.

A collection of 280 epiphytic bacteria, yeasts and fungal isolates from the phyllosphere of apple trees was investigated for phenotypic diversity. The antagonistic potential of these microorganisms against the apple scab pathogen *Venturia inaequalis* was demonstrated. The taxonomic diversity of antagonistic bacteria isolates was shown by phenotypic characteristics and the species identification was confirmed chemotaxonomically by polyamine and fatty acid analysis. Polyamines were also used to analyse the diversity and taxonomy of 91 strains from 30 taxa of soft rot pathogens and plant-associated members of the *Enterobacteriaceae*.

The diversity of the microbial community in a soil habitat of a natural apple orchard was investigated by cultivation-independent techniques using the metagenomic strategy. The PCR and expression cloning of metagenomic DNA extracted from soil samples made possible the identification of genes and microorganisms without their previous cultivation. The extracted metagenomic DNA of the microbial soil community was examined for phylogenetic diversity by sequence analysis of 16S rRNA genes. Bacteria of different phylogenetic groups including a majority of unidentified and uncultivated microorganisms were detected. Eight metagenomic libraries with averaged DNA inserts of 4.8 kb (in the range of 2.0 to 9.5 kb) were constructed. These harbored approximately 7.7 Gb of cloned metagenomic DNA. The biosynthesis of hydrolases and antimicrobial compounds by *Bacillus* spp. was proven by gene-specific PCR amplification and by direct cloning and expression of metagenomic libraries. The application of these methods enabled also the detection of novel genes which probably originate from hitherto unknown microorganisms.

A genomic cosmid library of an environmental isolate *B. subtilis* A1/3 with a broad antimicrobial activity spectrum was constructed to access the genes involved in the production of secondary metabolites. For cloning of this library an *E.coli-Bacillus* shuttle cosmid vector was constructed, that was useful in both host bacteria. The constructed cosmid library was used for the isolation of targeted genes implicated in the synthesis of an antifungal iturin-like lipopeptide antibiotic resulting in successful identification of the complete bacillomycin L gene cluster (16.5 kb).

For the discovery and expression analysis of the eukaryotic pathogenesis-related genes, a cDNA library was prepared from an *in vitro* established infection model or from plants infected with *V. inaequalis*. Two up-regulated fungal, hitherto unknown, potentially pathogenesis-related genes, *cin1* and *cin3*, were identified which encode the putative proteins with as yet unknown functions and are specifically expressed in the pathogen during biotrophic infection.

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Metagenomic expression libraries from soil DNA for accessing the diversity of a soil microbial community and new genes related to antibiotic and hydrolytic activities

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ABSTRACT

In order to access the genetic and functional potential of soil microorganisms culture-independent cloning techniques are increasingly explored. Here we constructed the metagenomic expression libraries with DNA directly extracted from soil of a natural habitat. The total genomic DNA from this soil microbial community, the source material for the metagenomic libraries construction, was examined for phylogenetic diversity by sequence analysis of 16S rRNA genes and was found to be from diverse bacterial species and most closely related to as yet uncultured organisms. The gene-specific PCR was used to probe this soil community for genes related to specific strain antimicrobial activities. The targeting of ericin and bacillomycin antibiotic genes of a distinct *Bacillus subtilis* has identified identical genes in this natural soil habitat.

The eight unamplified metagenomic libraries collectively comprised approximately 1.6 x 10^6 independent clones and harboured approximately 7.7 Gb of anonymous soil DNA in lambda phages. DNA inserts detected in 27 randomly chosen clones averaged 4.8 kb (range from 2 to 9.5 kb) in size. Initial functional screening of two libraries identified several phage clones that express heterologous genes from the inserts confirming that the ZAP Express vector can be used to maintain, express and analyse extracted soil DNA. The phenotypes expressed by these clones include glucanolytic and proteolytic activities detected by activity screening on lichenin, carboxymethylcellulose and gelatine as substrates. Two DNA inserts conferring either cellulolytic or proteolytic phenotypes after excision and subcloning into phagemids in *E.coli* were identified to carry genes of the endo-1,4-beta-glucanase or intracellular serine protease type, respectively. Other *E.coli* subclones revealed a number of putative genes without significant similarities to known sequences in the database. Many of these clones probably originated from uncultivated bacteria. Thus, the constructed metagenomic libraries are providing access to the genetic information of uncultured soil microorganisms and can serve as basis of new initiatives to conduct genomic studies. They link functional information about the soil microbiota dominated by microorganisms which are refractory to cultivation.

INTRODUCTION

Soil is one of the richest habitats, the main reservoirs of microbial diversity on Earth (Buckley and Schmidt, 2002). This potential resides on its extreme spatial heterogeneity and multiphase nature dominated by the soil solid phase. The architecture of the soil that is the

spatial arrangement of solids, pores, liquids, gases and solutes results in a wide variety of microbial niches with different suitable conditions for microbial growth (Ritz et al., 2003; Daniel, 2004, 2005). Microhabitats for soil microorganisms include the surfaces of the soil aggregates, as well as of the complex pore spaces between and inside the aggregates. Thus microorganisms are highly aggregated and adapted to various habitats in soil (Foster, 1988; Hassink et al., 1993; Ranjard and Richaume, 2001; Sessitsch et al., 2001). Because the microbial diversity in soils exceeds that of other environments and is far greater than that of eukaryotic organisms, soil microbial communities are among the most difficult communities to study. One gram of soil can contain up to 10 billion bacteria and hundreds of kilometres of fungal hyphae of possibly thousands of different prokaryotic and hundreds of fungal species (Rosello-Mora and Amann, 2001; Torsvik and Øvreås, 2002; Ritz et al., 2003).

The genetic complexity of microbial soil communities has been estimated by reassociation of community DNA isolated from various soil samples. According to such data, the soil community size has been estimated from 2,000 to 18,000 prokaryotic genomes per gram of soil (Torsvik et al., 1996, 1998; Doolittle, 1999). In contrast, the genomic complexity recovered by culturing methods was less than 40 bacterial genomes per gram of soil (Torsvik et al., 1998; Øvreås, 2000). The global fungal species richness was ‘conservatively’ estimated at 1.5 million species, alternative estimates ranging from 0.5-9.9 million (Hawksworth, 2001) and according to ITS-based estimates ranged from 3.5 to 5.1 million species (O’Brien et al., 2005). The number of known fungi species is estimated as at least 80000 (Kirk et al., 2001; Hawksworth, 2004). Only about 6720 prokaryotic species have been described (cultivated) and are available in the culture collections. That is because only about 0.1 to 1% of microbes are cultivable with current standard cultivation techniques (Torsvik et al., 1990; Amann et al., 1995; Torsvik and Øvreås, 2002). Mostly prokaryotes and many fungi (especially the symbiotic arbuscular mycorrizas, saprophytic basidiomycetes, rust and smut pathogens) are largely uncultivable. Consequently, the diversity of soil microbial communities remains still unexplored.

The application of cultivation-independent molecular approaches involving sequencing of ribosomal DNA fragments has accelerated the identification of uncultured microorganisms and the discovery of diverse new taxa. It has revolutionized the view of microbial diversity and revealed that the uncultured majority is highly diverse. About 41 new phyla have been added after environmental 16S rRNA gene sequencing from a wide range of habitats. Today, about 53 bacterial phyla were delineated and most are dominated by uncultured organisms. Among those contain 27 phyla cultivated microorganisms. 26 are candidate phyla, only known from DNA sequences, not containing any cultured members (Rappé and Giovannoni, 2003; Keller and Zengler, 2004). According to recent data, about 75% of deposited 16S rRNA sequences are from uncultured prokaryotes (Riesenfeld et al., 2004).

Methods based on rRNA gene sequences provided extensive information about the diversity of microbial populations, about the taxa and species present in an environment, but it does not reveal the functional role of the different microbes within the community and the genetic information they contain. Metagenomics is an alternative approach to accessing the 'metagenome' – the collective genomes of a microbial community (Handelsman et al., 1998). Metagenomic analysis provides some DNA sequence as well as functional information through sequences analysis and expression of traits. This involves the sequence-based or function-based approach after isolating DNA from an environmental sample, DNA cloning into a cultured host organism using a suitable vector, screening of clones for phylogenetic markers or for other genes by hybridization or PCR, or for expression of specific traits such as enzyme activity or production of antibiotics, or they can be sequenced randomly. Diverse activities have been discovered by functional analysis of soil metagenomic libraries such as new antibiotics (Wang et al., 2000; Brady et al., 2001, 2002, 2004; MacNeil et al., 2001; Gillespie et al., 2002), hydrolytic and degradative enzymes (Henne et al., 1999, 2000; Rondon et al., 2000; Knietsch et al., 2003; Voget et al., 2003), biosynthetic functions (Entcheva et al., 2001; Knietsch et al., 2003), antibiotic resistance genes (Courtois et al., 2003; Riesenfeld et al., 2004), or membrane proteins (Majernik et al., 2001). Some of the identified genes were extraordinary and novel and illustrated the power of functional metagenomics. Extracting the full value of metagenomic libraries requires applying functional, sequence and phylogenetic analysis (Handelsman, 2004).

The goal of this study was to study the accessibility of the genetic diversity in a natural habitat by direct cloning of DNA extracted from soil and to test the potential of the prepared soil metagenomic libraries to analyse their genetic contents. The DNA was prepared using different direct lysis methods and metagenomic libraries were constructed in *E.coli* using the high-efficiency Lambda Zap Express cloning vector. The phylogenetic diversity of the extracted soil DNA was assessed by sequence analysis of 16S rRNA gene clones. The potential for encompassing genes of functional relevance was investigated by sequence-driven approach using target gene-specific PCR to probe for microorganisms with specific metabolic capabilities or by functional screening of metagenomic libraries for the presence of genes conferring biological activities.

MATERIALS AND METHODS

Soil sample collection

Samples were collected in autumn 1999 and spring 2000 from soil areas of apple trees between agricultural fields of the Institute of Plant Genetics and Crop Plant Research (Gatersleben, Germany). Three 2-g subsurface soil samples were taken from each apple

tree with a sterile scalpel at a depth of 2 to 5 cm and placed in sterile, preweighed 15-ml polypropylene tubes containing 4 ml of phosphate-buffered saline (PBS: 10 mM $NaHPO_4$ [pH 7.4], 137 mM NaCl, 2 mM KH_2PO_4, 3 mM KCl) and one without PBS. These tubes were kept on ice and processed in the laboratory within 3 h. Upon return to the laboratory samples were adjusted to 2 g of sediment each as necessary by removing portions with a sterile scalpel.

Extraction of nucleic acid from soil

Nucleic acid was directly extracted from soil samples with use of a commercial FastDNA® SPIN Kit for Soil (Bio 101), except for the addition of vortexing the soil samples for 3 min in the presence of 0.1-mm diameter glass beads instead of bead beating, and by four different laboratory-devised direct soil DNA extraction procedures based on the *in situ* lysis of microorganisms in soil prior to DNA recovery and purification. Soil samples were suspended directly in lysis buffer and successively treated by four extraction procedures with various combination of physical disruption, chemical and enzymatic lysis, according to Zhou et al. (1996) with enzymatic and chemical lysis; Porteous et al. (1997) with ultrasonication and chemical lysis; Kuske et al. (1998) with freeze-thawing and chemical lysis or Edgcomb et al. (1999) with grinding under liquid nitrogen, freeze-thawing, enzymatic and chemical lysis. The purified soil DNA extracts from each procedure were pooled prior to PCR amplification and the cloning experiments to obtain representative extracts of nucleic acids from the entire microbial community and to reduce biases in DNA extraction from soil mainly due to incomplete cell lysis and DNA sorption at soil particles.

Purification of soil DNA samples

Crude soil DNA extracts were alternatively further purified on spin-filter using a commercial FastDNA® SPIN Kit for Soil (Bio 101), according to the manufacturer's instructions or by two rounds of ultrafiltration using Microcon-100 micro-concentrators (Amicon) or after caesium chloride-ethidium bromide density gradient ultracentrifugation (80,000 rpm for 20 h at 20°C). The purified DNA was recovered from gradient by butanol extraction and isopropanol precipitation according to standard protocol (Sambrook et al., 1989), but with a particular care to avoid mechanical shearing. The purified soil DNA extracts from each procedure were pooled prior PCR amplification and the cloning experiments in order to reduce sample number.

Size fractionation and cloning of soil DNA

The purified genomic environmental soil DNA was prepared for ligation by partial restriction digestion with the restriction enzymes *Eco*RI, *Bam*HI or *Sau*3AI. Spermidine was added to a final concentration of 4 mM to improve the digestibility of high-molecular-weight DNA. The

DNA fragment sizes were determined by standard electrophoresis or pulsed field electrophoresis on 0.8 or 1% agarose gel in 0.5 x TBE buffer for 10-24 h at 14°C, 170 V (6 V/cm) with 5-15 sec pulse time. In order to avoid cloning of very small DNA fragments, the digestion products were electrophoretically separated and restriction DNA fragments of >5 kb were excised from the gel and purified with QIAexII gel extraction kit (Qiagen) and then ligated with the *Eco*RI- or *Bam*HI-predigested Zap Express vector (Stratagene) by using T4 DNA ligase (Roche) according to the manufacturer's protocol. Recombinant lambda phage DNA was packaged by using Gigapack III Gold packaging kit (Stratagene). The cloning efficiency, the titer and fraction of phage containing inserts were determined by plaque assay with blue-white colour selection in the presence of Xgal and IPTG. The libraries were amplified according to the manufacturer's instructions and stored at -80°C.

Phylogenetic analysis of extracted DNA from soil by PCR amplification of 16S rRNA genes

Usefulness of the soil DNA extraction procedure for community analysis was tested by PCR amplification of rRNA gene fragments of several phylogenetic groups representing different components of the soil microbial community. Purified total DNA was used as template for PCR amplification of 16S rRNA genes. Five oligonucleotide primer sets were used for PCR that amplified either any bacterial genes coding for 16S rRNA genes (P3MOD, PC5B) or those of specific *Bacillus* species and relatives (BacF, BacR), high-G+C gram-positive bacteria (ActinoF, ActinoR), *Streptomyces* species and related taxa (StreF, StrepR) or fungi (ML7, ML8) with product size of 723, 600, 542, 1243 and 735 bp, respectively (described by Kuske et al., 1998).

PCR mixtures contained 50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 0.001% (wt/vol) gelatine, 2 mM each of the four deoxynucleoside triphosphates, 1 µM (each) oligonucleotide primer, 2.5 U of *Taq* DNA polymerase and 1 pg to 100 ng of template DNA in a total reaction volume of 100 µl. A negative control contained same mixture but no template DNA. PCR assays were conducted using the following cycling conditions: 2 min at 94°C; 35 cycles, with 1 cycle consisting of 1 min at 55°C, 1 min at 72°C and 30 sec at 94°C; 1 min at 55°C; and 5 min at 72°C.

In order to obtain a representative collection of 16S rDNA clones, the total DNA extracts were pooled before PCR from 4 samples obtained by different methods. Amplified products from four independent reactions using total DNA from soil as template were analysed by agarose gel electrophoresis for correct size, pooled, purified by using a Qiaquick PCR purification kit (Qiagen), ligated into the pGEM-T vector (Promega). This ligation mix was used to transform the *E. coli* host. Clones containing plasmids with inserts were identified by blue-white colour selection on agar plates in the presence of Xgal and IPTG. Individual white

colonies were subjected to PCR to ensure that an insert of the correct size was present in each clone, their DNA purified by QIAquick PCR purification kit (Qiagen) and the DNA subjected to sequencing with universal M13 primers. The partial 16S rDNA sequences were compared against those available in the database using BlastN provided by NCBI blast (http://www.ncbi.nlm.nih.gov/BLAST).

PCR-based identification of distinct biosynthetic genes in soil

In order to detect *B. subtilis* functional ericin- and iturin-like gene, e*riS* and *fenF* gene fragments were amplified from the soil DNA with primers designed based on either published (Stein et al., 2002) or unpublished genome sequences of *B. subtilis* A1/3. The size of the amplified DNA was 0.62 kb of a putative ericin and 0.66 kb of a putative iturin-like gene cluster, respectively. PCR amplification was carried out using 1 × *Taq* polymerase buffer (10 mM Tris-Cl [pH 9.0], 2.5 mM $MgCl_2$, 50 mM KCl, and 0.1% Triton X-100), 200 µM deoxynucleoside triphosphates, 100 pmol of each primer (orf1-eriS and fluoFfa-Ffs), 1-100 ng of soil DNA and 2.5 U of *Taq* DNA polymerase in a total reaction volume of 100 µl. Samples were initially denatured at 94°C for 2 min, followed by 30 sequential cycles at 94°C (30 s), 55°C (1 min) for *orf1-eriS* and *fenF* gene primer sets, 72°C (1 min), and a final extension at 72°C for 7 min. PCR products were analysed for correct size of an expected product by agarose gel electrophoresis and ethidium bromide staining. The PCR products were then purified using the QIAquick PCR Purification kit (Qiagen) according to the manufacturer's instructions and cloned using the pGEM-T vector (Promega). The desired inserts of the soil clones were amplified with vector-specific primers (T7, SP6) and sequenced.

Functional screening of soil DNA libraries for enzyme genes

The first was a plaque assay (10^5 pfu per 11 mm diameter NZY agar plate) performed. Plaques were incubated for 6-8 h at 37-42°C before performing overlays by spreading top agar medium containing assay-specific substrates onto the plaques as soon as they were 1 to 3 mm in diameter and incubated at 37°C until zones of phage lysis became visible.

For detecting β-glucanase activities, the plates with plaques of recombinant phages were overlaid with top agar containing 0.7% molten water agarose and either the substrate 0.5% lichenin or 0.5% CM-cellulose (carboxymethylcellulose) (Sigma). After overnight growth the plates were flooded with an aqueous solution of 0.1% Congo Red and incubated at room temperature for 30 min with slow rocking. Then they were washed twice with 1 M NaCl for 30 min. Positive clones were visible by a yellow zone of hydrolysis around the plaque against a red background. For detecting protease activity, plates were overlaid with top agar

containing 0.1% gelatine. A clear halo after flooding plates with saturated $(NH_4)_2SO_4$ solution indicates protease activity.

Amylase activity was detected by overlaying the plaques after overnight growth with top agar containing 0.5% starch followed by flooding plates with KJ solution. Plaques were scored for the presence of a bright orange halo. Phytase activity was assayed by overlaying with top agar containing 0.5% sodium phytate followed by successive flooding plates with 2% aqueous cobalt chloride solution and solution containing equal volumes of a 6.25% ammonium molybdate and 0.42% ammonium vanadate according to Bae et al. (1999). For detecting of amylase, pectinase and chitinase activities the library plates were overlaid with top agar containing 0.2% starch-RBB, amylopectin-RBB or chitin-RBB (soluble substrates covalently dyed with Remazol Brilliant Blue R) and scored after 3 days for the presence of a clear halo. DNAse activity was tested by plates overlayed with Bacto DNase methyl green agar (Difco). Screening for haemolytic activity was done by overlaying of sheep blood agar plates (Ovoid) with NZY top agar for growth of the library phages followed by 2 days of incubation at 28°C in order to obtain haemolysis.

All clones that were positive in an initial screen were retested at least once. Strains of the active phage were purified with two iterations of plaque isolation. The DNA purified from positive single plaque phage clones was *in vivo* excised using the ExAssist helper phage/XLOLR system according to the *in vivo* excision protocol (Stratagene) resulting in the transfer of DNA from positive phage clones onto the plasmid vector. *E. coli* clones containing the pBK-CMV phagemid plasmids were recovered after plating on LB containing kanamycin (50 μg/ml) and screened for enzyme activity by plating onto LB medium containing either 0.5% carboxymethylcellulose, 0.5% lichenin or 0.1% gelatine as substrate along with 15 μl of 50 mM IPTG. After overnight growth the plates were flooded with 0.1% Congo Red or saturated $(NH_4)_2SO_4$ solution. Positive clones were detected by a clear halo of hydrolysis after flooding plates. Plasmid DNA from excised recombinant pBK-CMV phagemid clones was extracted using 'QIAprep Spin Miniprep Kit' (Qiagen) according to the supplied protocol and sequenced with MVL (forward) and MVR (reverse) primers.

Antibacterial and antifungal screening of soil libraries

For antibacterial and antifungal assays, plaques were grown on NZY agar plates (120x120x17 mm) for 6-8 h at 37°C. Plates were then overlaid with 10 ml of TBY soft agar (0.7%) containing 0.5 ml of indicator bacteria, i.e. *Agrobacterium tumefaciens, Clavibacter michiganensis* subsp. *michiganensis* or *Staphylococcus aureus,* grown to an optical density at 600 nm of 0.2 for antibacterial assays or with soft agar containing of 2% malt extract and 0.5 ml of a *Saccharomyces cerevisiae* culture or 50 μl spores suspension (10^6/ml) of *Paecilomyces variotii* for antifungal assay and incubated overnight at 37°C or 28°C and then

at room temperature for several more days. Plaques producing antibacterial or antifungal activities were identified by a zone of inhibition in the bacterial or fungal lawn.

Sequencing and sequence analysis

DNA sequencing was carried out using an automated A.L.F system (Pharmacia) by the sequencing services of the Institute of Plant Genetics and Crop Plant Research (Gatersleben, Germany). Nucleotide and deduced amino acid sequences obtained from environmental clones were compared with GenBank databases of the National Center for Biotechnology Information (NCBI) server using BlastN or BlastX algorithms (http://www.ncbi.nlm.nih.gov/BLAST). Each sequence was submitted to the Check Chimera program of the ARB database to detect the presence of possible chimeric artifacts.

RESULTS

Extraction of DNA from soil

We chose soil from a natural habitat, the areas of apple trees between agricultural fields of the Institute of Plant Genetics and Crop Plant Research (Gatersleben, Germany), which were never treated with chemicals (pesticides or fungicides). To access genomic information from as large a pool of soil microbes as possible, including the uncultivated microorganisms, we extracted the DNA from soil using five different direct lysis methods with various combinations of physical disruption, chemical and enzymatic lysis as described in Materials and methods. The laboratory larger-scale methods (Zhou et al., 1996 and Edgcomb et al., 1999) yielded an amount of DNA that was significantly higher than those obtained with a commercial FastDNA® SPIN Kit for Soil (Bio 101) and the small-scale procedures (Porteous et al., 1997 and Kuske et al., 1998). The caesium chloride density gradient ultracentrifugation efficiently produced relatively pure DNA with high yield.

The purified soil DNA extracts from each procedure were pooled prior to PCR amplification and the cloning experiments in order to obtain representative DNA extracts from the entire microbial community and to reduce biases in DNA extraction from soil mainly due to incomplete cell lysis and DNA sorption to soil particles. Usefulness of the soil DNA extraction method for community analysis was tested by PCR amplification of 16S rRNA gene fragments of several phylogenetic groups representing different components of the soil microbial community, most of them uncultured.

Phylogenetic analysis of extracted soil DNA with a 16S rRNA gene library

The phylogenetic diversity represented in the isolated soil DNA was determined by 16S rRNA gene clone library, which was prepared from the same soil DNA extracts used for

constructing the metagenomic soil libraries. Group-specific primers for *Bacillus* species and relatives, high-G+C gram-positive bacteria, *Streptomyces* species and related taxa as well as primers conserved among all known bacteria or fungi were used to amplify rRNA genes. Amplified PCR products of the correct size were purified and cloned into the pGEM-T vector for establishment of 16S rRNA libraries. About 25 clones were sequenced. The obtained partial 16S rRNA sequences were compared to those available in the database using BlastN provided by NCBI. The majority (16 out 25) of the recovered 16S rRNA gene sequences showed the highest similarities (91-98%) to sequences of uncharacterized microorganisms, the uncultured environmental clones, which are only represented in the database by 16S rRNA sequences (Table 1).

The closest named species were *B. subtilis*, *B. niacini, B. infernus, B. mojavensis, B. litoralis* and *Sphingomonas* sp., with sequence similarities of 96-99%. Despite multiple attempts with the same DNA templates used successfully for amplification of bacterial rRNA genes, the primer sets specific to the fungi rRNA genes gave no PCR product or the amplification products of incorrect sizes. Not all of the bacterial diversity in the soil habitat could be revealed, because a number of 16S rDNA clones found no significant similarity to sequences in the database.

These data proved that isolated soil DNA used for construction of the metagenomic libraries to represent the genomic DNA of many bacterial types, and thus to serve for a valuable source of genes for novel enzymes and biologically active compounds.

PCR-based screening of extracted soil DNAs for ericin and iturin-like genes

In order to link the phylogenetic diversity analysis of our environmental soil DNA to its potential for encompassing genes of functional relevance, we the first used gene-specific PCR to amplify targeted gene sequences from soil DNA preparations. To detect the functional genes of the ericin and iturin-like biosynthetic pathway, known from the natural soil strain *B. subtilis* A1/3, the e*riS* and *fenF* gene fragments were respectively amplified from the different soil DNA preparations with primers based on published (Stein et al., 2002) or unpublished genome sequences of *B. subtilis* A1/3.

The ribosomally synthesized lantibiotic, ericinS, has antibiotic activities against several gram-positive bacteria (Stein et al., 2002). The ericin gene cluster of *B. subtilis* A1/3 is closely related to subtilin genes, but contains two structural genes *eriA* and *eriS* separated by *orf1* of unknown function. In this study for a specific search we used the orf1-eriS primer set for PCR amplification of a lantibiotic gen cluster region, containing the *orf1* and *eriS* genes. These unique nucleotide sequences were indeed obtained from the soil DNA samples. The alignment of the cloned PCR DNA to the NCBI using BlastN and BlastX revealed the identity

to the ericin gene cluster of *B. subtilis* A1/3 (Table 2). Moreover, the identity between the two sequences (clones E2A1, E5A), the two from different soil samples, was about 99% at the nucleotide level to the ericin cluster of *B. subtilis* A1/3.

Table 1. PCR identification of microorganisms in soil samples used the specific 16S rRNA primer*

Clone	Organism which gave best hit (blastn)	Gene bank accession no.	% identity to closest relative	Region of similar nucleotides	Score bits
	Primer pair for *Bacillus* spp. and relatives				
2B	*B. niacini* strain SAFN-019	AY167811	96	316	574
7B	*Bacillus* sp. ROO2A	AY188839	99	514	1006
10B	*B. mojavensis* JF-2	AY436360	99	515	997
12B	*B. subtilis*	AB016721	99	598	1173
19B	*B. subtilis* strain ZJY-116	AY897211	99	476	911
22B	*Bacillaceae* bacterium PH32	AF513474	99	481	940
TB2	*B. infernus* TH-23	BIU20385	96	582	1038
TB3	*B. litoralis*	AY608605	98	593	1134
	Primer for High (G+C) gram-positive bacteria				
1A	Uncultured actinobacterium	COF53574	95	447	745
1A1	Uncultured sludge bacterium A22	AF234701	92	277	385
2A	Uncultured bacterium clone AAE9	AY692242	95	477	788
2A1	Unidentified bacterial species clone mb1228	BSPZ9573	94	396	648
3A	Uncultured bacterium clone LCSA-SUC45	AJ616069	95	325	554
5A	Uncultured actinomycete	AOF55521	99	470	913
21A	Uncultured bacterium clone C1-K35	UBA42119	95	378	639
TA1	Uncultured *Planctomycetales* bacterium clone SM2F01	AF445727	93	324	505
TA3	Uncultured soil bacterium clone ABS-650	AY289480	92	339	476
	Primer for *Streptomyces* spp. and related taxa				
1S	Uncultured *Acidobacteria* bacterium clone BAC-14A1	AY214904	92	315	462
1S1	Agricultural soil bacterium clone SC-I-65	ASO25264	91	358	532
2S1	*Sphingomonas* sp. SIA181-1A1	AF395032	96	238	407
	Primer for bacteria				
1P	Uncultured bacterium clone oc11	AY491561	94	255	433
2P1	Uncultured bacterium clone 1973c-13	AY917793	97	380	711
13P	Uncultured *Acidobacteria* bacterium	UAC58204	96	287	500
15P	Uncultured soil bacterium clone 1213	AY493916	98	366	669
18P	Uncultured Green Bay *Ferromanganous micronodule* bacterium MND1	AF293006	97	473	876

* Primer according to Kuske C.R. et al., 1998, Appl. Environ. Microbiol. 64: 2463.

Table 2. Identified genes and similarities

Clone	Putative gene/ protein	Organism (accession number)	% identity	Region of similar amino acids or nucleotides	Score bits
E2A1	Subtilin-like gene cluster	*B. subtilis* A1/3 (AF233755)	99	594 nt	1162
	Unknown protein	*B. subtilis* A1/3 (AAL15568)	97	115 aa	238
E5A	Subtilin-like gene cluster	*B. subtilis* A1/3 (AF233755)	99	388 nt	754
	Unknown protein	*B. subtilis* A1/3 (AAL15568)	98	116 aa	241
F1A	Malonyl-CoA transacylase, *bmyD*	*B. subtilis* A1/3 (AF499447)	98	652 nt	1259
	Malonyl-CoA transacylase, BmyD	*B. subtilis* A1/3 (AAQ07409)	100	218 aa	430
C1	Endo-beta-1,4-glucanase	*B. subtilis* DLG (BACGLUB)	98	698 nt	1311
	Endo-beta-1,4-glucanase, cellulase (EC 3.2.1.4)	*B. subtilis* DLG (A26874)	97	98 aa	200
G1	Intracellular serine protease	*Bacillus* sp. WRD-2 (AY351917)	96	617 nt	1098
	Intracellular serine protease	*Bacillus* sp. WRD-2 (AAR12277)	98	113 aa	236
G2	Peptidase S1, chymotrypsin: TPR repeat	*Trichodesmium erythraeum* IMS101 (EAO28801)	50	31 aa	59
	UDP-N-acetylmuramoyl-alanyl-D-glutamate-2,6-diamin opimelate ligase	*Rhodopseudomonas palustris* CGA009 (CAE28976)	66	85 aa	171
L1	Similar to solute carrier family 8 (sodium/calcium exchanger)	*Danio rerio* (XP_695594)	42	31 aa	59
	Twin-arginine translocation pathway signal	*Burkholderia cenocepacia* AU 1054 (EAM14696)	49	62 aa	115
L2	Putative ubiquitin carrier	*Entodinium caudatum* (CAB57250)	77	17 aa	40
	TldD/PmbA family protein	*Caulobacter crescentus* CB15 (AK24788)	36	58 aa	99
C3	Hypothetical protein	*Nocardioides* sp. (BAA23266)	59	26 aa	57
C5	Probable N-methyl-hydantoinase B (EC 3.5.2.14)	*Bradyrhizobium japonicum* USDA110 (NP_769967)	41	59 aa	110
	Aerobic-type carbon monoxide dehydrogenase	*Chloroflexus aurantiacus* (ZP_00358607)	74	26 aa	57
C8	3-oxoacid CoA transferase (EC 2.8.3.5)	*Caenorhabditis elegans* (NP_496144)	35	44 aa	76
	VMP3 protein	*Volvox carteri* f. *nagariensis* (CAC39318)	49	25 aa	46
C15	Ubiquitin specific protease 52	*Homo sapiens* (NP_055686)	26	29 aa	32
L23	ABC-type antimicrobial peptide transport system, ATPase component	*Vibrio vulnificus* YJ016 (NP_935155)	65	42 aa	85

One of the various antifungal lipopeptide antibiotics produced by *B. subtilis* A1/3 strain is bacillomycin L, a member of the iturin group. Iturins are cyclic lipopeptides with a peptide moiety and β-amino fatty acid linked by amide bonds to the constituent amino acid residues (Moyne et al., 2001). A *bmyD* gene related to *fenF* (malonyl-CoA transacylase) seems to be indispensable for synthesis of an iturin group antibiotic (Duitman et al., 1999; Tsuge et al., 2001; Hofemeister et al., 2004). In an attempt to recover the *fenF*-linked genes of that antifungal lipopeptide from soil DNA we PCR amplified the 660 bp malonyl-CoA transacylase gene fragment using *fenF* specific primers. The amplified DNA was then cloned (F1A soil clone) and sequenced (Table 2). A BlastX search revealed the highest homology with *BmyD*, the malonyl-CoA transacylases of bacillomycin L of *B. subtilis* A1/3 (100%), as well as similarity to *fenF*-like genes of bacillomycin D of *B. amyloliquefaciens* (99%), *BamD* of *B. subtilis* (98%), and to other lipopeptide iturin-like gene clusters, such as *ItuD* of iturin A (98%) and *FenF* of mycosubtilin of *B. subtilis* (81%). This revealed strong conservation of the malonyl-CoA transacylase genes of the iturin group antibiotics, suggesting the gene a good target for the identification of these antibiotic genes.

Construction of metagenomic soil DNA libraries

We used the extracted soil DNA to construct the metagenomic expression libraries of soil community in a high-efficiency lambda Zap Express cloning vector. This maintains small fragments (up to 12 kb) linked to a *lacZ* promoter and facilitating functional analysis by increasing the chance of obtaining expression of cloned foreign genes. By independent attempts we prepared eight small insert DNA expression libraries. The primary titers were generally 10^5-10^6 pfu. Together, these unamplified libraries consisted of approximately 1.6 x 10^6 independent recombinant phages harbouring approximately 7.7 Gb of environmental DNA. The quality of the libraries was controlled by determination of the average insert size in randomly chosen clones and the percentage of recombinant plaques containing inserts. The libraries revealed average insert sizes of 4.8 kb (ranged from 2 to 9.5 kb). The percentage of plaques containing insert DNA was approximately 84 to 100% (Table 3). All libraries were amplified, in order to screen with several enzyme substrate analogue. The percentage of insert containing plaques of amplified libraries was approximately 75 to 99% (Table 3).

Partial restriction digestion with *Bam*HI was selected to improve constructing other metagenomic soil DNA libraries because initial cloning experiments using *Eco*R1 and *Sau*3A partial digests of soil DNA were less efficient. The reason for this failure was attributed to the contamination of the input DNA. Those coextracted contaminants present in the soil DNA extracts could not be entirely removed during purification and inhibited the restriction endonucleases, but apparently, *Bam*HI was most resistant and gave the higher cloning efficiencies.

Tabele 3. Characteristics of soil metagenomic libraries

Metagenomic library	Enzyme used for cloning	Total number of plaques	% recombinant plaques	Titer of amplified library, pfu/μl	% recombinant plaques of amplified library
1AE	*Eco*RI	0.8 x 10^5	100.0	2.4 x10^5	98.8
1S	*Sau*3A	0.2 x 10^5	93.5	2.9 x10^5	86.2
2S	*Sau*3A	0.3 x 10^5	83.6	1.6 x10^5	75.0
3S	*Sau*3A	0.8 x 10^5	84.9	7.9 x 10^5	75.9
4S	*Sau*3A	0.9 x 10^5	91.7	8.6 x 10^5	93.3
1AB	*Bam*HI	1.9 x 10^5	97.1	6.2 x 10^9	97.8
2BB	*Bam*HI	0.7 x 10^5	97.4	3.7 x 10^9	92.0
3TB	*Bam*HI	1.0 x 10^6	99.6	5.4 x10^9	99.4
BsA1/3E	*Eco*RI	3.0 x 10^5	94.2	3.5 x10^5	94.3

These libraries thereby created an archive of the soil community metagenome for functional studies, and served as source for the isolation of targeted genes. To analyse their genetic content and expression capabilities, two libraries were subsequently screened by appropriate enzymes and secondary metabolites of interest. This strategy has led to the identification of several clones which encode functional genes by expression of some enzyme activities.

Screening for expressed biological activities

Initial screens with one of the small libraries (1AE) for gene expression were started in order to investigate the functional diversity of the metagenomic DNA captured in our libraries by exploring the expression of heterologous DNA. The library clones were screened by plaque assay for various biological activities such as glucanase, protease, amylase, chitinase, pectinase, DNase, phytase and proteins conferring haemolytic, antibacterial and antifungal activities. For this aim the agar plates with plaques were overlaid with top agar containing an assay-specific substrate for detecting the enzyme activity, or overlaid with an indicator organism such as *Agrobacterium tumefaciens*, *Clavibacter michiganensis* or *Staphylococcus aureus* or with *Saccharomyces cerevisiae*, *Paecilomyces variotii* in order to detect antibacterial and antifungal activities, respectively (see Materials and methods).

Finally, we recovered four clones expressing glucanase and two clones with protease activities on lichenin, carboxymethylcellulose and gelatine as substrates. This confirmed efficiency of the strategy used for detection of genes encoding natural products. The larger libraries and those with larger inserts were expected greatly to enhance this chance. Indeed, yielded a screen of an unamplified 3TB library of about 150,000 pfu for a given phenotype, i.e. glucanolytic activity, five positive clones which were quite diverse at the nucleotide sequence level. All phage clones that expressed enzyme activities were purified, the inserts

excised in the form of phagemids, which were used to transform *E. coli* in order to examine their gene expression in a plasmid system and to characterize the inserts.

Characterization of clones with gene expression

Protease-producing clones.

With initial activity-screen of first metagenomic library 1AE for enzyme activity we have identified two clones (G1, G2) that expressed proteolytic activity from library plaques overlaid with top agar containing gelatine (Fig. 1). However only the G1 clone still conferred the protease phenotype after excision of the phagemid DNA. The DNA insert of the G1 clone of approximately 2 kb in size was sequenced and compared to the sequences in the NCBI databases. Sequence analyses revealed the predicted gene encoding an intracellular serine protease which possessed the highest similarities of 96-98% at both the nucleotide and protein level to a gene of *Bacillus* sp. WRD-2 (Table 2). The phagemid containing *E. coli* subclone G2, lacking proteolytic activity, was also sequenced. The deduced protein revealed low sequence similarity (50%) to peptidase S1 encoded by *Trichodesmium erythraeum*. The insert DNA from this clone thus probably originated from an unidentified bacterium.

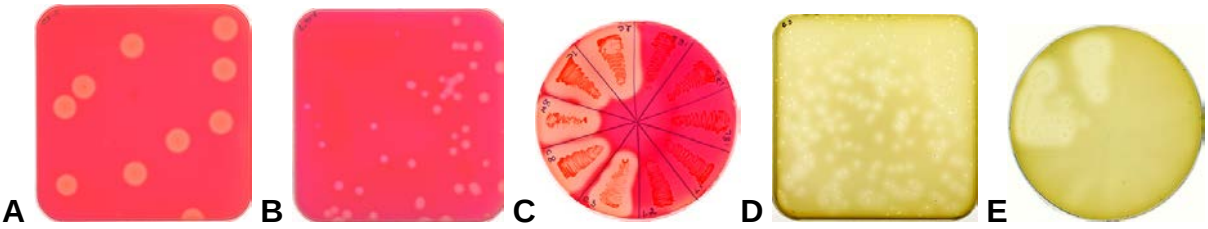

Figure 1. Activity-based plate screening of metagenomic library clones. Cellulase activity of phage clones on agar media containing 0.5% carboxymethyl cellulose (**A**) or lichenin (**B**), and of *E. coli* clones harbouring recombinant phagemids after *in vivo* excision (**C**). Proteolytic activity of phage clones isolated from soil metagenomic library (**D**) and of *E. coli* clones after *in vivo* excision (**E**) detected on agar media containing gelatine by zones of clearance around the colonies.

Cellulase-producing clones.

Activity-based screening of the 1AE library on either carboxymethylcellulose (1,4-β-glucan) and lichenin (1,3-1,4-β-glucan) as assay-specific substrates for detecting glucanolytic activity (Fig. 1) resulted in four positive clones, C1 and C2 onto CM-cellulose (CMC), and L1 and L2 onto lichenin plates. All of these clones however hydrolysed CMC as well as lichenin, with larger zones on CMC plates. After excision the resultant *E. coli* phagemid clones were verified for glucanase activity by plating onto LB medium, containing CMC or lichenin substrate. The clones C1 and C2 retained cellulase activities. Sequencing revealed that the inserts were almost identical (99%), indicating that the same cellulase gene was present in

each, which resulted from duplicate cloning. The highest identity (97-98%) was found to an endo-1,4-beta-glucanase (cellulase) from *B. subtilis* DLG strain at both the nucleotide and protein level (Table 2). Other *E. coli* subclones L1 and L2, not expressing the glucanolytic activity, were also sequenced and although no significant similarities to known nucleotide sequences in the database were apparent, the deduced proteins revealed intermediate sequence identity (36-77%) with database entries, indicating that the insert DNA from these clones probably originated from unknown, hence uncultivated bacteria.

A part of unamplified 3TB library was screened for glucanolytic activity. Within we identified out of 150000 clones five positive (C3, C5, C8, C15, L23) containing inserts of approximately 6 kb in size as estimated by PCR followed by agarose gel analysis. However, no glucanolytic active phagemid *E. coli* subclones were obtained from those positive phage clones. Glucanase activity was not detected in the medium even after cell lysis. The sequences analyses again suggesting that five independently derived clones had been obtained. BlastN analysis of all sequenced ends did not verify any similarity, but the BlastX searches revealed similarities with only low levels of identity (26-74%) to known protein sequences in the databases (Table 2). This again confirms the cloning of DNA from likely yet unknown genomes.

DISCUSSION

The present work describes the construction of metagenomic libraries of DNA which was extracted from a natural soil habitat to explore the genetic and metabolic diversity of its microbial community. The soil DNA samples, the source material for the metagenomic libraries construction, were examined for phylogenetic diversity by sequence analysis of 16S rRNA genes. These data proved the detection of diverse bacterial species and most closely related to as yet uncultured organisms.

Although the 16S rRNA gene analysis provides valuable information about diversity and evolution of microbial populations, it does not reveal the full complexity of prokaryotic diversity and the role of the different microorganisms within the natural population. The 16S gene itself accounts for approximately 0.05% of the average prokaryotic genome and cannot be used to obtain information about the physiology of the microorganism from which it was obtained (Rodríguez-Valera, 2002). Since it has been demonstrated that microorganisms with identical 16S rDNA sequences can have very different overall genomes and display significant differences in physiologies and growth temperature optima (Jaspers and Overmann, 2004; Hahn and Pöckl, 2005). One of the main aims of microbial ecology today is to link the identity of the different microorganisms within a habitat to their activities in it.

We assessed the antimicrobial potential of indigenous community populations by screening metagenomic soil extracts for the presence of antibacterial ericin and antifungal bacillomycin antibiotics of *B. subtilis* using the gene-specific PCR approach. The targeting of such functional genes has identified an environmental *B. subtilis* strain with apparent similarity to strain A1/3, with identity of 99% for the ericin genes and 100% for the bacillomycin genes. Such gene-specific PCR is currently used to probe communities for microorganisms with specific metabolic or biodegradative capabilities as well as in order to detect new enzyme and antimicrobial activities from environmental samples. So far it only accessed genes that are similar to known sequences (McDonald et al., 1995; Watanabe et al., 1998; Hallin and Lindgren, 1999; Sheu et al., 2000). To merely retrieve conserved genes from soil habitats by PCR, the construction of libraries is not a prerequisite. This approach require suitable (often degenerated) primers that are derived from conserved regions of known genes and gene products. This approach, however, is essentially limited to the identification of new members of known gene families. Moreover, often it results in the amplification of only partial genes and requires additional steps (primer/genome walking, hybridization, random primed PCR, etc.) to access the full-length genes (Cowan et al., 2004, 2005). That subsequent recovery of full-length genes from the isolated complex soil DNA by PCR appeared to be difficult, whereas an insert from a metagenomic library that contains the gene sequence of interest, might harbour the full-length gene (Morris et al., 1995). Thus, metagenomic analysis is an appealing alternative and has advantages over PCR-based methods for isolating new genes. It also provides access to uncultured microorganisms, but without prior knowledge of gene sequences offers the chance to recover complete genes (Handelsman, 2004; Streit and Schmitz, 2004; Schloss and Handelsman, 2005).

Such approach has been intended in our study by making eight metagenomic libraries from total DNA extracted from soil using a high-efficiency Zap Express cloning vector. The resulting clones were screened for expression of enzymes and antibiotics. Since, the average size of the structural genes for most enzymes is around 1 to 2 kb, small-insert libraries are useful for the isolation of single genes or small operons encoding metabolic functions such as biodegradation (Tyson et al., 2004; Venter et al., 2005). In contrast, biosynthetic pathways require big gene clusters and hence cloning libraries with larger inserts (Eyers et al., 2004).

With our functional screening approach we have detected various enzyme activities, but no antimicrobial activities. To test the validity of antimicrobial activity screening of these metagenomic libraries, and to optimise the conditions for this activity screen, we first made a genomic library from *B. subtilis* A1/3 strain (BsA1/3E, Tab. 3), a strain with various antimicrobial activities (Hofemeister et al., 2004), in the same Zap Express cloning vector, which was also used for construction of the soil metagenomic expression libraries. This

genomic library was screened by function-based approach for the expression of the lantibiotic ericin which displays antibiotic activity against various gram-positive bacteria (data not shown). Unexpectedly however, no antibacterial activity of library clones was obtained, although a PCR analysis showed that the genes were present. This illustrated the problems involved in expression screening or non-optimal assay conditions.

In another study, enzymatic activity was detected by means of low-level hydrolysis of a fluorogenic chitin analogue by the microtiter assay of excised phagemid clones from the Lambda ZAP library (Cottrell et al., 1999). This, however, failed at the plaque assay. The greater sensitivity of the microtiter assay was likely because the microtiter assay uses intact cells containing excised expression plasmids, whereas cells in plaques have been lysed. In addition to likely higher enzyme production in the microtiter assay with intact cells, it was found that positive clones lysing by sonification, although the effect is analogous to viral lysis, greatly reduced the hydrolysis of chitin (Cottrell et al., 1999).

The use of *E. coli* as the host might also limit the chance to express DNA from soil microorganisms, because the function is not always expressed in this host cell. Recently, the potential success of functional screening for gene expression in a heterologous *E. coli* was analysed using the complete genome sequences from 32 prokaryotes and bioinformatics tools. About 40% of the enzymatic activities were predicted to be readily recovered by random cloning in *E. coli* (Gabor et al., 2004). A range of alternative heterologous hosts, particularly from taxonomic groups that contain few expression signals functional in *E. coli*, made it possible to detect the expression of a larger number of genes from metagenomic libraries. Shuttle cosmid or BAC vectors have been used that allow the construction of soil libraries using the standard host *E. coli*, and subsequently the screening in other hosts which harbour different gene expression machineries, such as *S. lividans* or *P. putida* (Courtois et al., 2003; Martinez et al., 2004), *R. leguminosarum* (Wexler et al., 2005) and *Bacillus* (Handelsman et al., 2002). But there are no reports of soil libraries in eukaryotic hosts. A candidate eukaryotic host would be the yeast *Schizosaccharomyces pombe* and vector derived from the yeast artificial chromosome (YAC) (Pettit, 2004).

Despite these limitations we readily detected by functional screening protease and cellulase enzyme activities from clones in two metagenomic libraries. In this approach we discovered an endo-1,4-beta-glucanase (cellulase) and an intracellular serine protease, both were closely related to known enzymes of *Bacillus* spp. Genes of the *Firmicutes* group were predicted to be most readily expressed in *E. coli*, since they have the largest fraction of independently expressible genes (73%), compared to other taxonomic groups (Gabor et al., 2004). Polymer-degrading enzymes such as proteases, lipases and cellulases are most frequently isolated from *Bacillus* or related species (Rees et al., 2003). Bacilli are biologically

quite active in soil, and appear numerically abundant with standard soil plating methods (Pettit, 2004). *Bacillus* spp. likely therefore predominated in the 16S rRNA gene analysis of our soil DNA samples, and were thus also most frequently identified by gene-specific PCR of soil DNA and by functional screening of the metagenomic soil libraries. It is also likely that the higher relative representation abundance of *Bacillus* DNA reflects the lysis of spores as well as vegetative cells during the isolation of genomic DNA from soil as well, possibly in proportion to their natural abundance. During the course of sequencing we also found putative ORFs with recognisable identity to the variety of other proteins. We identified clones with relatively low similarity to known sequences (26-65%) at the protein level to data base entries, which were lacking any significant identity at the nucleotide level.

These results show that constructed soil-derived metagenomic libraries contain heterologous anonymous soil DNA sequences that can be expressed in *E. coli* at detectable levels. Such we found proteolytic and cellulolytic enzyme activities of clones in two of the libraries, apparently encoded by anonymous DNA. The fact that two of the screened activities were identified by initial screening suggests the method could successfully extract and identify also genes from anonymous soil microorganisms of soil habitats.

So far, the metagenomic libraries are clearly a rich and widely unexplored resource for the identification of novel genes and natural products. The first positive discovery of protease through activity-based screening of soil-derived plasmid library was described by Santosa et al. (2001). A novel alkaline EDTA-sensitive metalloprotease with highest similarity of 30% to an enzyme from starfish was recovered by activity screening of a soil metagenomic library (Gupta et al., 2002). The first successful expression screening of metagenomic libraries constructed with DNA derived from a mixture of cellulose-enrichment cultures of thermophilic anaerobic digesters of lignocellulosic feedstocks recovered two unidentified cellulase genes which encoded an endoglucanase C and β-glucosidase, both of which were distinctly different (28% and 40% identity) from all previously reported homologues (Healy et al., 1995). A number of new cellulase genes with identity of 29-51% were discovered by activity screening of the ZAP Express libraries constructed from cellulose-enrichment cultures of soda lakes, lake sediments and soda soil (Rees et al., 2003, Grant et al., 2004). New cellulase activities were identified in libraries prepared from DNA isolated from enrichment cultures with a higher frequency (fourfold increased) compared to libraries made directly from the total environmental DNA itself (Rees et al., 2004).

The novelty of the biocatalysts discovered by metagenomic approach arises from the enormous genetic and metabolic diversity of uncultured soil microorganisms. In fact, rediscovery of genes is rarely reported, indicating that the soil DNAs cloned and screened up to now represent only a very small portion of the entire genetic diversity found in soil samples

(Daniel, 2004). An ‘average’ soil DNA extract containing, conservatively, about 500 bacterial 5-Mb genomes and about 100 fungal 20-Mb genomes will therefore constitute around 5 Gb of DNA sequence which is rather larger than a human genome (Cowan et al., 2004). According to other conservative estimates the soil metagenomes could constitute between 20 and 2000 Gb of DNA sequences (Cowan et al., 2005). Correspondingly, metagenomics is becoming synonymous with 'megagenomics' - genomics on a massive scale, necessitating large libraries to achieve complete coverage (Handelsman, 2005). About 1 million clones, each containing 50 kb of DNA, would be required in order to provide single-fold coverage of the metagenome of about 10^4 different species in a gram of soil. So far, none of the libraries constructed to date approach this size (Handelsman, 2004).

The metagenomic approach can be used to answer ecological questions as well. By examining cellulase and protease genes including their frequency and expression in natural samples, it is possibly to learn more about the ecology and the relative abundance of cellulose- and protein-degrading microorganisms in soil systems. Hydrolysis of cellulose, protein and other high-molecular-weight biopolymers by hydrolases is still an essential first step in the degradation of organic material in nature and the nutrient turnover processes in soil, for example in the degradation of plant leaf litter. Cellulose, the major component of the plant cell wall, is the most abundant renewable biomass in nature. Approximately 4-9 x 10^{10} metric tons of cellulose are produced each year by photosynthesis (Coughlan, 1985; Schultz and Taylor, 1989). A corresponding amount is degraded through the microbial cellulases in a variety of environments (Ljungdahl and Erikkson, 1985). However, very little is known about the microorganisms and their genes involved during the course of leaf litter decomposition (Aneja et al., 2004). Habitat-specific functional fingerprints can be inferred on the basis of the identification of predominant gene families (functional inventories). For example, cellobiose phosphorylase, involved in degradation of plant material, is found in the soil, but none are found in the Sargasso Sea or in the deep-sea whale falls sequences (Tringe et al., 2005).

Protease production is an inherent capacity of all microorganisms. They elaborate a large array of intracellular and/or extracellular proteases (Kalisz, 1988; Gupta et al., 2002). Microbial proteases are among the most important hydrolytic enzymes and were initially characterized as nonspecific degradative enzymes that are associated with protein catabolism. Now it is becoming increasingly recognized that proteolysis represents another mechanism for achieving precise control of biological processes in living organisms, through the hydrolysis of specific substrates (Ballinger et al., 1998). The intracellular proteases are important for various cellular and metabolic processes, such as sporulation and differentiation, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool (Gupta et al., 2002; Lee et al., 2004).

Our understanding of soil fungal community diversity and functioning remains poor relative to that of soil bacterial communities. Fungi are a diverse component of soil microbial communities. Here they function as decomposers, mycorrhizal mutualists and pathogens (O'Brien et al., 2005). However, little information exists about the distribution, diversity and activities of fungi in soil (Kowalchuk, 1999; van Elsas et al., 2000; O'Brien et al., 2005). There are a limited number of fungal-specific markers and those only provide an estimate of fungal biomass in soil (Anderson and Cairney, 2004). In contrast to bacteria, taxonomic identification of fungi based on sequences of eukaryotic ribosomal small subunit, the 18S rRNA, is more problematic due to the relative lack of variation within 18S rRNA genes between closely related fungal species as a result of the relative short period of evolution of the fungi kingdom compared with bacteria (Hugenholtz and Pace, 1996). This is compounded by the lack of an exhaustive database of fungal reference sequences. Despite these limitations, the 18S rRNA gene has been the most widely used, exploiting both conserved and the variable regions containing within it. In addition, the internal transcribed spacer (ITS) region located between the 18S rRNA and 28S rRNA genes, and incorporating the 5.8S rRNA gene, has also been targeted. Conserved PCR primers for amplification of fungal rRNA and ITS regions were designed by White et al. (1990). We have used primers ML7 and ML8 for amplifying segment of the mitochondrial large rDNA genes of fungi from extracted soil DNA, but this might be not a good target region for use with environmental samples. We have obtained either no PCR product or, in case of amplification, the products were incorrect in size and corresponding non-fungal rRNA sequences. This lack of specificity for fungal templates limits their usefulness in mixed environmental samples, such as soil, particularly where the ratio of fungal DNA to non-fungal DNA is low and results in co-amplification of similar target DNA from non-fungal sources (Harris, 1994). This may also reflect primer bias, or might have resulted from other factors, including bias associated with the DNA extraction procedure. Although we used the different chemical soil DNA extraction procedures in this study, the extracts in fact include DNA from only a subset of the organisms. The extent to which fungal mycelium and spores from different fungal taxa are lysed by these techniques remains to be analysed. It is known that spores are lysed differently than mycelia and mycelia of different ages also have different lysing efficiency (Prossser, 2002). The technique of bead beating, which applies high shear forces to cells, is more effective and recovers more diversity than chemical lysis methods (Miller et al., 1999; Niemi et al., 2001), was not used in this study.

Expression in bacterial hosts is usually limited to prokaryotic genes, but soil DNA can, depending on the isolation method, contain a substantial amount (>60%) of eukaryotic DNA (Gabor et al., 2003). This can lead to an increase in the required library size due to the formation of non-productive clones with eukaryotic inserts, virtually diluting the desired

prokaryotic recombinants. Owing to the presence of intron-containing sequences, metagenomic expression libraries are generally not suitable for mining eukaryotic genes (Cowan et al., 2005). Eukaryotes such as fungi are also an important component of the soil ecosystem, but their genetic potential has not been fully integrated into soil-based gene discovery. Using eukaryotic hosts for activity-based screening could also be useful for function-driven screens of soil-based libraries.

In addition another option is the construction of soil metagenomic libraries from cDNA to exploit uncultivable eukaryotic genomes, which would allow the discovery of novel genes derived from eukaryotes. This approach suffers from the technical difficulties associated with mRNA recovery from environmental samples, and efficient methods for the lysis of eukaryotic microorganisms in soil samples. The conservation of intact poly(A) mRNA would also be needed. The isolated poly(A) mRNA can then be used for cDNA synthesis and for library construction in expression vectors for the further identification of functional eukaryotic genes. The use of RNA seems to be more effective than DNA for profiling functional microbial communities because RNA is quite a more sensitive biomarker owing to its high turnover (Manefield et al., 2002). RNA would cover structural genes from lower eukaryotes as well as from prokaryotes. A particularly effective approach would be to use one of several differential expression technologies that rely on the isolation of mRNA to target transcriptional differences in gene expression (Carulli et al., 1998; Green et al., 2001; Kuhn, 2001).

The expression profile of microorganisms from metagenomic samples can be compared pre- and post-exposure to specific substrate, than the expression of genes up-regulated in any specific activity can be identified. However, metagenomic cDNA libraries cannot be as comprehensive as genomic libraries because the former can never represent non-expressed genes. In addition, the process of RT–PCR amplification limits the size of inserts and could impose a large sequence-dependent bias on the library (Beja et al., 2000).

Taken together, metagenomics provides an important tool in investigations of the genetics and biochemistry of soil microbiota from both ecological and biotechnological perspectives. A polyphasic study with different kinds of approaches can provide a basis for conducting genomic analyses of uncultured microorganisms and understanding their functions within the environment.

The present study was just an approach to ‘bring’ metagenomic DNA of soil samples in the ‘house’ of *E.coli* in order to explore the activities of the newly acquired genes. In fact, we found some new activities of already well known genes and also those of likely yet unknown organisms. However, various host organisms are likely required in order to find more of them.

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Construction and use of a genomic cosmid library of an environmental *Bacillus subtilis* A1/3 strain to explore its genetic capacity

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ABSTRACT

An exceptional broad spectrum of inhibitory activities of a natural *Bacillus subtilis* A1/3 strain against phytopathogenic viruses, fungi and bacteria led us to propose a wide spectrum of efficient and eventually new genes contributing to its antibiotic activities. In order to explore the promising genetic capacity of a *B. subtilis* A1/3 strain and to access the genes involved in the production of secondary metabolites we constructed and screened a genomic cosmid library of a surfactin-deficient mutant of this strain.

An *E. coli-B. subtilis* shuttle cosmid vector was constructed for the use in both host bacteria. For two-stage cloning experiments, primary cloning occurs in *E. coli*, followed by the transfer of selected hybrid cosmids containing large DNA inserts (up to 40 kb) to a secondary host *B. subtilis* that is more appropriate for analysing the biological activities of the products of cloned genes. The unamplified library consisted of approximately 5000 independent recombinant clones with inserts in the range of 30 to 40 kb in length, and revealed average insert sizes of approximately 34 kb. This equals to approximately 170 Mb of *B. subtilis* BC51 genomic DNA and a calculated 40-fold coverage of the genome. A total of 4608 cosmid-containing clones of the amplified library were isolated and stored individually in twelve 384-wells microtiter plates at -80°C.

This *B. subtilis* strain has shown a broad antifungal activity spectrum with strong inhibitory activity against the five target plant pathogenic fungi tested. When used against *Venturia inaequalis* in conidial germination inhibition assay, the germlings exhibited morphological changes and hyphal swellings. To identify the gene cluster implicated in the synthesis of antifungal iturin-like lipopeptide antibiotic the constructed cosmid library was the source for the isolation of targeted genes by subsequently hybridization screening resulting in successful identification of a clone carrying the complete bacilomycin L gene cluster. These results revealed a powerful application of the constructed cosmid library for the identification of genes encoding the entire pathway for synthesis of secondary metabolites of interest.

INTRODUCTION

The virtuosity of bacteria as synthetic chemists is unparalleled in nature (Emmert et al., 2004). Bacteria produce structurally diverse compounds with a wide spectrum of activities

including antimicrobial activities. Antibiotics are among the most important compounds of microbial origin. The rich diversity of the microbial world provides a seemingly endless resource for this purpose. One natural reservoir of bioactive compounds are the microbes in the soil. Most of the antibiotics used today come from cultured soil bacteria. Rhizosphere, the layer of soil around roots, is a 'microbial hot-spot' where diverse interaction between organisms take place and the highest diversity and richness of antagonistic bacteria were found (Whipps, 2001; Berg et al., 2005).

One of the main representatives of soil antagonists, *Bacillus subtilis*, is commonly associated with soil and plant roots and is the most commonly studied with regards to its wide spectrum of antibiotic activity. Several hundred wild-type *B. subtilis* strains have been collected and shown the potential to produce more than two dozen antibiotics with diverse structures (Stein, 2005). All of the genes specifying antibiotic biosynthesis combined amount to about 350 kb, almost about 10% of the annotated ORFs, but no strain possesses them all. All studied *B. subtilis* strains produce individual antibiotic cocktails. The average of about 4–5% of a *B. subtilis* genome is devoted to antibiotic production (Stein, 2005). The produced anti-microbial active compounds include predominantly peptides, as well as non-peptidic compounds such as polyketides, amino sugars, phospholipids and phenolic derivatives. *B. subtilis* is one of the best known peptide-forming cell factories hitherto known (Steller et al., 1999), and biosynthesis of antimicrobial peptides is a common phenomenon among many Gram-positive bacteria (Hancock, 1997).

Peptide antibiotics of *B. subtilis* are either ribosomally synthesized and post-translationally modified lantibiotics and lantibiotic-like peptides or non-ribosomally generated lipopeptides. Lantibiotics are synthesized as precursor peptides and post translationally modified into biologically active forms (Jack and Jung, 2000; McAuliffe et al., 2001). They antibiotically act against several Gram-positive bacteria by the formation of voltage-dependent pores into the cytoplasm membrane that causes the efflux of ions and metabolites, and finally, the collapse of the membrane potential (Breukink et al., 1999). The lipopeptides belong to the most frequently produced *B. subtilis* antibiotics which are non-ribosomally synthesized by large megaenzymes, the multifunctional peptide synthetases. The lipoheptapeptide surfactin is the most powerful biosurfactant known, it exerts a detergent-like action on biological membranes (Carrillo et al., 2003) and displays exceptional anti-viral and anti-mycoplasma activities (Peypoux et al., 1999). Fengycin specifically acts against filamentous fungi by inhibition of phospholipase A_2 (Vanittanakom et al., 1986; Nishikori et al., 1986). The iturin family encompass the closely related cyclic lipoheptapeptides mycosubtilin, the iturines and the bacillomycins with strong anti-fungal, haemolytic and only limited anti-bacterial activities (Thimon et al., 1995). Those interact with membranes to form pores by complexing sterols

(Maget-Dana et al., 1985; Volpon et al., 1999). The dipeptide antibiotic bacilysin displays activity against some bacteria and fungi, then blocks the glucosamine synthetase, and hence, bacterial peptidoglycan or fungal mannoprotein biosynthesis that leads to cell protoplasting and lysis (Kenig et al., 1976; Chmara, 1985; Milewski, 1993).

The large number of its known antibiotics might reflect the numerousness of natural isolates and their genetic diversity. *B. subtilis* isolates are ubiquitous in soil and on plants, occurring in all plant microenvironments: rhizosphere, endorhiza, phyllosphere and endosphere (Berg et al., 2005). In one study 47 of 51 *Bacillus* strains isolated from different habitats were identified as *B. subtilis*. Its diverse biochemical activities and genetic diversity were not correlated with their habitat (Pinchuk et al., 2002). The frequent occurrence of *B. subtilis* among other natural isolates might be also a consequence of the produced antimicrobial and fungicidal compounds that benefit its survival in their environment by elimination of competitors in the same habitat (Stein, 2005). Beyond the antimicrobial activities distinct *B. subtilis* antibiotics and antibiotic-like compounds play crucial roles in communal development and are involved in the morphology and physiology of *B. subtilis*. Non-ribosomally produced lipopeptides are involved in biofilm and swarming development (Kinsinger et al., 2003; Yan et al., 2003; Hofemeister et al., 2004). Lantibiotics function as pheromones in quorum-sensing (Stein et al., 2002; Kleerebezem, 2004). The antibiotic-like killing factor Skf is produced in sporulating cells and some antibiotics (subtilin, subtilosin, bacilysin, surfactin) are consequently induced and likely involved in the action against non-sporulating sister cells (Gonzalez-Pastor et al., 2003).

Some antibiotics are produced by a great variety of *B. subtilis* strains (subtilosin, surfactin, bacilysin), others, however, are strain-specific (subtilin, ericin, mersacidin) (Stein, 2005). The completely sequenced *B. subtilis* 168 strain, the model microorganism, cultivated in the laboratory for several decades, did harbour genes but due to mutations does not produce the various lipopeptide or polyketide antibiotics compared to other environmental strains. The natural *B. subtilis* A1/3 strain has exceptionally diverse antibiotic capacities, exceeded that of strain 168 and other strains. At least eight distinct antibiotics were identified: Lantibiotic-like peptides ericin A and S (Stein et al., 2002), lipopeptides surfactin, fengycin and bacillomycin L (Steller et al., 1998; Hofemeister et al., 2004), the dipeptide bacilysin (Steinborn and Hofemeister, 1998/2000), the polyketides bacillaene and difficidin (Hofemeister et al., 2004).
To explore the wide genetic capacity of this strain and to access the genes involved in the production of secondary metabolites we constructed a *B. subtilis* A1/3 genomic cosmid library described in this report. Here we present the construction of cosmid shuttle vector and its use for constructing in *E. coli* a library containing large DNA inserts of up to 40 kb, which

can then be transferred to *B. subtilis* by transformation (of competent cells as well as protoplasts).

The purpose of this study was also to assess the antifungal spectrum of the produced compounds and to identify a gene cluster implicated in the synthesis of the antifungal iturin-like lipopeptide antibiotic of *B. subtilis* A1/3 strain that was before still unknown. Using the constructed cosmid library as source for the isolation of targeted genes by hybridization we identified one clone carrying the genes that harbour the complete bacilomycin L gene cluster.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

The strains and plasmids used are listed in Table 1. *Escherichia coli* cultures were propagated in LB (1% tryptone, 0.5% yeast extract, 1% NaCl) and *Bacillus subtilis* in TBY medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37 °C. Antibiotics were used at the following final concentrations: ampicillin 100 μg/ml for *E. coli* and erythromycin 5 μg/ml for *B. subtilis*. Recombinant plasmids were amplified in *E. coli* XL1-blue MR, *B. subtilis* GSB26 and GSB285. The arrayed library cosmid clones were stored at –80°C in 384-well microtiter plates contained 40μl of the growth medium 2YT (1.6% tryptone, 1% yeast extract, 0.5% NaCl) supplemented with 1/10 vol of 10x HMFM freezing medium (36 mM K_2HPO_4, 13.2 mM KH_2PO_4, 0.4 mM $MgSO_4$, 6.8 mM $(NH_4)_2SO_4$, 1.7 mM Na_3-citrate, 4.4% glycerol) and 100 μg/ml ampicillin.

Table 1. Bacterial strains and vectors used in this study

Strain or vector	Relevant characteristics	Reference or source
B. subtilis A1/3	Wild type, isolated from tomato hydroponic cultures	Huber et al. (1991)
B. subtilis GB709	Plasmid cured derivative of *B. subtilis* A1/3	Stein et al. (2002)
B. subtilis BC51	GB709 disruption mutant *srfC*::pMEsd51	Hofemeister et al. (2004)
B. subtilis GSB26	*str* ^{r} mutant of QB1133 *amyE aroI906 metB6 sacA321 sfp*^{0}	Steinborn and Hofemeister (1984)
B. subtilis GSB285	GSB26 derivative with enhanced stabilization of recombinant plasmids	Steinborn (1996)
E.coli XL-1 Blue MR	recA1 endA1 gyrA96 thi-1 supE44 relA1 lac	Stratagene
SuperCos 1	Amp^r, Kan^r, pUCori	Stratagene
pSB 681	*E. coli/Bacillus* shuttle cosmid vector, SuperCos 1 derivative, Amp^r, Kan^r, Em^r, pUCori, theta-rep	This study
pSB 595	*Bacillus* cloning vector, Em^r, theta-rep	Steinborn et al. (2005)

Antifungal activity

Antifungal activity of *B. subtilis* A1/3 was evaluated *in vitro* using a dual culture on malt agar containing 2% of malt extract and adjusted to pH 6.8. For antifungal activity tests the following plant pathogenic fungi were used: *Botrytis cinerea* B05.10 (B.Tudzynski, Münster), *Venturia inaequalis* ZH1 (C. Gessler, ETH Zürich), *Cladosporium lagenarium, Fusarium solani* and *Phytophthora sojae* (syn. *P. megasperma* f. sp. *glycinea*) (from collection of fungi, Dep. of Biotechnology, TU Kaiserslautern). After 10 days of incubation at 20°C, zones of inhibition were measured as described previously (Kucheryava et al., 1999). The assay for testing inhibition of *V. inaequalis* conidial germination was performed with the bacterial culture and cell-free culture supernatants of *B. subtilis* A1/3 as described by Kucheryava et al. (1999).

For mass production of conidia the *V. inaequalis* was cultured on malt agar plates covered with cellophane as reported by Parker et al. (1995) at 18°C for 2 to 3 weeks under near-UV light to induce sporulation. A spore suspension was prepared in sterile water by vortexing the fungus grown in cellophane, the suspension was subsequently filtered through a 20 μm nylon filter, centrifuged at 3000g for 10 min, the pellet with the spores resuspended in water and adjusted to 10^5 conidia per ml determined by using a haemocytometer. The germination of 100 conidia was microscopically examined 24 h after incubation at room temperature after mixing of equal volumes of the conidial suspension and bacterial culture or cell-free culture supernatant of *B. subtilis* A1/3. The extraction of *B. subtilis* A1/3 culture supernatant was as described by Hofemeister et al. (2004).

DNA manipulation

Chromosomal DNA from *B. subtilis* BC51, a *srfC* disruption mutant of plasmid cured derivative of *B. subtilis* A1/3, was isolated with the Qiagen Genomic-tip 500/G. Plasmid DNA was purified using a QIAprep Spin Miniprep or Plasmid Midi isolation kit (Qiagen), except that *B. subtilis* cells were incubated in buffer P1 supplemented with 5 mg/ml lysozyme for 30 min. Restriction enzyme analysis, ligations, etc. were performed with commercially available enzymes according to the manufacturer's recommendations.

PCR or restriction DNA fragments were isolated from agarose gels and purified using QIAquick PCR purification kit or QIAexII gel extraction kit (Qiagen).

Competent cells of *E. coli* XL1-blue MR were prepared and transformed following the procedure of Hanahan (1983). Competent cells or protoplasts of *B. subtilis* GSB26 and GSB285 were performed and transformed by the protocol of Dubnau and Davidoff-Abelson (1971) or using the protoplast transformation protocol of Chang and Cohen (1979), respectively.

Construction of *E. coli-B. subtilis* shuttle cosmid pSB 681

The novel cosmid shuttle vector pSB 681 was constructed from cosmid SuperCos 1 vector (Stratagene) by replacing the neomycin resistance gene with the erythromycin resistance gene and introduction of *Bacillus* replication origin both from the *Bacillus* cloning vector pSB 595 as follows: The cosmid vector SuperCos 1 was digested with restriction endonucleases *Sma*I and *Bgl*II. This releases a DNA fragment containing the truncated neomycin resistance gene. The vector DNA was purified by agarose gel electrophoresis and the QIAEX II gel extraction procedure (Qiagen). *Bacillus* cloning vector pSB 595 was digested with restriction endonucleases *BamH*I and *EcoR*V. A DNA fragment of approximately 6800 bp containing the *Bacillus* replication origin and the erythromycin resistance gene was purified, mixed with the SuperCos1-Neo$^-$ (without neomycin marker) vector and ligated using T4 DNA ligase. The ligation mix was transformed in Epicurian Coli supercompetent cells (*E. coli* XL1-blue MR strain) (Stratagene) according to the manufacturer's protocol. The transformants were plated on LB agar containing 100 µg/ml of ampicillin. Cosmid DNA was isolated and purified by using the Qiagen plasmid isolation kit and tested with restriction enzymes. The resulting 13.6 kb cosmid shuttle vector is named pSB 681. For the large-scale preparation of cosmid DNA the amplification of cosmids was performed in the presence of chloramphenicol to achieve high yield of cosmids. The *E. coli* culture carrying the cosmid was grown in LB broth containing 100 µg/ml ampicillin to late log phase (OD_{600} of ~0.6), then chloramphenicol was added (170 µg/ml) and the culture incubated for a further 3 hours. Cosmid DNA was extracted by using a plasmid isolation kit (Qiagen) according to the manufacturer's recommendations.

Transformation of *B. subtilis* with shuttle cosmid pSB 681

Cosmid pSB 681/5 DNA extracted from *E. coli* clones was used for the transformation of *B. subtilis*. Competent cells of *B. subtilis* GSB26 and the protoplasts of *B. subtilis* GSB285 were transformed by the method of Dubnau and Davidoff-Abelson (1971) or Chang and Cohen (1979), respectively. Transformants were selected on TBY agar containing 5 µg/ml of erythromycin. Cosmid DNA from *B. subtilis* clones was extracted by a plasmid isolation kit (Qiagen) and specified after cuting with restriction enzymes.

Construction of a *B. subtilis* BC51 genomic cosmid DNA library

A genomic library of *B. subtilis* BC51 strain, a *srfC* disruption mutant of plasmid cured derivative of *B. subtilis* A1/3, was constructed using the *E. coli-B. subtilis* cosmid shuttle vector pSB 681 as follows: Chromosomal DNA of *B. subtilis* BC51 was digested partially with *Mbo*I to the average size of 30 to 50 kb. Spermidine was added to the final concentration of 4

mM to improve the digestibility of high-molecular-weight DNA. The DNA fragment sizes were determined by pulsed field gel electrophoresis on 1% agarose gel in 0.5 x TBE buffer for 20 h at 14°C, 170 V, 6 V/cm with 5-15 sec switch time. DNA fractions enriched in 30–50 kb fragments were dephosphorylated with shrimp alkaline phosphatase to avoid ligation of non-contiguous fragments into concatamers (i.e. formation of chimeras of insert genomic DNA).

The cosmid vector DNA was digested with *Xba*I to linearize it in between the two cos-sites, dephosphorylated with shrimp alkaline phosphatase and again digested with *Bam*HI. Dual *cos*-sites eliminate the need to dephosphorylate the vector cloning site prior to ligation with insert DNA and allow the simple and efficient cloning using non size-selected on gel or gradients insert DNA, since the packaging system only accepts inserts in a certain size range (30-42 kb). This vector DNA was mixed and ligated with the dephosphorylated enriched 30-50 kb fragments of genomic DNA. The ligation mixture was packaged in bacteriophage heads using a lambda Gigapack III Gold packaging extract (Sratagene) and infected into *E. coli* XL-1 Blue MR cells according to the manufacturer's protocol. The transformants were plated on LB agar containing 100 µg/ml ampicillin. After overnight incubation at 37°C the single colonies were transferred into 384-well microtiter plates contained 40µl of the growth medium 2YT supplemented with 1/10 vol. of 10x HMFM freezing medium and 100 µg/ml ampicillin using the automated colony picking system BioPick (BioRobotics Ltd.). Freshly inoculated 384-well plates were overnight incubated at 37°C t and then frozen at -80°C until further use.

Screening of genomic cosmid library by colony hybridization

High-density colony filters for hybridization were prepared from thawed 384-well microtiter plates using the robotic gridding system BioGrid (BioRobotics Ltd.). Each of the 384-well plates was replicated twice onto a sterile 22x22 cm LB-Amp-agar plate covered with 22 cm square nylon filter Hybond N+ (Amersham). Arrayed colonies from 6 microtiter dishes were spotted onto nylon Hybond N+ membranes. Plates were incubated at 37°C until the colonies were clearly visible. Filters were removed from the agar surface and placed, colony-side-up, onto Whatman 3 mm paper saturated with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for two minutes, and then neutralized for five minutes in 1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0, rinsed in 2x SSC (0.3 M NaCl, 0.5 M sodium citrate) for a few minutes and baked at 80°C for 1.5-2 hours. One high-density nylon filter containing the immobilized DNA from 2300 clones was spotted out of six 384-well microtiter plates and the duplicate colony filters were used for hybridization.

The DNA probes used for hybridization screening of the library were labelled using the following non-radioactive methods. The fluorescein-labelled DNA probes (a 485 bp fragment

of *fen4* gene or about 11 kb fragment of ericin gene cluster) were prepared using the isolated target gene DNA fragments, Klenow polymerase and the ECL random prime labelling system (Amersham), followed by hybridization performed according to the supplied protocol. The digoxigenin-labeled DNA probe (a 660 bp fragment of *bmyLD* gene) was prepared with the PCR DIG probe synthesis kit (Roche) using *Taq* polymerase and DIG-11-dUTP, which was incorporated during the PCR with *B. subtilis* BC51 chromosomal DNA. The hybridization experiments were performed according to the manufacturer's instructions.

PCR amplification

PCRs were performed using *Taq* DNA polymerase or Expand long template PCR kit (Roche) and the reaction conditions were as recommended by the supplier. PCR was run using the following protocol: 100 to 200 ng of genomic DNA, a 0.2 mM concentration of each deoxynucleoside triphosphate, the required concentration (100-300 pmol) of each primer and 1.25 U of DNA polymerase. The samples were amplified after incubation at 94°C (2 min), followed by 30 cycles at 94°C (30 s), 55°C (30 s), and 72°C (1 min) for amplification of small (0.5 kb) and at 72°C (3 min) for amplification of larger (>3 kb) DNA fragments, followed by 1 cycle of 72°C (7 min), respectively.

DNA sequencing and analysis of cosmid end sequences

The plasmid templates were purified from *E. coli* cultures by using a plasmid isolation kit (Qiagen). The inserts of selected clones were sequenced from both ends by using the T3 and T7 primers that flanked the insert. The insert DNA of cosmid pSC9i10 was sequenced by primer walking. Nucleotide sequences were determined with an automatic A.L.F. DNA Sequencer (Pharmacia) by sequencing services of the Institute of Plant Genetics and Crop Plant Research (Gatersleben, Germany). The sequence data were evaluated by a nucleotide and translated-protein search compared with GenBank databases using BlastN and BlastX server (http://www.ncbi.nlm.nih.gov/BLAST) of NCBI (National Centre for Biotechnology Information).

RESULTS

Antifungal activity

The five target plant pathogenic fungi *B. cinerea, C. lagenarium, F. solani, P. sojae* and *V. inaequalis* were used to find out if *B. subtilis* A1/3 strain has a broad antifungal activity spectrum. The bacterial culture and cell-free culture supernatants of *B. subtilis* A1/3 showed strong inhibitory activity against all fungal plant pathogens tested. In dual-culture agar plate assays (Fig. 1), *B. subtilis* A1/3 exhibited maximal activity against *V. inaequalis* (57.1%

mycelial growth inhibition), followed by *C. lagenarium* (53.1%), *B. cinerea* (30%), *F. solani* (22.1%) and *P. sojae* (13.2%). Analysis of mycelial growth inhibition revealed *V. inaequalis* to be the most sensitive fungus against the *B. subtilis* A1/3 samples.

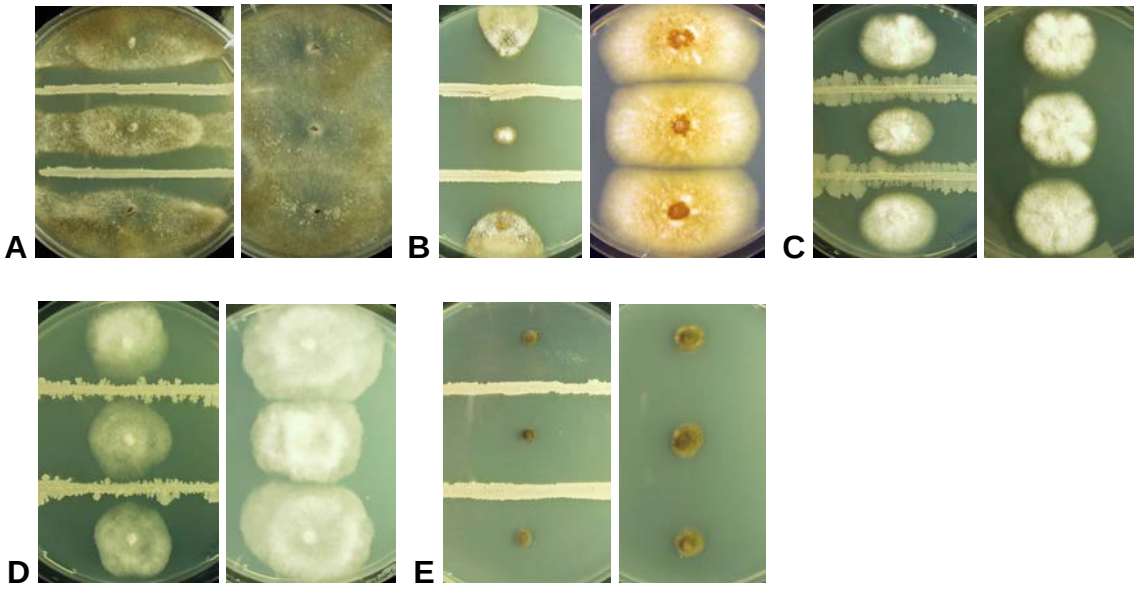

Fig. 1. Antifungal activity of *B. subtilis* A1/3 strain against plant pathogens: *B. cinerea* (**A**), *C. lagenarium* (**B**), *F. solani* (**C**), *P. sojae* (**D**) and *V. inaequalis* (**E**) in a dual-culture agar plate assay. The fungal growth inhibition was observed after 10 days incubation at 20°C.

Inhibition of conidial germination of *V. inaequalis* was analysed, since germination is the starting event of the asexual life cycle of this fungus. Antifungal compounds should preferably prevent germination that is also an important mechanism of fungal inhibition. The same inhibitory activity of *B. subtilis* A1/3 against *V. inaequalis* was found for mycelial growth and spore germination (Fig. 2).

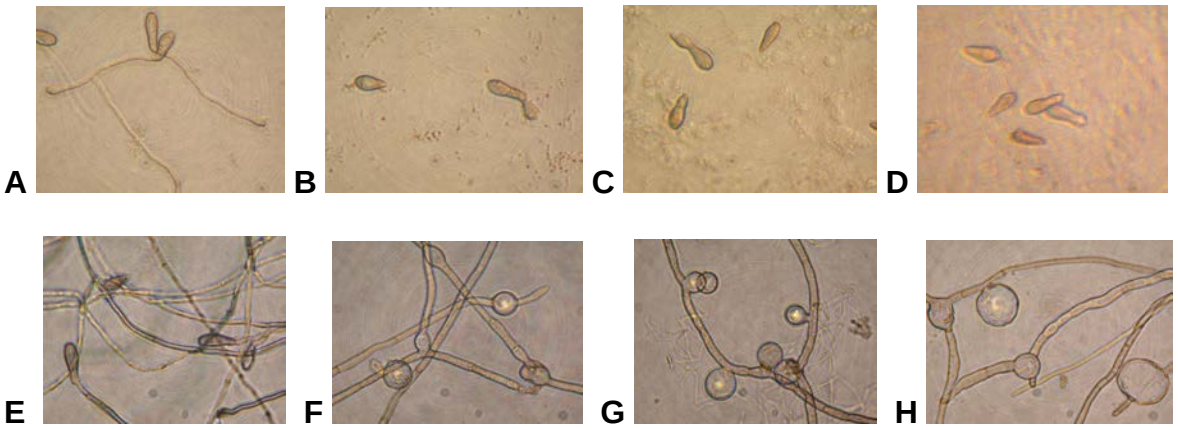

Fig. 2. Antifungal activity of *B. subtilis* A1/3 strain against *V. inaequalis* in conidial germination inhibition assay. Germination of *V. inaequalis* conidia observed after 24 h incubation in the absence (**A, E**) and presence of cells (**B**) or cell-free culture supernatants obtained from 48 h (**C**) and 72 h (**D**) culture of *B. subtilis* A1/3. Effect of culture supernatants from 30 h (**F**), 48 h (**G**) and 72 h (**H**) culture of *B. subtilis* A1/3 on the hyphae of germinated *V. inaequalis* conidia exposed for 24 h.

Microscopical analysis of conidia exposed for 24 h to the bacterial culture revealed 53.2% inhibition of germination and cell-free culture supernatants prepared from 30, 48 and 72h culture of *B. subtilis* A1/3 showed the increased to 64.9, 75.4 and 89% conidial germination inhibition, respectively. Although these culture supernatants did not completely inhibit germination, they prevented growth of hyphae after conidia germination. The morphology changes, swelling and deforming hyphae of the germinating conidia were documented in the presence of all tested *B. subtilis* A1/3 supernatants after 24h incubation (Fig. 2, F-H).

The *B. subtilis* A1/3 strain is known to produce a number of antifungal compounds including the dipeptide antibiotic bacilysin (Steinborn and Hofemeister, 1998/2000), fengycin (Steller at al., 1999) and iturin-like lipopeptide antibiotics, but the corresponding genes of a last antimycotic lipopeptide were still unknown before. Their identification was a goal of this work.

Construction of a novel *E. coli-B. subtilis* shuttle cosmid vector pSB 681

For the construction of a *Bacillus* genomic library it was decided to use a shuttle vector, which can be replicated and maintained in *E. coli* and *B. subtilis.* For this the recombinant cosmid pSB 681 (Fig. 3) carrying both replicons was constructed by replacing the neomycin resistance gene with the erythromycin resistance gene and introducing the theta-replication origin from the *Bacillus* cloning vector pSB 595 used for cloning in several *Bacillus* species.

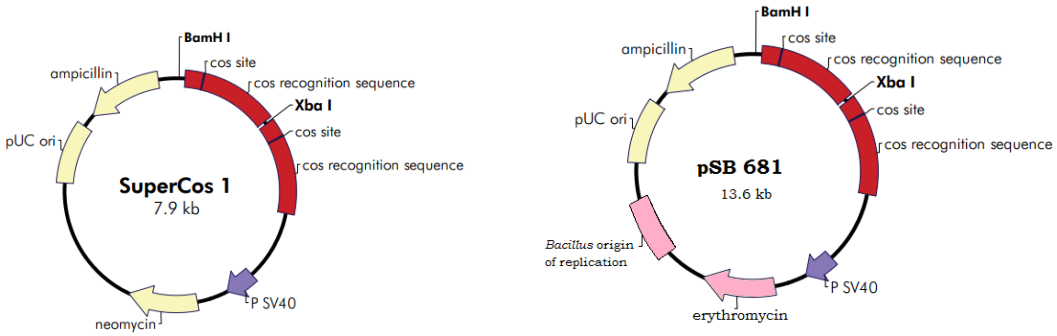

Fig. 3. Construction of the *E. coli-B. subtilis* shuttle cosmid vector pSB 681 by replacing the neomycin resistance gene in SuperCos1 with the erythromycin gene as antibiotic resistance marker for *Bacillus* and introducing the theta-replication origin from the *Bacillus* cloning vector pSB 595.

In this way, *Bacillus* insert DNA of the library clones could readily be mobilized from the *E. coli* host to an alternative *B. subtilis* strain. SuperCos1 cannot replicate in *Bacillus* and carries by side of the Amp^R a neomycin resistance gene under control of an SV40 promoter not useful in *B. subtilis*. Therefore, it was necessary to introduce a second origin of replication and an antibiotic resistance marker for *Bacillus*. To achieve this goal, SuperCos 1 was digested with restriction endonucleases *Sma*I and *Bgl*II which released a fragment containing a truncated neomycin resistance gene, and ligated with pSB 595 previously

linearized by *Bam*HI/*Eco*RV treatment. After transformation into *E. coli* cells, the transformants were selected by resistance to ampicillin. All transformants examined contained a 13.6 kb plasmid. The cosmid isolated from these transformants was designated as pSB 681.

The constructed shuttle cosmid pSB 681 was used to transform competent cells and protoplasts of *B. subtilis* GSB26 and GSB285 strains, respectively. *B. subtilis* transformants were selected by resistance to 1-5 μg/ml erythromycin and analysed for their plasmid content. The restriction enzyme digestion of the vectors from a representative number of the selected colonies from either *E. coli* or *B. subtilis* revealed same patterns, i.e. structural identity (results not shown). The constructed vector replicated in *E. coli* as well as in *B. subtilis*, and conferred ampicillin resistance to *E. coli,* but erythromycin resistance to *B. subtilis*. Due to its ColE1 replication origin, the cosmid could be amplified by chloramphenicol resulting in much higher DNA yields of hybrid cosmids and enhanced transformation capacity. This was of significant advantage in transferring a *Bacillus* gene library from *E. coli* back into competent *B. subtilis* cells.

Construction of cosmid DNA library from *B. subtilis* BC51 strain

The genomic library of *B. subtilis* BC51, a *srfC* disruption mutant of plasmid cured derivative of *B. subtilis* A1/3, was constructed using the *E. coli-B. subtilis* shuttle cosmid pSB 681 as described in Materials and Methods. Primary titer was 10^4 cfu/ml, the primary unamplified library consisted of approximately 5000 independent recombinant clones with inserts in the range of 30 to 40 kb in length. The average insert size was estimated to approximately 34 kb by *Not*I restriction digests of 10 randomly chosen clones. Thus, the library harboured a total of approximately 170 Mbp of *B. subtilis* BC51 genomic DNA, equivalent to a calculated 40-fold coverage of the genome. A total of 4608 cosmid-containing clones of amplified library were isolated and stored individually in twelve 384-wells microtiter plates at -80°C.

The cosmid DNA isolated from randomly chosen *E. coli* library clones was transformed into *B. subtilis* and compared with cosmid DNA extracted from *B. subtilis* clones by *Not*I and *Eco*RI restriction enzymes digestion. Comparative restriction analyses revealed identical digestion patterns of all examined inserts in the *B. subtilis* hosts and the parent *E. coli* library clone (data not shown). This demonstrates that the library clones were sufficiently stable in both host bacteria and even large-insert (up to 40 kb) library clones were stable transferred and maintained in *B. subtilis.* This was especially important for the two-stage cloning experiments, i.e. primary cloning in a convenient host such as *E. coli*, and the transfer of selected hybrid cosmids to a secondary host *B. subtilis* that is more appropriate for analysing the biological activities of the products of cloned genes.

Screening of *B. subtilis* BC51 genomic cosmid library by colony hybridization

To investigate the genomic information captured in our library we carried out initial screening for various genes by colony hybridization. The library was stored in an ordered array performed using a picking robot by transfer of individual colonies into the wells of 384-well master plates which were used as an archival source to replicate each clone separately into nylon Hybond N+ filter. Thus, each clone in the library could be handled and assayed individually. One high-density nylon filter contained the immobilized DNA of 2300 clones spotted out of six 384-well microtiter plates. Duplicate colony filters were used for hybridization.

The first hybridization screening of the library clones from six archival master microtiter plates (no. 1 to 6) was conducted with the fluorescein-labeled DNA probes prepared using the isolated target gene DNA fragments. Genes from antibiotic synthesis gene clusters, namely *eriB-eriK* genes of ericin, *fen4* of fengycin and *bmyLD* of bacilomycin L were subsequently used as probes for capturing the corresponding clones in the library by colony hybridization screening. Positive recombinant clones were selected and cosmids were purified and end sequenced. This has led to the identification of several clones which encode various *Bacillus* genes (Table 2). Two of the sequenced clones were found to harbor DNA from *E. coli* indicating the presence of contaminating host chromosomal DNA in the cosmid vector preparation. Treatment of purified cosmids with PlasmidSafe DNase is recommended for eliminating *E. coli* DNA contamination.

The second hybridization screening of the library clones, from master microtiter plates no. 7 to 12 was performed with the digoxigenin-labeled 660 bp PCR fragment of *bmyLD* gene as probe. Primers were designed according to the sequence of the transposon mutant BA13-20 deficient in production of bacillomycin L (Hofemeister et al., 2004). The disrupted gene encoded malonyl-CoA transacylase which is necessary for the synthesis of bacillomycin L as well of other antifungal lipopeptide antibiotics of the iturin family. This was used as probe for capturing the corresponding, so far unknown, gene cluster in *B. subtilis* A1/3. In this way, 11 positive clones were recovered by screening of approximately 2300 *E. coli* colonies. We selected one clone containing a designated pSC 9i10 cosmid for further study to identify the complete bacilomycin L operon. The insert was completely sequenced, and the obtained sequence found to encompass a 16.5 kb region harbouring complete bacilomycin L gene cluster (Hofemeister et al., 2004).

Thus, these results revealed that the constructed cosmid library represented an archive of the genome of *B. subtilis* BC51, and a useful source for the isolation of targeted genes by hybridization, resulting in successful identification of genes encoding the entire pathway for synthesis of secondary metabolites of interest.

Table 2. Genes and similarities identified by hybridization screening of cosmid library

Clone	Putative gene/ protein	Organism (accession number)	Identity %	Region of similar amino acids	Score bits
Fengycin *fen4* gene as probe used for hybridization screening					
5F17	YngH protein	*B. amyloliquefaciens* (CAE11268)	74	69	126
5D17	Polyketide synthetase pksP	*B. subtilis* (E69679)	31	71	97
	Polyketide synthase pksL	*B. subtilis* (CAA78479)	39	34	60
4I20	FenD protein	*B. amyloliquefaciens* (CAE11274)	62	128	214
	YoeA protein	*B. amyloliquefaciens* (CAE11280)	95	98	184
3L16	YngB protein	*B. amyloliquefaciens* (CAE11263)	98	115	229
Ericin genes as probe					
2N12	Putative SpaB	*B. subtilis* (AAL15564)	96	27	63
3J16	Polyketide synthase pksR	*B. subtilis* (NP 389604)	67	57	123
4D15	Polyketide synthase	*B. subtilis* (CAB13606)	45	63	103
Bacillomycin L *bmyLD* gene as probe					
4B23	Sterol-sensing 5TM box, membrane protein ydfJ	*B. licheniformis* (AAU25508)	82	89	182
	Membrane protein ydfJ, similar to antibiotic transport-associated protein	*B. subtilis* (BAA19377)	83	183	291
3L24	Polyketide synthase pksR	*B. subtilis* (NP 389604)	68	51	116
	Polyketide synthase pksM	*B. subtilis* (NP 389601)	64	57	101
2J23	Putative N-acetylmannosaminyl-transferase (major teichoic acid biosynthesis protein A)	*B. subtilis* (NP 391456)	70	68	157
	N-acetylglucosaminidase (major autolysin)	*B. subtilis* (NP 391459)	52	41	87
9I10	Bacillomycin L	*B. subtilis* (AF499447)	100	3982	7907
Random sequencing					
3B	Polyketide synthase pksM	*B. subtilis* (NP 389601)	70	56	119
	Polyketide synthase pksJ	*B. subtilis* (NP 389598)	80	225	470

DISCUSSION

Biological control agents which can control more than one pathogen are particularly interesting. The five target plant pathogenic fungi *Botrytis cinerea, Cladosporium lagenarium, Fusarium solani, Phytophthora sojae* and *Venturia inaequalis* were used to analyse the antifungal spectrum of *B. subtilis* A1/3 strain which has previously shown to encompass *F. oxysporum*, *Penicillum chrysogenum*, and *Paecilomyces variotii* (Hofemeister et al., 2004). In this study the bacterial culture and cell-free culture supernatant of *B. subtilis* A1/3 showed

weak to moderate inhibitory activity in dual-culture agar plate assays against all of the fungal plant pathogens tested. The weak inhibitory activity (13.2%) was found against *P. sojae* which belong to oomycetes that have a fungus-like morphology but are phylogenetically distinct from the true fungi. They differ from fungi in many aspects, such as production of wall-less motile zoospores and the no-chitinous nature of the cell wall, i.e. in contrast to all other fungi cellulose is present in their cell walls (Judelson, 1997; Govers, 2001). Furthermore, it has been well documented that *Phytophthora* spp. lack a complete sterol synthesis pathway and require an exogenous source of these lipids for normal growth and development (Nes, 1987). One consequence of their unique features is that most fungicides are not inhibitory for oomycete species and notably for *Phytophthora*. A new antibiotic, zwittermicin A, is identified in soil bacterium *B. cereus*. It has a broad spectrum of activity, including inhibition of oomycete pathogens of plants and is synthesized by a mixed NRPS/PKS pathway (Emmert et al., 2004).

The significant inhibitory activity of *B. subtilis* A1/3 was detected against *V. inaequalis*, both against mycelial growth and conidial germination. So far, antifungal activities of *B. subtilis* have been tested mainly against mycelial growth, whereas less information is available about their effect on fungal spore survival and germination. Since germination represents the starting event of the fungal life cycle, germination inhibitors are desired as antifungal compounds (Chitarra at al., 2003). The morphological changes, swelling and deforming hyphae of the germinating conidia were exhibited in the presence of *B. subtilis* A1/3 supernatants from late stationary phase. This indicates that the antifungal compounds produced may belong to the iturin group of antibiotics, which are known to be the main products during the stationary phase, in contrast to other antifungal lipopeptides such as surfactin and fengycin, which are produced, earlier, namely during transition between exponential and stationary growth phases (Hofemeister et al., 2004; Koumoutsi et al., 2004; Vater et al., 2002).

The action of iturin-like compounds produced by *B. subtilis* against *Penicillium roqueforti* conidiospores was assessed by fluorescence staining techniques that revealed labelling of damaged spores, indicating permeabilization and irreversible damage (Chitarra at al., 2003). Even after one month, spores of *Aspergillus flavus* had not germinated in the presence of bacillomycin D produced by *B. subtilis* (Moyne et al., 2001). The mode of action, namely permeabilization of plasma membranes, is common for all antibiotics of the iturinic group including mycosubtilin, iturins and bacillomycins. All these lipopeptides are very potent against a broad range of fungi (Moyne et al., 2001; Chitarra at al., 2003) and different yeasts (Latoud et al., 1987, 1988, 1990; Thimon et al., 1995). In addition, they are active against only a few bacteria (Peypoux et al., 1979), and are also haemolytic (Latoud et al., 1986;

Thimon et al., 1994; Hofemeister et al., 2004). They act on the biomembrane with an established sterol dependency. Insertion in the cytoplasmic membrane is followed by auto-aggregation to form a pore which causes cellular leakage, resulting in the loss of essential macromolecular compounds (Maget-Dana et al., 1985; Volpon et al., 1999). According to Latoud et al. (1986), at a concentration inducing 100% hemolysis, bacillomycin L leads to the release of about 1.9% of cholesterol from erythrocytes into the supernatant. The antifungal activity is believed to rely on a better interaction with ergosterol, which is more abundant in fungal membranes, than cholesterol present in other organisms (Volpon et al., 1999). *A. flavus* conidial membranes contain ergosterol and cholesterol, which could both be the target of bacillomycin D (Moyne et al., 2001).

The environmental *B. subtilis* A1/3 strain has diverse antibiotic capacities and eight antibiotics were identified (Steller et al., 1998; Steinborn and Hofemeister, 1998/2000; Stein et al., 2002; Hofemeister et al., 2004), three of them, bacilysin, fengycin and bacillomycin L, with anti-fungal activities. The genes encoding the biosynthesis of the dipeptide bacilysin were detected previously (Steinborn and Hofemeister, 1998/2000; Steinborn et al., 2005). This antibiotic displays activity against some bacteria and fungi. It blocks glucosamine synthetase, and hence, bacterial peptidoglycan or fungal mannoprotein biosynthesis that leads to cell protoplasting and lysis (Kenig et al., 1976; Chmara, 1985; Milewski, 1993). The gene cluster involved in fengycin synthesis was identified in an earlier study (Steller et al., 1999). Fengycin is known to act specifically against filamentous fungi by inhibiting phospholipase A_2 (Vanittanakom et al., 1986; Nishikori et al., 1986). Fengycin acts in a synergistic manner with bacillomycin D of *B. amyloliquefaciens*, both antibiotics act in a synergistic manner against phytopathogenic fungi as shown by Koumoutsi et al. (2004). In contrast to the single mutants, a double mutant lacking both bacillomycin D and fengycin did not develop antifungal activity. This phenomenon has been described also for surfactin and iturin A of *B. subtilis* (Maget-Dana et al., 1992) and for secondary metabolites produced by actinomycetes, and has been interpreted as an adaptation of the bacterium to compete with other microorganisms (Challis and Hopwood, 2003). In addition, enzymes such as proteases, chitinases and glucanases which accomplish degradation of host cell walls often have antifungal activity individually and act synergistically with antibiotics (Lorito et al., 1994, 1996; Chen et al., 2004). Thus, *B. subtilis* possesses the attractive feature of a biocontrol agent by the production of a variety of antimicrobial compounds. Resistance of the target organism should occur only at very low frequency (Handelsman and Stabb, 1996).

Genes involved in synthesis of antifungal iturin-like lipopeptide by *B. subtilis* A1/3 were not yet identified. Only one gene has been tagged in a transposon mutant lacking hemolytic and anti-fungal activities due to the loss of bacillomycin L synthesis detected by MALDI-TOF-

mass spectrometry (Hofemeister et al., 2004). The gene (*bmyLD*) disrupted by the transposon encodes malonyl-CoA transacylase that is indispensable for the synthesis of the iturin family lipopeptides (Duitman et al., 1999; Tsuge et al., 2001). The purpose of this study was to assess the other genes harbouring complete bacilomycin L gene cluster.

Our approach to the study of *B. subtilis* A1/3 genome was the construction of a cosmid library to access large, contiguous segments of genomic DNA and whose heterologous expression in *E. coli* as well in *B. subtilis* eventually allows the detection of biological activities. Because of the low transformation efficiency using *Bacillus* as a primary cloning host, the possibility of using *E. coli* was an attractive alternative. The advantages of the shuttle vector which can be replicated and maintained in both host organisms and the use of both host systems thus increasing the chances of detecting expression of genes of interest.

As a starting point for the construction of the shuttle vector, the *E. coli* cosmid SuperCos1 (Stratagene) was used. This vector accepts DNA inserts ranging from 30-42 kb in size and the host organism due to its deficiency in certain restriction systems cannot cleave inserted DNA carrying cytosine and/or adenine methylation, additionally it is endonuclease-deficient and recombination deficient. These advantages of the system also include the utilization of highly efficient lambda in vitro packaging systems for initially generating a library in *E. coli* host, since packaged genomic DNA inserts may be protected against degradation. Once inside an *E. coli* host cell, damaged inserts may be repaired by the host's cellular DNA repair mechanisms. For the ligation and packaging the system requires only small amounts of starting genomic DNA (5-10 µg), and size selection may not be required since the packaging system only accepts inserts in a certain size range (30-42 kb). The initial library in *E. coli* may be amplified to produce supercoiled cosmid DNA which may be used in high efficiency transformation methods for introduction into *Bacillus* expression host organisms.

SuperCos1 was modified by introduction of *Bacillus* replication origin and selectable marker genes. The constructed *E. coli-B. subtilis* cosmid vector pSB 681 thus contains a multiple cloning site, a ColE1 origin and the ampicillin resistance gene respectively for replication and selection in *E. coli*, an theta-replication origin and erythromycin resistance genes for maintenance and selection in *B. subtilis*, an SV40 origin and dual cos sites for *in vitro* packaging in lambda phage.

Natural and industrial isolates of *B. subtilis* species frequently harbour large plasmids. In a collection of *B. subtilis* environmental strains (Titok at al., 2003), large (~90 kb) plasmids were found to be widely represented in the collection. They replicate via a theta mode, as theta circles, in contrast to other small plasmids (<10 kb) which belong to the pC194 family and replicate as rolling circles. The theta-replicating-based vectors may be much more useful for large DNA cloning purposes than rolling circle-based vectors. This was shown by

Steinborn (1996) and was confirmed by our cosmid shuttle vector which efficiently transformed *B. subtilis* competent cells and protoplasts.

The use of *E. coli* as the more convenient host limits the ability to express DNA from *Bacillus*. The potential success of functional screening for gene expression in a heterologous *E. coli* was analysed using the BAC chromosome library of *B. cereus* and it was shown that only 6 of 10 activities tested were detected using *E. coli* (Rondon et al., 1999). It was shown that cloned cereolysin from *B. cereus* is poorly expressed in *E. coli*, but exhibited hemolytic activity at levels comparable to those of the *B. cereus* donor strain after subcloning into *B. subtilis* by using a bifunctional plasmid shuttle vector. Synthesis and transport across the cytoplasmic membrane was quite efficient in *B. subtilis* (Kreft et al., 1983).

Moreover, shuttle *E.coli-Bacillus* promoter-trap plasmid vector containing an improved *gfp* gene was constructed for identifying regulatory sequences of *Bacillus* which could be transferred into *B. subtilis* as well into *B. cereus* (Dunn and Handelsman, 1999). A shuttle *E.coli-Bacillus* superBAC1 vector with inducible copy number in *E.coli* was also developed for large-insert cloning (Handelsman et al., 2002). Other shuttle vectors with wider host-ranges have been made, one such vectors greatly extend the range of phenotypes that can be screened by providing a different cellular environment, but do function in other bacteria and enable gene expression that was absent in *E. coli* (Joseph et al., 2001; Martinez et al., 2004; Li et al., 2005; Wexler et al., 2005).

The constructed *B. subtilis* BC51 genomic cosmid library was stored in an ordered array followed by transfer of individual library colonies into the wells of coded archival 384-well master plates. These can subsequently be used as an archival source to replicate each clone separately into one or more working plates or filters. Thus, each clone in the library may be handled and assayed individually. Replication and transfer of the clones are performed preferably by laboratory robots. High-density nylon filters containing the immobilized DNA from 2300 clones were used for hybridization screening. In this way, the occurrence of different genes in the library was analysed, and a clone was identified with an insert that encompassed a 16.5 kb region harbouring complete bacilomycin L gene cluster (Hofemeister et al., 2004). These results suggest a powerful application of the constructed *B. subtilis* BC51 genomic cosmid library for the identification of single genes and complete gene clusters encoding the entire pathway for synthesis of secondary metabolites of interest.

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SYSTEMATIC AND APPLIED MICROBIOLOGY

Isolation and Characterization of Epiphytic Bacteria from the Phyllosphere of Apple, Antagonistic *in vitro* to *Venturia inaequalis*, the Causal Agent of Apple Scab

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Summary

Bacteria from the phyllosphere of an apple orchard in North Germany ("Altes Land"), transferred to non-chemical pest management ten years ago, were obtained in June and September 1995. One hundred and fifty epiphytic isolates were tested for inhibition of mycelial growth and conidial germination of *Venturia inaequalis*, the causal agent of apple scab. Twenty seven epiphytes displayed a distinct antagonistic activity *in vitro*. However, more than one third of these antagonists induced necrotic lesions on apple seedlings and hypersensitive reaction on tobacco. The other were ranked as to their efficacy. The antagonistic epiphytes were also taxonomically characterized by a polyphasic approach based on polyamine and fatty acid profiles and biochemical reactions. Eighteen Gram-negative strains belong to the genus *Pseudomonas* and two strains to the genus *Erwinia*. Six Gram-positive strains were allocated to the genus *Bacillus* and one to the genus *Curtobacterium*. Due to their strong inhibition of mycelial growth and conidial germination five strains of *P. fluorescens* and one strain of *B. pumilus* which display no plant-deleterious effects are suggested as potential biocontrol agents of *V. inaequalis*.

Key words: Apple scab – biological control – phyllosphere – epiphytic bacterial antagonists – polyphasic taxonomy – *Pseudomonas* – *Bacillus*

Introduction

Apple scab, caused by the fungus *Venturia inaequalis* (Cooke.) Winter amend. Aderhold (anamorph *Spilocaea pomi* Fries), is the most important disease of apple worldwide, and it very likely occurs in every country where cultivated apple (*Malus x domestica* Borkhausen) is grown. Although scab has a broad geographical distribution, it is a serious problem mainly in temperate regions with cool, moist weather in early spring (MCHARDY, 1996). Chemical control of the fungus *V. inaequalis* requires between 10 to 20 applications each season (KOLLAR, 1997) and consumes roughly two thirds of the total costs for chemical pest management in fruit growing (MCHARDY, 1996). Several factors make the development of biological scab control agents an attractive alternative to chemical control: the developing resistance against fungicides in *V. inaequalis* and the growing concern about effects of fungicides on food safety and environmental pollution. However, the main efforts in control of apple scab within the last decade have been aimed at modifications in the usage of fungicides (MCHARDY, 1996).

Biological control of apple diseases by epiphytic organisms is a strategy with great potential (SPURR and KNUDSEN, 1985). Ideally, indigenous bacteria should be used. The region "Altes Land" in Northern Germany, the largest fruit-growing area in Western Europe, was selected for isolation of potential antagonists. Epiphytic bacteria were isolated from leaves of apple trees in an orchard in Hechthausen neither treated with organic fungicides nor with copper- and sulphur-containing agents since 1989.

The apple scab pathogen *V. inaequalis* is a highly sophisticated parasite which must maintain a compatible relationship with its host for at least several days in order to establish a stroma and sporulate. Initial establishment of *V. inaequalis* on the leaf surface requires germination of arriving conida, formation of an appressorium followed by mycelial growth. Thus, inhibition of conidial germination and/or reduction of vegetative growth of *V.*

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inaequalis with foliar microorganisms appears attractive. Of course, rapid selection of efficient antagonists from a large collection of epiphytes (when available) is the initial step of such an approach. For this purpose screening in the laboratory is preferred to lengthy experiments with plants in the greenhouse. The selected antagonists were taxonomically characterized by a polyphasic approach according to WAYNE et al. (1987).

Materials and Methods

Isolation of epiphytes: In order to enlarge the ecological variety for sampling of potential antagonists, the orchard was devided in 14 different micro-habitats (see Discussion). Isolations were made from healthy and scab-infected leaves obtained from different cultivars of *Malus x domestica* (Gloster, Ingrid Marie, Holsteiner Cox, Jamba, Boskoop). Twenty leaves were collected randomly from each tree. The phylloplane bacteria were detached from the leaves by using the leaf-imprinting-on-agar-method. For this purpose apple leaves were cut with a corkborer (diameter 2 cm) into four disks, placed on tryptic soy broth (TSB) agar (Difco) containing 50 µg cycloheximide per ml. After incubation at 18 °C for 2 to 5 days in the dark, leaf pieces were removed. Alternatively, 5 to 10 g of cut leaves were submerged in sterile saline and homogenized with an Ultra-turrax. Suitable dilutions were plated on (TSB) agar and incubated as above. The resultant colonies were re-streaked three times to ensure purity, isolates were selected on the basis of their different colony morphology on TSB agar, and designated with small letters (Tab. 1), accordingly. For maintenance purified isolates were stored at 4 °C on TSB slants or transferred every six weeks.

Pathogen isolation and cultivation: The reference strain *Venturia inaequalis* DSM 1002 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig). Strain V71, isolated from leaf lesions of apple, was a gift of A. Kollar (Biologische Bundesanstalt, Dossenheim). Monoconidial isolates of *V. inaequalis* were isolated from sporulating scab lesions on leaves and fruits of different apple cultivars.

For the mycelium inhibition test the strain DSM 1002 or a mixed culture of recently obtained isolates of *V. inaequalis* were grown on potato dextrose agar (PDA, DIFCO) at 18 °C in the darkness for 2 to 3 weeks. Mass production of conidia of *V. inaequalis* was carried out exactly as described by PUTTOO and BASU CHAUDHARY (1988). The resulting suspension of harvested conidia was filtered through Miracloth (Calbiochem, pore size 22–25 µm) and diluted with sterile water to a concentration of 10^5 conidia per ml as determined with a hematocytometer.

Inhibition of mycelial growth: Antibiosis against *V. inaequalis* strain DSM 1002 or a mixed culture of 30 recently obtained isolates of *V. inaequalis* was examined by inhibition of radial growth using a bioassay of ZÄHNER (1965) modified to a dual culture test as follows. Two plugs of *V. inaequalis* (5 mm in diameter) were transferred onto PDA, one bacterial isolate was streaked in between. After a 2 weeks-incubation at 18 °C radial growth (mm) of *V. inaequalis* was measured using plates with *V. inaequalis* only as a control. The percentage of inhibition was calculated using the following formula:

$$G_v - G_{v+b} / G_v \times 100$$

where G_v is the growth of *V. inaequalis* alone, and G_{v+b} is the growth of *V. inaequalis* in the presence of the bacterial isolate. The tests were repeated twice.

Inhibition of conidial germination: One hundred microlitre of a mixture of equal volumes of conidia of *V. inaequalis*, adjusted to 10^5 per ml as above, and putative antagonistic bacteria, adjusted to 10^8 cfu per ml, were pipetted onto a microtiter-plate. After incubation at 18 °C for 48 h the settled conidia were resuspended before aliquots (10 µl) were withdrawn for microscopical observation. Conidia were considered as having germinated when the germ tube was at least half the length of the conidia. Total numbers and numbers of non-germinated conidia were recorded for each well. The percentage of inhibition were calculated using samples of *V. inaequalis,* treated in the same way but no bacteria added, as a control.

Pathogenicity tests: Hypersensitive reaction (HR) was assayed with tobacco plants (KLEMENT et al., 1990). Alternatively, induction of necrosis on apple seedlings, *Malus* x *domestica* cv Golden Delicious, was assayed by tissue infiltration using a high pressure sprayer (KLEMENT et al., 1990). The seedlings were examined for necrotic lesions after one week. Suspensions of the putative antagonists were adjusted to an optical density (OD_{578nm}) of 1.0 in the first and to an OD_{578nm} of 0.06 in the second series.

General tests for identification: The bacterial isolates that showed inhibition of both mycelial growth and conidial germination were submitted to Gram-differentiation (GERHARDT et al., 1994). Gram-positive strains were microscopically examined for sporulation, too. King's medium B was used for detection of fluorescent pigments (Klement et al., 1990). After growth at 27 °C, colonies were visualized for pigmentation and additionally examined in the dark for fluorescence with a ultraviolet lamp (366 nm). The biochemical features (API 20NE) of the fluorescent pseudomonads were examined according to the instructions of the manufacturer (API bioMerieux GmbH, Nürtingen, Germany), as were identification and probability calculation. The Enterotube II multiple-test system (Becton Dickinson and Company, Cockeysville Maryland 21030, USA) was used for identification of two yellow Gram-negative strains.

Chemotaxonomic analysis: Polyamines were extracted and determined according to SCHERER and KNEIFEL (1983). The concentrations of polyamines were calculated according to BUSSE and AULING (1988).

Fatty acid methyl esters were obtained from 40 mg cells scraped from Petri dishes by saponification, methylation and extraction using minor modifications of the method of MILLER (1982). The fatty acid methyl esters mixtures were separated using a model 5898A microbial identification system (Microbial ID, Newark, DE 19711 U.S.A.) which consisted of a Hewlett-Packard model 5980 gas chromatograph fitted with a 5% phenylmethyl silicone capillary column (0.2 mm × 25 m), a flame ionization detector, a Hewlett-Packard model 3392 integrator, Hewlett-Packard model 7673A automatic sampler, and a Hewlett-Packard model 216 computer (Hewlett-Packard Co., Palo Alto, California, U.S.A.) Peaks were automatically integrated and fatty acid names and percentages calculated by the Microbial Identification System Standard Software (Microbial ID). The gas chromatographic parameters were as follows: carrier gas, ultra-high-purity hydrogen; column head pressure 60 kPa; injection volume 2 µl; column split ratio, 100:1; septum purge 5 ml/min; column temperature, 170 to 270 °C a 5 °C/ min; injection port temperature, 250 °C; and detector temperature, 300 °C.

Results

Isolation and screening for antagonistic activity *in vitro*

One hundred and fifty bacteria corresponding to fifteen different colony morphotypes were obtained from

an orchard without chemical pest management. The majority of isolates was obtained by leaf-imprinting on agar. This procedure favoured growth of bacteria resident on the apple leaf surface. Roughly one tenth of the colony morphotypes originally observed on petri dishes from inoculation with homogenized plant leaves (possibly allowing the isolation of endophytic bacteria) were later lost during re-streaking for purification.

One fifth of the isolates exerted antibiosis against the reference strain *V. inaequalis* DSM 1002 in the dual culture-test (Table 1). The most distinct inhibition was shown by the eight isolates IBa4, IBa9, IBa10, IBa15, IBi44, IBi56, IIBk9, and IIBk16. The pathogenicity of the reference strain *V. inaequalis* DSM 1002 has not been re-examined during maintenance and was thus unknown. Therefore, the putative antagonists had to be confirmed in a test with strains of *V. inaequalis* resembling more closely an infectious natural population than using merely a single strain of *V. inaequalis* (QUIMET et al., 1997). Remarkably, the most efficient antagonists of the first assay were also able to inhibit radial growth of a mixture of 30 hypervirulent monoconidial cultures, recently isolated. Strain IBa 4 was an exception.

The build-up of scab in regions with conducive climates relies on the massive distribution of conidia from *V. inaequalis* caused by wind. This early conidial phase of infection (Fig. 1) is extremely sensitive. Consequently, another approach for screening of potential antagonists in the laboratory was conceived under the assumption that the progression of scab infections from germinating conidia of *V. inaequalis* should be susceptible to interfering microorganisms. Therefore, the antagonists were also examined to see whether they would be able to inhibit conidial germination of *V. inaequalis*. As strain DSM 1002 appeared to be unable to form conidia, the virulent isolate *V. inaequalis* strain V71 was used for this test. Some of the twenty five antagonists (Table 1) had a very high, others a moderate capability to inhibit conidial germination.

Table 1. Properties and identification of epiphytic bacteria antagonistic towards *Venturia inaequalis*.

Strain[a]	In-vitro inhibition (%) by selected bacteria of			Induction of		Production of		Identification
	mycelial growth of		germination of V71-conidia	HR[b]	necrosis on apple[c]	Endo-spores	fluorescent pigments	
	DSM 1002	a mixture						
IBa9	64	53	41	–	–	–	y.g.	*P. fluorescens*[d,f,g]
IBa10	64	61	83	–	–	–	y. g.	*P. fluorescens*[d,f,g]
IBa13	36	20	91	–	–	–	y. g.	*P. putida*[f,g]
IBa15	64	54	66	–	–	–	y. g.	*P. putida*[f,g]
IBi44	70	49	46	–	–	–	y. g.	*P. fluorescens*[d,f,g]
IBi58	32	n. i.	57	–	–/+	–	y. g.	*P. fluorescens*[d,f,g]
IIBk3	28	n. i.	81	–	–	–	y. g.	*P. fluorescens*[d,f,g]
IIBk16	58	38	93	–	–	–	y. g.	*P. fluorescens*[d,f,g]
IIBo43	12	n. i.	31	–	–	–	y. g	*P. fluorescens*[d,f,g]
IBi60	34	n. i.	89	+	+	–	b. g.	*P. syringae*[f,g]
IBi61	28	n. i.	70	+	+	–	b. g.	*P. syringae*[f,g]
IBa19	28	n. i.	89	+	+	–	b. g.	*P. syringae*[f,g]
IBa24	22	14	87	+	+	–	b. g.	*P. syringae*[f,g]
IBb31	50	11	86	+	+	–	b. g	*P. syringae*[f,g]
IBi46	38	9	33	–	+	–	b. g.	*P. syringae*[f,g]
IBi51	52	n. i.	46	+	+	–	b. g.	*P. syringae*[f,g]
IBi56	58	61	87	+	+	–	b. g.	*P. syringae*[f,g]
IBi59	36	n. i.	59	+	+	–	b. g.	*P. syringae*[f,g]
IBa4	66	n. i.	n.d.	–	–	–	–	*E. agglomerans*[e,f,g]
IBa5	24	n. i.	n.d.	n.d.	–	–	–	*E. agglomerans*[e,f,g]
IBa20	36	18	86	n. d.	–	–	–	*Curtobacterium* sp[f,g]
IIBk9	56	32	57	n. d.	–	+	–	*B. pumilus*[f,g]
IIBj47	25	17	67	n. d.	–	+	–	*B. mycoides*[f,g]
IIBj48	31	10	86	n. d.	–	+	–	*B. mycoides*[f,g]
IIBj49	29	27	69	n. d.	–	+	–	*B. mycoides*[f,g]
IBj63	36	44	49	n. d.	+/–	+	–	*B. mycoides*[f,g]
IBj64	48	27	51	n. d.	–	+	–	*B. mycoides*[f,g]

[a] Designation according to the period of sampling (Roman numerals: I – early, II – late season) and morphotype (small letters)
[b] Hypersensitive reaction (HR) on tobacco leaves
[c] Induction of necrosis on apple seedlings ('Golden Delicious') by tissue infiltration
[d] Identification according to API 20NE
[e] Identification according to Enterotube II
[f] Identification according to MIDI, also allowing allocation of strains IBa10, IBi58, IIBo43 to *P. putida*
[g] Identification supported by polyamine profile
Abbreviation: y. g – yellow-green; b. g. – blue-green; n. d.– not determined; n. i. – no inhibition.

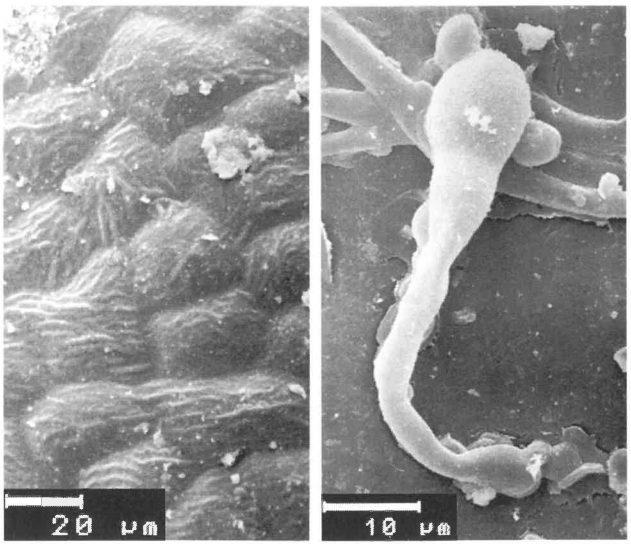

Fig. 1. Scanning electron micrograph of an uninfected (left) apple leaf surface (bar ≙ 20 µm) and a leaf surface with a germinated conidium of *Venturia inaequalis* (right) invading the plant cuticle with its appressorium (bar ≙ 10 µm).

Examination of antagonists for absence of plant-damaging effects

Antagonists would not be harmful to plants. Thus, the hypersensitive reaction (HR) on tobacco plants was used for quick detection of pathogenic pseudomonads and other Gram-negative bacteria (KLEMENT et al., 1990). Eight of the twenty Gram-negative isolates induced HR (Table 1). Using the additional tissue infiltration test the same isolates were found to induce necrosis on apple seedlings. Merely, isolate IBi46 was an exception (Table 1) inducing deleterious effects in the test with apple seedlings, only. The isolates IBi58 and IBj63 showed a variable reaction.

Taxonomic characterization

The twenty-seven bacteria shown to be antagonistic against *V. inaequalis* were further characterized by a polyphasic taxonomic approach. For a primary differentiation, the KOH-test (Suslow et al., 1982) and the common Gram-staining revealed twenty strains with a Gram-negative and seven strains with Gram-positive reaction. Six strains of the latter group were shown to produce endospores. Eighteen Gram-negative strains produced diffusable pigments on King's medium B (Table 1). The multiple-test system API 20NE identified the producers of an intense yellow-green pigment as *Pseudomonas fluorescens* but led to misidentification (see DISCUSSION) of the strains producing a blue-green fluorescent pigment. Two further Gram-negative isolates displayed a distinct yellow colony morphology. The results from the Enterotube II multiple-test system allocated both to *Enterobacter agglomerans* (a synonym of *Erwinia herbicola*).

Table 2. Polyamine pattern of antagonistic bacterial isolates.

Strain	Polyamines (µmol/g of d. w.)				
	DAP	PUT	CAD	SPD	SPM
I Ba 9	1.4	87.2	3.6	18.2	1.1
I Ba 10		78.9		7.8	
I Ba 13	0.7	95.9	3.7	19.0	
I Ba 15		90.2		22.9	
I Bi 44		83.7		10.3	
I Bi 58		19.1		3.5	
II Bk 3	2.1	44.8	4.9	4.0	
II Bk 16		74.4	0.9	17.6	
II Bo 43	2.8	61.2	24.5	18.9	
I Bi 60		64.6		6.4	
I Bi 61		68.2		12.5	
I Ba 19		67.4		7.6	
I Ba 24		87.8		17.3	1.0
I Bb 31	1.6	77.4		18.3	1.1
I Bi 46	1.7	86.5		16.0	1.4
I Bi 51		74.0		4.8	
I Bi 56		40.2		2.3	
I Bi 59		57.0		12.1	
IBa4	35.7	36.4	4.1	4.3	0.7
IBa5	13.5	25.2	3.4	3.2	
I Ba 20		1.3		0.5	1.3
II Bk 9				25.3	
II Bj 47				14.9	
II Bj 48				16.2	
II Bj 49				13.4	
I Bj 63				21.6	
I Bj 64				25.0	

Abbreviations: DAP – diaminopropane; PUT – putrescine; CAD – cadaverine; SPD – spermidine; SPM – spermine.

Chemotaxonomy is a rapid and simple means for reliable classification of new bacterial isolates to either the genus (WAYNE et al., 1987) or eventually to the species level. The polyamine approach was successful for differentiation of plant-associated Proteobacteria (AULING et al., 1991) and is also applicable to Gram-positive bacteria (BUSSE et al. 1996). Here, four different types of polyamine patterns were observed among the twenty-seven antagonists (Table 2). The eighteen producers of fluorescent pigments had a homogeneous profile characteristic of authentic pseudomonads (BUSSE and AULING, 1988; AULING, 1993). The two yellow strains IBa4 and IBa5 had diaminopropane and putrescine as major polyamines, a pattern observed previously in *Erwinia herbicola* (ZHEREBILO et al., 1997a;b). The Gram-positive strain IBa20 repeatedly displayed a very small polyamine pool with putrescine, spermidine and spermine. The remaining Gram-positive strains had significant amounts of spermidine, a polyamine profile reported for bacilli (HAMANA et al., 1989).

Two main groups were obtained from fatty acid analysis. Members of the first group, branching into the true pseudomonads (OYAIZU and KOMAGATA 1983) and the Enterobacteriaceae, synthesized unbranched saturated, unsaturated, and hydroxy fatty acids (Tab. 3).

Table 3. Fatty acids of Gram-negative antagonistic bacterial isolates (% fatty acid of total)[1].

	10:0 3OH	12:0 ALDE	12:0 2OH	12:0 3OH	12:0	ECL 13.96	14:0	ECL 14.50	14:0 3OH	16:1 cis-9	16:0	17:0 cycl	18:1 cis-11	18:0
IBa9	3.9		4.4	3.8	2.3					37.4	28.3	1.6	17.5	0.5
IBa10	4.7		5.1	4.6	2.3					37.0	28.9	2.0	15.6	
IBa13	4.5		5.1	4.8	2.2					35.3	29.7	3.1	15.3	
IBa15	4.5		5.3	4.7	2.2					35.5	29.8	3.3	14.8	
IBi44	3.7		4.5	4.3	2.3					37.2	29.4	2.0	16.3	
IBi58	4.5		5.2	4.9	2.8					36.3	28.4	2.2	15.7	
IIBk3	3.8		4.6	4.2	2.2					35.5	28.4	1.7	18.9	0.7
IIBk16	4.6		5.9	4.7	1.6					36.3	32.2	3.1	11.6	
IIBo43	3.0		4.5	4.1	2.5					35.1	28.6	1.3	20.9	
IBi60	3.1		3.2	4.4	4.7					40.3	27.1		16.2	0.8
IBi61	3.6		3.3	4.7	4.9					40.9	28.7		13.9	
IBa19[2]	2.7		2.7	3.3	4.2					36.9	28.3	0.8	15.6	4.9
IBa24[2]	2.8		2.9	4.2	4.4					40.4	25.9		17.8	0.9
IBb31[2]	3.2		3.1	4.2	4.5					39.8	28.6		15.7	0.7
IBi46[2]	2.9		2.9	3.8	4.6					41.8	26.3		16.2	0.5
IBi51[2]	2.8		2.9	3.9	4.4					38.4	29.0	0.6	17.1	0.8
IBi56	3.3		3.0	4.7	4.7					41.0	28.9		14.2	
IBi59[2]	3.5		3.3	4.7	4.7					38.9	27.7		16.2	1.1
IBa4		3.2			3.1		4.5	1.1	5.4	24.7	32.2	10.6	14.9	
IBa5		2.6			3.1	0.6	4.6	0.9	4.9	26.7	31.4	10.0	14.6	

The abbreviation for the fatty acids are illustrated by the following examples: 16:0, hexadecanoic acid (palmitic acid); cis-9 16:1, cis-9 hexadecenoic acid (palmitoleic acid); 3OH-12:0, 3-hydroxy dodecanoic acid (β-hydroxy lauric acid) anteiso 13:0, 11methyl dodecanoic acid.; cis-916:1 alc, hexadecenol.

[1] Only fatty acids > 0.5% are listed.

[2] These strains contain a 10-methyl 18:1 fatty acid (on average 1% of total fatty acids), in addition.

Members of the second group possessed iso- and anteiso branched acids as the principal fatty acids (Tab. 4). The pseudomonads were characterized by the 3-hydroxy decanoic/dodecanoic acid (10:0 OH-3 + 12:0 OH-3) and the 2-hydroxy dodecanoic acid (12:0 OH-2) as their diagnostic fatty acids. The pseudomonads were further divided in one subgroup with less than 4% of dodecanoic acid (12:0) and more than 1% of a cyclopropane fatty acid (17:0 cycl. 7) and another subgroup with more than 4% of dodecanoic acid and less than 1% of the 17:0 cycl.7 acid. Most strains of this subgroup had a fatty acid with ECL of 18.08, previously identified as 10-methyl 18:1 (AULING et al., 1986). The two members of the Enterobacteriaceae taxon had substantial amounts of the 3-hydroxy myristic acid (14:0-3OH) and some unknown lipids with an equivalent chain length (ECL) of 13.96 and 14.50, respectively. Relatively high amounts of the 17:0 cyclopropane fatty

Table 4. Fatty acids of Gram-positive antagonistic bacterial isolates (% fatty acid of total)[1].

	12:0 iso	12:0	13:0 iso	13:0 ante-iso	14:0 iso	14:0	15:0 iso	15:0 ante-iso	16:1 cis-9 alc.	16:1 iso	16:0 iso	16:1 cis-7	16:1 cis-9	15:0 iso 2OH	16:0	15:0 2OH	17:1 iso	17:1 ante-iso	17:0 iso	17:0 ante-iso	18:1 cis9
IIBa20						1.9		23.8			3.6	3.9			6.5					6.9	53.3
IIBk9			0.8		1.1	1.2	48.7	29.9			1.6	1.5			2.4		1.5		6.4	4.9	
IIBj47			10.2	4.2		4.8	29.3	2.4	3.0	1.7	6.2	3.4	1.8	6.9	5.8		11.5	4.5	4.4		
IIBj48		0.5	8.3	0.7	2.8	6.1	32.2	3.2	1.3	1.3	5.1	1.5	1.6	13.1	3.4	1.2	13.2		4.5		
IIBj49	1.3		12.0	1.6	4.8	3.9	26.0	3.4	3.9		7.6	5.2		7.3	6.5		9.8	1.9	4.8		
IBj63			8.8	1.3	3.4	5.0	29.1	4.4	2.1		5.1	4.3		6.3	7.7	0.8	13.1	2.3	5.3	1.1	
IBj64	0.8		9.6	0.7	4.0	4.6	30.4	2.2	3.1	1.1	6.4	4.8	1.0	7.1	5.7	0.5	11.2	2.8	4.0		

Abbreviations as for Table 3.

acid and the 7,8 methylene-hexadecanoic acid were found in addition.

The fatty acids profiles allowed identification of the fluorescent isolates either as *Pseudomonas fluorescens*, *Pseudomonas putida* or *Pseudomonas syringae* and of the two yellow strains as *Pantoea agglomerans (Enterobacter agglomerans, Erwinia herbicola)* with high correlation (Tab. 1).

A *Bacillus*-type profile was observed in six Gram-positive strains. The fatty acid pattern of strain IIBk9 was composed of iso/anteiso-branched fatty acids only allocating this isolate to *Bacillus pumilus*. The isolates IIBj47, IIBj48, IIBj49, IB63 and IBj64 had branched 2-hydroxy fatty acids and an unsaturated alcohol cis-9 16:1 in addition and were assigned to *Bacillus mycoides*. Finally, the deviating fatty acid pattern of strain IBa20 with iso/anteiso branched fatty acids but a high amount of oleic acid is quite diagnostic for some species of the genus *Curtobacterium*.

Discussion

Recovery of an orchard from a severe outbreak of apple scab after complete discontinuation of chemical pest management was observed (A. WREDE, personal communication). This encouraged a strategy of biological control through enhancing the establishment of indigenous microorganisms from this particular orchard. In the epidemiology of a leaf disease like apple scab the early and epiphytic steps of a pathogenic infection are important. This suggests to exploit antagonistic microorganisms from the phyllosphere of apple (but not from soil) as the most promising source for control of *V. inaequalis*. The availability of a large collection of indigenous epiphytes was a prequisite in our biological control programme but indispensably led to a first selection of potential antagonists in a rapid laboratory screening. Remarkably, the different isolates did not display the same antagonistic activity in the two tests applied here (Table 1). This emphasizes the importance of applying different selection methods to ensure that candidates with inhibitory potential are not discarded too early. Furthermore, the necessity to include virulent strains in the initial laboratory screening is indicated by our finding that some antagonists merely inhibited vegetative growth of the reference strain DSM 1002 but not of a mixture of recently isolated strains of *V. inaequalis*.

Biological control agents would not be plant pathogens. Lengthy greenhouse trials may be circumvented by another approach for rapid exclusion of plant-deleterious bacteria. Remarkably, fatty acid profiles identified a larger group of our antagonists as *P. syringae*, a well-known phytopathogen which embraces numerous pathovars (YOUNG et al., 1996). Here, the multiple test-system failed due to the absence of any *P. syringae* reference strain in the API 20NE analytical profile index. On the other hand, a good correlation of the conclusions drawn from both the fatty acid and polyamine data was observed.

Seasonal variation of the epiflora from the phyllosphere of apple has been reported by NAUMANN and GIERZ (1992) based on a three-years study using colony traits and pigmentation. Here potential biocontrol agents were sampled twice in 1995 and our taxonomic characterization revealed the presence of *P. syringae* only in the early season. Prevailence of members of the genus *Pseudomonas* in spring only and later decline of their population numbers appears to be a characteristic feature of the phyllosphere in German orchards (Steinbrenner, 1991, F. Berger, personal communication). This seasonal variation is of great interest for future design of biocontrol programmes for apple scab. Furthermore, our sampling procedure allowed to exploit ecological niches by defining micro-habitats combining spatial distribution of scab in the orchard under study and susceptibility of the five cultivars grown there, i. e., Holsteiner Cox and Boskoop are slightly, Jamba and Ingrid Marie are moderately and Gloster is highly susceptible. The underlaying idea was that even the microflora from apple leaves infected with scab to variable degrees might contain organisms with suitable antagonistic activities. We also thought to warrant biodiversity of the potential control agents due to this isolation from 15 micro-habitats. This aim was achieved as a variety of twenty seven bacterial antagonists belonging to different genera (*Pseudomonas, Erwinia, Bacillus,* and *Curtobacterium*) was obtained. The identification to species level of the three isolates allocated to *Erwinia* (*Pantoea*, GAVINI et al., 1989) and *Curtobacterium*, respectively, may be completed given confirmation of their antagonistic efficacy *in planta*.

It is of interest to compare our results with those of recent biocontrol studies (BURR et al., 1996; KALENICH and PADALKO; 1996; PADALKO, 1996). In the North American study, strains of *P. syringae* were the most efficient bacterial antagonists. In the Ukrainian studies, antagonistic strains of *B. cereus, B. subtilis, P. syringae*, and *P. putida* were reported. On the other hand, strains of *P. fluorescens* with an antagonistic potential against *V. inaequalis,* as isolated here from the phyllosphere of an apple, have not previously been reported.

The current investigation of our bacterial and fungal (FISS et al., 1999) epiphytes in greenhouse and field trials is confined to those antagonists which are not plant-deleterious. They constitute only a limited, but valuable pool (roughly 7%) within the indigenous epiflora of the particular orchard studied here.

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Isolation and characterization of epiphytic fungi from the phyllosphere of apple as potential biocontrol agents against apple scab *(Venturia inaequalis)*

Isolierung und Charakterisierung von epiphytischen Pilzen aus der Phyllosphäre von Äpfeln zur biologischen Bekämpfung des Apfelschorferregers *Venturia inaequalis*

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Summary

From the phyllosphere of an apple orchard in North Germany ("Altes Land"), which had been transferred to management without pesticide treatments 10 years ago, 107 epiphytic fungi were isolated within the season 1995. The observation of a relatively high number of antagonistic interactions within the fungal community of this non-sprayed apple phylloplane suggested exploitation of natural antagonists for biological control of apple scab. This concept was further supported by the finding of 25 antagonists of the apple scab pathogen in an *in vitro* screening. Among them, six isolates of *Aureobasidium, Botrytis, Cladosporium, Epicoccum* and *Fusarium* inhibited mycelial growth of *Venturia inaequalis* (Cooke) G. Winter more than 80 % and conidial germination more than 50 %. When applied to apple seedlings 24 h prior to inoculation with *V. inaequalis,* eight isolates effectively suppressed scab to a level comparable to the fungicide control. As these effective fungi of the class Hyphomycetes had no adverse effects on the plant, they are suggested as potential biocontrol agents against *V. inaequalis* in its biotrophic phase.

Key words: Phyllosphere; antagonism; apple scab; biological control of *Venturia inaequalis;* fungal antagonists; seedlings of cv. 'Golden Delicious'

Zusammenfassung

Aus der Phyllosphäre einer Obstanlage in Norddeutschland („Altes Land"), die seit 10 Jahren ohne jede Behandlung mit Fungiziden betrieben wird, wurden insgesamt 107 Pilze während der Saison 1995 isoliert. Die Beobachtung einer relativ hohen Anzahl antagonistischer Wechselwirkungen zwischen epiphytischen Pilzen aus dieser nicht-gespritzten Apfelphyllosphäre regte dazu an, Antagonisten zur biologischen Bekämpfung von *Venturia inaequalis* (Cooke) G. Winter zu nutzen. Dieses Konzept

erschien noch aussichtsreicher, nachdem bei einem *in vitro*-Screening 25 Isolate mit antagonistischer Wirkung gegen den Apfelschorferreger gefunden wurden. Davon zeigten sechs Isolate von *Aureobasidium, Botrytis, Cladosporium, Epicoccum* und *Fusarium* eine starke Hemmung des Myzelwachstums (über 80 %) und der Konidienkeimung (über 50 %). Bei einer Applikation auf Apfelsämlingen im Gewächshaus 24 h vor der Inokulation mit *V. inaequalis* reduzierten insgesamt acht der antagonistischen Isolate die Schorfentwicklung auf Apfelsämlingen im gleichen Maße wie die Fungizidkontrolle. Weil die wirksamen Pilze aus der Klasse der Hyphomyceten keine pflanzenschädlichen Effekte zeigten, bieten sie sich als Antagonisten zur biologischen Bekämpfung des Apfelschorferregers *V. inaequalis* in der biotrophen Phase an.

Stichwörter: Phyllosphäre; antagonistische Pilze; Apfelschorf; biologische Bekämpfung von *Venturia inaequalis;* Screening für epiphytische, pilzliche Antagonisten; 'Golden Delicious'-Sämlinge

1 Introduction

Apple scab, caused by the fungus *Venturia inaequalis* (Cooke) G. Winter amended Aderhold, is the economically most important apple disease around the world. If not controlled, the pathogen can cause extensive losses in regions with humid, cool weather during the spring months. Despite the tremendous amount of research on scab management (MacHardy 1996), its control still requires up to 20 fungicide applications annually (Kollar 1997). Independent of the cost of chemical control, seasonal plasticity of fungicide sensitivities (Schulz et al. 1986; Köller et al. 1995) is known and the long-term efficacy of fungicides is questionable. Resistance to benomyl, dodine, and sterol demethylation inhibitors has been detected in several countries (MacHardy 1996; Kunz et al. 1997). These findings increase the pressure for introduction of new fungicides and an assessment of the baseline sensitivity in *V. inaequalis* populations is reasonable in order to be able to monitor shifts in sensitivity levels after application (Kunz et al. 1998). Moreover, apple pesticide programmes have been shown to have a substantial quantitative and qualitative effect on non-target epiphytic micro-organisms (Andrews 1981), which might be antagonistic to foliar pathogens. Therefore, the application of natural microbial antagonists to control apple scab could be a promising alternative to application of synthetic fungicides. Two main approaches have been considered (reviewed in the Introduction by Quimet et al. 1997). The first, inhibition of the sexual stage of *V. inaequalis* for prevention of primary scab infection, was studied by the group of Andrews (Andrews et al. 1983; Heye and Andrews 1983) and others (Miedke and Kennel 1990). The second approach of inhibiting the asexual summer stage by foliar application of fungal antagonists has been initiated but was not successful with fungal antagonists (Boudreau and Andrews 1987). Recently, the effectiveness of epiphytic bacteria and yeasts from apple orchards as biological control agents against *V. inaequalis* has been described (Burr et al. 1996; Kucheryava et al., 1999). Epiphytic fungi from the phyllosphere of apple have been isolated with a focus on control of the postharvest pathogens *Botrytis cinerea, Monilinia fructigena* and *Penicillium expansum,* but not for control of apple scab (Falconi and Mendgen 1994).

Here, we report on the isolation and characterization of epiphytic fungi from a particular apple orchard which had been transferred from conventional fruit production to management without any fungicide treatments. The fungi of the epiphytic community obtained here share the same habitat and eventually compete for limited resources in order to survive. Such strategies for survival may be also defensive against *V. inaequalis* during an outbreak of apple scab. Therefore, it appeared necessary to determine first the antagonistic interactions within this specialized fungal community in qualitative and quantitative tests in the laboratory in order to learn whether such reactions are only rare events or occur frequently. The data obtained encouraged us to initiate an approach to the exploitation of our foliar fungal isolates as biological control agents. Subsequently, they were studied in culture and *in planta* for their ability to reduce the development of *V. inaequalis,* respectively its symptoms.

2 Materials and methods

2.1 Sampling of epiphytic fungi

Isolations were made from healthy and scab-infected leaves obtained from different cultivars of *Malus domestica* (Borkh.) cv. 'Gloster', 'Ingrid Marie', 'Holsteiner Cox', 'Jamba', 'Boskoop' using the leaf-imprinting-on-agar-method. Twenty leaves were collected randomly from each tree used for sampling in an orchard near Hechthausen in Lower Saxony twice (June, September) during the season 1995. From each leaf, four disks were cut with a corkborer (diameter 2 cm), placed on potato dextrose agar (PDA, DIFCO) containing 50 µg tetracycline per ml. After incubation at 18 °C for 2 to 5 days in the dark, leave pieces were removed. Alternatively, 5 to 10 g of leaf disks were submerged in sterile saline and homogenized with an Ultra-Turrax and suitable dilutions were plated and incubated as above. Small plugs were cut from the edge of the resultant fungal colonies and transferred to fresh PDA three times. Pure isolates were selected on the basis of their different colony morphology. For maintenance, the isolates were either transferred every 6 weeks or stored at 4 °C on PDA slants.

2.2 Test for detection of antagonistic interactions within the fungal epiflora

Antagonistic activity was assayed by using a modification of a test for screening of Actinomycetes for production of antibiotics (ZÄHNER 1965). Growth inhibitory activity of six epiphytic fungi per Petri-dish, cross-streaked against a central fungal strain preincubated for 3 days, was recorded after further incubation at 18 °C in the dark for 1 or 2 weeks.

2.3 Source of *V. inaequalis* and culture condition

V. inaequalis DSM 1002 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). The pathogenicity of this reference strain has not been re-examined and conidia formation was never observed during maintenance (G. STOSCHEK, Braunschweig, personal communication). Conidiation was also not observed in our laboratory neither by the procedure of WASSERMANN (1969) nor by the method of ZOBRIST and BOHNEN (1958). Therefore, *V. inaequalis* DSM 1002, grown on PDA at 18 °C and 3 weeks, was only used for screening of antagonistic fungi which inhibit mycelial growth of the apple scab pathogen.

Conidia of *V. inaequalis* for screening of antagonistic fungi, which inhibit conidial germination of the apple scab pathogen, were obtained by shaking apple leaves with sporulating scab leasions from different cultivars in sterile water for 15 min. Upon microscopical control, the resulting suspensions were highly enriched for conidia of *V. inaequalis* and almost free of contaminants. These conidial suspensions were filtered through Miracloth (Calbiochem) and diluted with sterile water to a concentration of 10^5 conidia per ml as determined with a hematocytometer. Monoconidial isolates of *V. inaequalis* were isolated from scab lesions on leaves and fruits of various apple cultivars.

2.4 Test for inhibition of mycelial growth of *V. inaequalis*

Antagonistic activity was examined by inhibition of radial growth of DSM 1002 in a dual culture test as described by MELGAREJO et al. (1985). Agar plugs of *V. inaequalis,* 5 mm in diameter cut from the edge of a 14-day-old culture, were transferred to the centre of assay plates (PDA). Two further plugs, excised from colonies of epiphytic fungi growing actively on PDA, were placed on both sides of *V. inaequalis* at a distance of 2 cm. Control plates contained *V. inaequalis* only.

Radial growth of *V. inaequalis* was measured after 3 weeks incubation at 18 °C. The percentage of inhibition was calculated using the following formula: $(G_v - G_{v+b} / G_v) \times 100$
where G_v is the growth of *V. inaequalis* alone, and G_{v+b} is the growth of *V. inaequalis* in the presence of the fungal isolate. All tests were conducted twice.

2.5 Test for inhibition of conidial germination of *V. inaequalis*

Plugs of epiphytic fungi, 4 mm in diameter, were placed in the centre of plates with Bacto agar (DIFCO) and incubated at 18 °C for 4 days. Around the resulting colony, 200 µl of the conidial

suspension of *V. inaequalis* adjusted as above was spread with a glass rod. The controls contained only conidia of *V. inaequalis*. Incubation at 18 °C was continued for further 48 h. Then 100 conidia were microscopically examined for germination. Numbers of non-germinated conidia were recorded and the percentage of inhibition were calculated. Conidia were considered as having germinated when the germ tube was at least half the length of the conidium.

2.6 Identification of antagonistic isolates

Fungal isolates were identified whenever possible to the species level according to Domsch et al. (1980). The identification of isolate IIPn24 was confirmed by the Centraalbureau voor Schimmelcultures (CBS, Baarn, the Netherlands).

2.7 Disease reduction on apple seedlings

Apple seedlings were grown from seeds collected from open-pollinated 'Golden Delicious' trees. Seeds were stratified in glass Petri dishes that were partially filled with moistened quartz sand at 4 °C for 2 months. Germinating seeds were transplanted into 8-cm diameter plastic pots with a peat-based substrate and grown for 3 weeks in the greenhouse at about 21 °C. When the seedlings had developed 4 or 5 leaves, they were used to test the antagonistic potential. The two youngest fully expanded leaves on each seedling were marked, then the suspensions of putative antagonists were sprayed onto the seedlings until runoff by means of an atomizer at a volume of about 0.3 ml per plant. The inoculum of hyphal propagules was produced from PDA-grown cultures (18 °C, 7 days) by homogenization with a sterile Ultra-Turrax as described by Pilz et al. (1991). In order to maintain high humidity for 24 h, sprayed seedlings were kept at 18 °C under polyethylene bags. Then they were inoculated with a mixture of *V. inaequalis* conidia. Mass production of conidia from monoconidial isolates was carried out in 250 capped prescription bottles on muselin cloth wetted in 10 ml of 4 % malt extract broth (DIFCO) according to Puttoo and Chaudhary (1988). Incubation was at 18 °C in the dark for 3 to 6 weeks. For harvest of conidia, the used medium was poured off and the capped bottles were agitated with 20 ml of H_2O. The resulting suspension was filtered through Miracloth (Calbiochem) and adjusted by dilution with sterile potassium phosphate buffer, pH 7.0, to a final concentration of 10^5 conidia per ml. A mixture of equal volumes of the 18 isolates of *V. inaequalis* was applied as inoculum of the pathogen to the apple seedlings which were kept again in polyethylene bags for an 48-h infection period. Then the bags were removed, the seedlings were further incubated at ambient temperature in the greenhouse for 21 days, and finally rated for scab severity. Each fungal antagonist was tested with four seedlings. Disease incidence was expressed as the number of seedlings infected. Controls were seedlings infected with *V. inaequalis* alone or treated with water, Omnex (Penconazol, curative, applied at 0.3125 g/l) and Dithane Ultra (Mancozeb, systemic, applied at 2.5 g/l), both supplied by Urania Agrochem GmbH (Hamburg).

The pathogenicity of the isolates IPm34 and IPm41 was tested on detached leaves from M9 rootstocks. Small plugs of mycelium were inoculated onto the leaf surface and incubated in polyethylene bags at 18 °C for 2 days.

3 Results

3.1 Antagonistic interactions within the fungal community of the phyllosphere

Hundred seven indigenous fungi were isolated from the phyllosphere of an orchard without pesticide management. The pure cultures obtained were designated according to the period of sampling and the thirteen colony morphotypes recognizable. A greater variety of morphotypes was obtained from sampling in early compared to late summer (eight versus five morphotypes) indicating seasonal variation of the fungal epiflora. Only the 56 isolates obtained in the second isolation series were studied in order to determine the amount and mode of antagonistic interactions within the fungal epiflora of the non-sprayed orchard (Table 1). Among 1540 combinations, a total of 8 % antagonistic interactions

was found. Most isolates inhibited mycelial growth of at least one or a few members of the epiphytic community. Some were found to over-grow most of the other isolates.

Table 1. Sources of fungal isolates and antagonistic interactions within the fungal community of the apple-phyllosphere

Tab. 1. Herkunft der Pilzisolate und antagonistische Wechselwirkungen innerhalb der pilzlichen Gemeinschaft aus der Apfelphyllosphäre

Fungal Isolate[a]	Source of isolation			Antagonistic action	
	Cultivar	Microhabitat[b]	Scab symptoms	Range[c]	Mode of action action[d]
IIPk1	'Ingrid Marie'	f	+		
IIPk2	'Holsteiner Cox'	r	+	2	IN
IIPk3	'Jamba'	r	+	1	IN
IIPk4	'Jamba'	r	–[e]		
IIPk5	'Jamba'	r	–[e]	3	IN
IIPk6	'Konferenz[P]'	r	+		
IIPk7	'Gloster'	G	++		
IIPk8	'Gloster'	G	–	1	IN
IIPk9	'Boskoop'	G	–	3	IN
IIPk10	'Boskoop'	G	–		
IIPt11	'Ingrid Marie'	f	–	3	IN
IIPt12	'Ingrid Marie'	f	–	2	IN
IIPt13	'Holsteiner Cox'	r	–	1	IN
IIPt14	'Jamba'	r	–	3	IN
IIPt15	'Holsteiner Cox'	f	+	3	IN
IIPt16	'Holsteiner Cox'	f	+	2	IN
IIPt17	'Holsteiner Cox'	r	+	3	IN
IIPt18	'Holsteiner Cox'	r	+	2	IN
IIPt19	'Jamba'	r	–[e]	1	NI
IIPt20	'Konferenz[P]'	r	+		
IIPt21	'Gloster'	G	++	32	OG
IIPt22	'Boskoop'	G	+	2	IN
IIPn23	'Ingrid Marie'	f	+	4	IN
IIPn24	'Ingrid Marie'	f	+	4	IN
IIPn25	'Holsteiner Cox'	f	+	4	IN
IIPn26	'Holsteiner Cox'	f	+	5	IN
IIPn27	'Jamba'	r	+	6	IN
IIPn28	'Jamba'	r	+	2	IN
IIPn29	'Gloster'	G	++	6	IN
IIPm30	'Holsteiner Cox'	f	–	52	OG
IIPm31	'Jamba'	r	–[e]	26	OG
IIPm32	'Jamba'	r	–[e]	35	OG
IIPm33	'Boskoop'	G	+	26	OG
IIPl34	'Ingrid Marie'	f	+	2	IN
IIPl35	'Holsteiner Cox'	f	–		
IIPl36	'Holsteiner Cox'	f	–	6	IN
IIPl37	'Holsteiner Cox'	r	–	3	IN
IIPl38	'Holsteiner Cox'	r	–	5	IN
IIPl39	'Jamba'	r	–		
IIPl40	'Ingrid Marie'	f	+	36, (5)	OG, (IN)
IIPl41	'Holsteiner Cox'	f	+	1	IN
IIPl42	'Holsteiner Cox'	r	+	2	IN
IIPl43	n. k.	n. k.	n. k.	4	IN
IIPl44	n. k.	n. k.	n. k.	2	IN
IIPl45	n. k.	n. k.	n. k.	1	IN
IIPl46	n. k.	n. k.	n. k.	4	IN

Continuation Table 1/Fortsetzung Tab. 1

IIPl47	n. k.	n. k.	n. k.	3	IN
IIPl48	'KonferenzP'	r	+	43, (2)	OG, (IN)
IIPl49	'Gloster'	G	++	3	IN
IIPl50	'Gloster'	G	++	2	IN
IIPl51	'Gloster'	G	–	5	IN
IIPl52	'Gloster'	G	–		
IIPl53	'Boskoop'	G	+	4	IN
IIPl54	'Boskoop'	G	+		
IIPl55	'Boskoop'	G	–	7	IN
IIPl56	'Boskoop'	G	–	3	IN

[a] Designation according to morphotype (small letters) and the period of sampling (I = early season, II = late season)
[b] The orchard was devided into three quarters: f = in front, r = in the rear, G = Glind (this area was extremly infested with apple scab)
[c] Only isolates from the late season were examined. The number of different isolates susceptible towards the antagonistic action of a particular strain is indicated while each isolate was tested against 55 other isolates
[d] Mode of action: ability to reduce mycelial growth (IN) or to over-grow (OG)
[e] Young leaves
[P] Pear-cultivar 'Conference'
Abbreviation: n. k. = not known

3.2 *In vitro* inhibition of *Venturia inaequalis*

The apple scab pathogen is a highly sophisticated parasite, and it must maintain a compatible relationship with its host for at least several days to establish a stroma and sporulate (MacHardy 1996). Thus, for a first screening the isolated phylloplane fungi were submitted to a dual culture-test to examine their inhibitory activity against mycelial growth of *V. inaequalis*. Twenty-three of the 107 isolates were able to inhibit radial growth on PDA (Table 2) of the reference strain *V. inaequalis* DSM 1002. A distinct inhibition was exerted by six fungi (IPk14, IPl24, IPm33, IPm34, IPm41, and IIPl34), which inhibited the growth of *V. inaequalis* by more than 80 % compared to the control.

The early phase of infection from conidia of *V. inaequalis* is thought to be extremely sensitive and thus progress of the infection on the plant leaves should be susceptible to interferring micro-organisms. Therefore, it appeared necessary to compare the growth-inhibiting ability of the antagonists identified in the dual culture-test also by their inhibitory effect on the conidial germination of *V. inaequalis*. Using a conidial suspension composed of monoconidial strains of *V. inaequalis*, a total of 21 out of 107 epiphytic isolates was able to inhibit germination of *V. inaequalis* (Table 2). The isolates collected in June 1995 had intermediate effects on the germination of the apple scab pathogen. Five isolates (IPk15, IPl20, IPl26, IPm30, and IPm40) suppressed conidial germination by at least 50 % compared to the control. Among the isolates from September 1995, only strain IIPn24 effectively suppressed conidial germination of *V. inaequalis*.

The 25 isolates displaying repeatedly the highest antagonistic activity against *V. inaequalis* in both *in vitro* tests were identified on the basis of their conidial states. As no sexual states were observed even under a variety of culture conditions, the antagonists apparently belong to the class of Fungi imperfecti. Nevertheless, the observed anamorphs allowed a tentative allocation of the antagonists to six different species and five genera (Table 2).

3.3 Control of scab on apple seedlings

The above *in vitro* tests had eliminated the bulk of organisms which showed neither inhibition of vegetative growth nor of conidial germination of *V. inaequalis*. As next step, the antagonistic activity of the reduced number of promising organisms had to be confirmed *in vivo*. For this purpose, 'Golden Delicious' seedlings were selected due to the susceptibility of this cultivar towards apple scab. The scab symptoms appear as lesions with indefinite margin of a netlike pattern of diffused fungal growth., mostly along the midrib and major lateral veins (MacHardy 1996). Eight of the 25 isolates that showed *in vitro* antagonism caused an effective reduction in scab on apple seedlings (Table 2). Of these, three were isolates that had efficiently suppressed mycelial growth or spore germination in the previous

Table 2. Effect of 25 selected fungi on mycelial growth and conidial germination of *Venturia inaequalis in vitro* and on scab severity on apple seedlings
Tab. 2. Einfluß von 25 ausgewählten Pilzen auf Myzelwachstum und Konidienkeimung von *Venturia inaequalis* und Schorfausbildung auf Apfelsämlingen

Isolate	Identification	Inhibition (%)[a]		Disease incidence upon apple seedlings[b]
		Mycelial growth	Conidial germination	
I Pk 8	*Cladosporium (herbarum)*	71	15	1
I Pk 9	*Cladosporium (herbarum)*	71	15	2
I Pk 10	*Cladosporium (herbarum)*	79	50	1
I Pk 14	*Cladosporium (herbarum)*	93	19	3
I Pk 15	*Cladosporium (herbarum)*	71	63	1
II Pt 14	*Cladosporium (herbarum)*	36	–	2
I Pl 18	*Epicoccum nigrum Link*	71	16	4
I Pl 19	*Epicoccum nigrum Link*	71	21	2
I Pl 20	*Epicoccum nigrum Link*	57	72	3
I Pl 21	*Epicoccum nigrum Link*	71	14	1
I Pl 24	*Epicoccum nigrum Link*	97	36	3
I Pm 28	*Epicoccum nigrum Link*	71	38	2
I Pm 29	*Epicoccum nigrum Link*	71	18	1
I Pm 30	*Epicoccum nigrum Link*	57	52	2
I Po 50	*Epicoccum nigrum Link*	50	50	1
II Pn 28	*Epicoccum nigrum Link*	75	42	4
II Pl 34	*Epicoccum nigrum Link*	89	–	2
I Pl 26	*Aureobasidium (pullulans)*	–	56	n.d.
I Pm 33	*Aureobasidium (pullulans)*	86	49	1
I Pm 40	*Aureobasidium (pullulans)*	–	54	n.d.
II Pt 13	*Aureobasidium (pullulans)*	43	10	2
II Pn 24	*Fusarium graminum*[c]	64	71	1
II Pn 26	*Fusarium* spp.	64	48	2
I Pm 34	*Botrytis cinerea*	86	–	2
I Pm 41	*Botrytis cinerea*	86	–	3

[a] Data calculated from two independent experiments.
[b] Numbers indicate the incidence of infections. 0 = no seedling with scab lesions, 4 = all seedlings with scab lesions. Total: four seedlings per isolate.
[c] Identified by CBS

in vitro tests: *Cladosporium* spp. (IPk15), *Aureobasidium* spp. (IPm33), and *Fusarium graminum* (IIPn24).

Finally, it was necessary to exclude any harmful effects of the putative antagonists. None of the antagonistic isolates (Table 2) displayed deleterious effects on apple seedlings, even not the two isolates IPm34 and IPm41, allocated to *Botrytis cinerea,* which developed distinct necrotic lesions on detached leaves from M9 rootstocks.

4 Discussion

Biological control of apple diseases by management of epiphytic organisms is a strategy with great potential (Spurr and Knudsen 1985; Andrews 1990). If successful colonization by non-pathogens is intended for foliar disease management, microbial interactions should be exploited using indigenous micro-organisms from the phyllosphere of a particular agricultural area. We have chosen the region "Altes Land" in Northern Germany, for two series of isolation of potential antagonists under the

assumption that different antagonistic abilities to control apple scab would be developed in the microflora of early and late summer. In retrospective, a greater antagonistic potential of the fungal epiflora collected in early summer is emerging from the laboratory and glasshouse screening (cf. Table 2). In addition, we learned that antagonists were obtained from leaves with and without scab symptoms. A particular apple orchard in Hechthausen, where treatments with fungicides had been discontinued 10 years ago, was selected for isolation. In view of a microbiologist, such an orchard constitutes an undisturbed habitat of epiphytic micro-organisms, many of which may be capable of influencing the growth of pathogens (Blakeman and Fokkema 1982). Our sampling procedure in late summer reflected the heterogeneity of this habitat by dividing the orchard into three quarters (see legend to Table 1). It is of interest that no antagonistic isolate was obtained from the particular microhabitat extremely infested with apple scab (Glind). Here, we focussed on fungi which constitute an important component of the microbial population of aerial plant surfaces (Dickinson and Bottomley 1980). Any approach to exploit antagonistic effects of foliar micro-organisms against the apple scab pathogen requires a basic understanding of the nature of their specialized community in the phyllosphere, i. e., the ability of a fungus to exist in a particular habitat such as on the surface of a plant organ is largely determined by its ecological relationship with other micro-organisms which may be beneficial or harmful. That our isolates from the non-sprayed apple orchard may be harmed or have their activities curtailed, an observation which provides an approach to the manipulation of fungi as biological control agents (Burge 1988), is shown by the data in Table 1. The amazingly high number of antagonistic interactions detected *in vitro* resulted from two different reactions, inhibition and over-growing. The first reaction appeared to be important for biocontrol of apple scab, too. The latter reaction, although striking, was not observed in the subsequent *in vitro* tests with *V. inaequalis*.

In spite of different methods for isolation of epiphytic fungi from the phyllosphere of apple (detachment by shaking, Falconi and Mendgen 1994; leaf-imprinting and blending with Ultra-Turrax, this study), the taxa of antagonistic fungi identified here were shown to be major members of the fungal community in the population studies of Falconi and Mendgen (1994). The use of a rich complex medium (PDA) in both studies finally led to isolates which can be cultivated and handled under laboratory conditions and are not too fastidious for the biocontrol approach envisaged. On the other hand, it is not amazing that different antagonists were obtained to some extent when isolation was started from leaf litter (Quimet et al. 1997).

From our collection of 107 fungal isolates, only 12 very effective fungi (11 %) were selected finally for their inhibitory activity against either mycelial growth or conidial germination of *Venturia inaequalis*. With regard to the percentage of antagonists which can be found in such screenings, our findings are in agreement with other published results (cf. extensive discussion of Quimet et al. 1997). The purpose of the two *in vitro* tests applied here was to eliminate those organisms in our collection which showed no inhibition of *V. inaequalis* in the laboratory so that a reduced number of promising organisms could be examined for the capability to suppress apple scab on plants. However, the two *in-vitro* tests provided different results. Although initially only those fungal isolates displaying at least mycelial inhibition of 30 % had been submitted to further screening for inhibition of conidial germination of *V. inaequalis,* we later learned that some antagonists (IPl26, IPm40), not detected in the first test, were able to inhibit the apple scab pathogen in the second *in vitro* test. Conversely, a number of isolates with distinct inhibition of mycelial growth (IIPl34, IPm34, IPm41, cf also Table 2), were unable to inhibit germination of conidia. Thus, different screening methods are recommended to ensure that candidates with antagonistic potential are not discarded too early in the selection procedure.

Obviously, the degree of inhibition detectable *in vitro* and *in vivo* was also not identical although a few isolates were found to be effective throughout (*Cladosporium* spp., IPk15; *Aureobasidium* spp., IPm33; *Fusarium graminum,* IIPn24). A number of isolates inhibited mycelial growth and conidial germination, but did not affect considerably the development of scab lesions on seedlings (*Cladosporium* spp., IPk14; *Epicoccum* spp., IPl24). The antagonistic potential of other isolates emerged only in the plant trials as they inhibited *V. inaequalis* merely at an intermediate level *in vitro,* but effectively suppressed scab development on seedlings (*Epicoccum* spp., IPo50).

In conclusion, the eight isolates which suppressed scab on apple seedlings at the highest level in the greenhouse are suggested as potential biocontrol agents of *V. inaequalis* as they had no adverse effects on the plants. It is promising that two of these antagonistic isolates were also able to suppress apple scab

in initial field trials (unpublished results). Clearly, further effort is required to demonstrate applicability of epiphytic organisms for management of apple diseases. In our view, a neglected perspective is that biological control using foliar antagonists may supplement not only chemical control of apple diseases but also substitute copper treatments in ecological management (Boss et al. 1998) which cause accumulation of this heavy metal in the environment.

The eight antagonists remaining from the above three-step screening protocol were allocated by their anamorphs to different genera of the class Hyphomycetes *(Aureobasidium, Cladosporium, Epicoccum, Fusarium).* Due to their additional adverse effects on plants, the antagonistic *Botrytis*-isolates appeared of less value and no further effort was put into an investigation of their teleomorph. *Aureobasidium pullulans* and *Cladosporium herbarum* have been described as plant-beneficial due to production of the plant growth hormone indole acetic acid (Bhatt and Vaughan 1962; Buckley and Pugh 1971). Likewise, antagonistic properties of members of both species directed against diverse plant-pathogenic fungi but not *V. inaequalis* have been reported (Newhook 1957; Baigent and Ogawa 1960; Pace and Campbell 1974; Blakeman and Fokkema 1982). *A. pullulans* produces antifungal compounds (Takesako et al. 1991) but it is less clear which role these compounds may play on the leaf surface. Members of *Epicoccum* have been successfully used on pruning cuts against the apple canker pathogen, the ascomycete *Nectria galigena* (Corke and Hunter 1979). Antifungal and antibacterial activities were found in strains of *Epicoccum purpurascens* (Mallea et al. 1991). Among the species of *Fusarium* which are known to produce a number of secondary metabolites (Nelson et al. 1983), *Fusarium roseum* has been described to be antagonistic against *Claviceps purpurea* (Mower et al. 1975). However, as to our knowledge, the species *Fusarium graminum* detected here has neither been studied for antagonistic properties nor for the production of secondary metabolites.

In view of the complexity of interactions which may occur on the surface of apple leaves, the mechanisms of antagonistic action of the fungal isolates identified by us need to be carefully studied before these isolates can be applied in biological control of *V. inaequalis.*

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Literature

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SYSTEMATIC AND APPLIED MICROBIOLOGY

Diversity of Polyamine Patterns in Soft Rot Pathogens and Other Plant-Associated Members of the Enterobacteriaceae

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Summary

Polyamine profiles of 91 pectolytic and other plant-associated strains from 30 taxa of the Enterobacteriaceae were obtained by gradient high performance liquid chromatography (HPLC). *Pectobacterium carotovorum*, basonym *Erwinia carotovora*, contained a high amount of putrescine and less diaminopropane. Diaminopropane was absent in *Pectobacterium chrysanthemi*, basonym *E. chrysanthemi*, whereas cadaverine was present in addition to the major compound putrescine. This chemotaxonomic difference reflects the deepest phylogenetic branching point within the recently emended genus *Pectobacterium* which lies between the two species *P. carotovorum* and *P. chrysanthemi*. Both important soft rot pathogens are easily distinguishable from each other and from the type species of the genus *Erwinia* as diaminopropane is the only major polyamine compound in *E. amylovora*. Chemotaxonomic heterogeneity is also emerging with respect to DYE's Amylovora group proposed in an early phytopathological concept.

Key words: pectolytic erwinias – *Pectobacterium carotovorum*, basonym: *Erwinia carotovora* – *Pectobacterium chrysanthemi*, basonym: *E. chrysanthemi* – *Erwinia amylovora* – chemotaxonomy – HPLC – polyamines – diaminopropane – putrescine

Introduction

Gram-negative, non-sporeforming, peritrichous, fermentative, and rod-shaped bacteria are united in the genus *Erwinia* as proposed by WINSLOW et al (1920) for plant-associated bacteria that are pathogens, saprophytes and epiphytes. The tendency to preferentially place strains of interest to phytopathologists into the genus *Erwinia* is reflected by an early phytopathological proposal to retain one genus in spite of its heterogeneity and separate the organisms into three groups, namely the Amylovora, Carotovora, and Herbicola group (DYE, 1968, 1969a,b).

E. amylovora is the type species of the genus (LELLIOTT and DICKEY, 1984) and a typical representative of Dye's Amylovora group, also referred to as „true erwinia" which cause dry necrosis or wilt disease on plants. Dye's Carotovora group is synonymous with the terms „soft rot erwinias" (PÉROMBELON 1991) or „pectobacteria" (ZHEREBILO, 1997) comprising those species which macerate plant tissues. Following this view *E. chrysanthemi* (LELLIOTT and DICKEY, 1984), and *E. cacticida* (ALCORN et al.,1991) were recognized as further species. Researchers' opinions about the number of existing *Erwinia* species which cause soft rot disease and their phylogenetic positions within the genus are very contradictory (WALDEE,1945; BRENNER et al., 1972, 1973; SKERMAN et al., 1980; LELLIOTT and DICKEY, 1984; ALCORN et al.1991). Recent phylogenetic efforts (KWON et al., 1997; HAUBEN et al., 1998; SPRÖER et al., 1999) culminated in the separation of the „pectobacteria" (ZHEREBILO, 1997) from the genus *Erwinia* and a reclassification leading to the emended genera *Erwinia* and *Pectobacterium* and to a new genus *Brenneria*.

Previous efforts to use polyamines as biomarkers of plant-associated bacteria revealed that this chemotaxonomic approach was helpful and indicated a great heterogeneity of members of the genus *Erwinia* and a polyamine composition different from other taxa (GVOZDYAK et al., 1982a, b, ZHEREBILO et al., 1984; GVOZDYAK et al., 1985; ZHEREBILO et al., 1985; GVOZDYAK and ZHEREBILO, 1990). However, within the course of these studies the limitations of the method used (paper electrophoresis, ZHEREBILO and VISHTALYUK, 1985) became obvious. Here we report on separation and identification of polyamines by high performance liquid chromatography (HPLC) with a focus on „soft rot erwinias" versus *E. amylovora.*

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Materials and Methods

Bacterial strains, media and growth conditions:

The description of strains studied in this work is given in Table 1. The pathovar designation of YOUNG et al. (1996) was applied for the plant pathogenic strains adapted with respect to the reclassification by HAUBEN et al. (1998). The pectolytic activity was examined according to GVOZDYAK et al., 1982a. The abbreviations of culture collections are as follows: ATCC = American Type Culture Collection, Rockville, Md.; CCM = Czechoslowak Collection of Microorganisms, Brno, Czechoslovakia; CFBP = Collection Francaise de Bacteriés Phytopathogènes. Station de Phytopathologie Végétale et Phytobactériologie, Institut National Reserche Agronomique, 49071 France, LMG = Culture Collection Laboratorium voor Microbiologie, Gent, Belgium; CPPB IMV = Collection of Phytopathogenic Bacteria of the Institute of Microbiology and Virology of Ukr. Acad. of Sci.; ICMP = International Collection of Microorganisms from Plants, Landcare Research, Private Bag 92170, Auckland, New Zealand, NCPPB = National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom.

Strains were cultivated in modified Omelianski mineral medium (0.1% K_2HPO_4, 0.1% $(NH_4)_2HPO_4$, 0.05% $MgSO_4$, 0.01% $CaCl_2$, 0.1% NaCl, 0.006% Fe_2SO_4, 0.5% glucose, 0.3% yeast extracts, 0.3% peptone). Strains of *E. carotovora* obtained from S. Priou were grown in King's medium B (KING et al., 1954). The bacteria were cultivated in 100 ml portions in 500 ml Erlenmeyer flasks on a reciprocal shaker at 160 r.p.m. and 28 °C. The biomass was harvested in the logarithmic phase of growth by low-speed centrifugation (5000 x g, 30 min), washed once with saline and lyophilized.

Extraction and analysis of polyamines:

Polyamines were extracted with perchloric acid from lyophilized cells and analysed as described by SCHERER and KNEIFEL (1983) with the modification according to BUSSE and AULING (1988) using a linear gradient of 40 to 85% acetonitrile-water for high performance liquid chromatography. The identity of separated polyamines was confirmed by comparing their retention times with those of authentic samples purchased from Sigma, Deisenhofen, Germany.

Results

HPLC analysis of polyamine pools in LMG 2404^T, the reference strain of *Pectobacterium carotovorum* subsp. *carotovorum*, basonym: *E. carotovora* subsp. *carotovora*, and sixteen other isolates from six different host plants allocated to this subspecies (Tab. 1) revealed putrescine as the dominant and diaminopropane as the next major compound. Minor amounts of spermidine were observed throughout. A similar content and polyamine composition was found in strains of *Pectobacterium carotovorum* subsp. *atrosepticum*, basonym: *E. carotovora* subsp. *atroseptica* (NCPPB 549, NCPPB 3386, NCPPB 3406, 36A, NB 1526), in *Pectobacterium carotovorum* subsp. *betavasculorum*, basonym: *E. carotovora* subsp. *betavasculorum*, (NCPPB 2795, NB 2122) and in *Pectobacterium carotovorum* subsp. *odoriferum*, basonym: *E. carotovora* subsp. *odorifera* (CFBP 1878, CFBP 3259, NB 1892). Merely, strain NCPPB 3390, received as *E. carotovora* subsp. *atroseptica*, had a deviating pattern with putrescine as the dominant and cadaverine as the next major compound. Thus, members of *Pectobacterium carotovorum* are chemotaxonomically homogeneous. Basically the same polyamine pattern was also found in another species of this genus, emended by Hauben et al. (1998), namely *Pectobacterium cacticidum*, basonym: *E. cacticida.*

A polyamine pattern different from *Pectobacterium carotovorum* was found in the other important species of pectolytic bacteria, *Pectobacterium chrysanthemi*, basonym *E. chrysanthemi* (Tab. 1), i. e., putrescine was the only major polyamine compound and diaminopropane was absent whereas cadaverine was found in addition. Four isolates received as *E. chryanthemi* pv. *dieffenbachiae* (G261 and ICPB ED 107) and pv. *dianthicola* (ICPB EC 174 and 258) contained a high amount of cadaverine but not exceeding that of the main component putrescine. Finally, the distinct amount of diaminopropane detected in three isolates tentatively identified as *E. cytolitica* (CPPB IMV 8449) or received as *E. chrysanthemi* pv. *zeae* (G147) and pv. *dieffenbachiae* (ICPB ED 102), respectively, excludes theses strains from *Pectobacterium chrysanthemi* (Tab.1). A polyamine pattern clearly different from that of *P. carotovorum* or *P. chrysanthemi* was found in the type strain of *Pectobacterium cypripedii*, basonym *Erwinia cypripedii*, with cadaverine as the main polyamine and putrescine and diaminopropane as the next major compounds.

The five strains of the type species of the genus, *E. amylovora* LMG 2024, NCPPB 595, NCPPB 8507, 427, and 554 displayed diaminopropane as the only major compound. Minor amounts of putrescine, cadaverine, spermidine, and spermine were also detected (Tab. 1). This polyamine pattern clearly distinguishes *E. amylovora* from the above species which cause soft rotting. However, when further strains from Dye's Amylovora group were analysed they showed a polyamine pattern different from the type species of the genus. For example, the type strains of the species *E. tracheiphila* (LMG 2906), *E. mallotivora* (LMG 2708), and *E. rhapontici* LMG (2688) had a nearly identical content and composition with diaminopropane and putrescine as the two major polyamines easily distinguishable from *E. amylovora*. Four additional isolates received either as *E. toxica* or *E. rhapontici* displayed a pattern with a high putrescine content characteristic of *Pectobacterium carotovorum* and may be misidentified. In addition, with exception of strain LMG 2698, members of *Brenneria*, studied here, *B. nigrifluens*, basynom *E. nigrifluens*, LMG 2694, *B. rubrifaciens*, basonym *E. rubrifaciens*, LMG 2709^T, LMG 2710 and ICPB ER 104, *B. paradisiaca*, basonym *E. paradisiaca* LMG 2542^T, and *B. salicis*, basonym *E. salicis* ATCC 29294, and *B. quercina*, basonym *E. quercina*, LMG 2724^T, ATCC 29282, CPPB IMV 8453 were also distinguished from *E. amylovora* by a high putrescine and a low diaminopropane content. Cadaverine and spermidine were also found in *Brenneria*. The strain received as *E. quercina* Ac.1 had no pectolytic activity and displayed an elevated level of putrescine and spermidine allowing an allocation to the authentic genus *Pseudomonas* (AULING, 1993), also suggested by the pattern

Table 1. Bacterial strains and polyamines patterns of *Erwinia* and related genera[a, b]

Species name	Strain	Host	Origin	Received from	Polyamines ((mol/g dry weight)				
					DAP	PUT	CAD	SPD	SPM
Erwinia:									
E. amylovora (Burrill 1882) Winslow, Broadhurst, Buchanan, Krumwiede, Rogers & Smith 1920	LMG 2024^T = ATCC 15580 = NCPPB 683	*Pyrus communis*	UK, 1959	J. De Ley	20.5	1.2	2.6	1.7	0.1
	NCPPB 595	*Pyrus communis*	UK	R. A. Lelliott	32.9	0.6	0.6	0.4	0.2
	427	*Pyrus communis*	USA	N. W. Schaad	40.7	1.9	3.5	3.7	
	554	*Pyrus communis*	USA	N. W. Schaad	39.1	4.3	3.2	4.4	
	NCPPB 8507				33,0	1,0	0,6	0,4	
	ICPB EA 164		USA	N.W. Schaad	7,3	29,0	1,0	3,2	
	ICPB EA 107		USA	N.W. Schaad	15,2	21,7	19,4	3,5	
	ICPB EA 177		USA	N.W. Schaad	9,5	20,9	38,2	4,2	
	ICPB EA 174		USA	N.W. Schaad	7,9	20,9	34,5	2,6	
	ICPB EA 114		USA	N.W. Schaad	t	26,1	43,6	2,5	
	ICPB EA 137		USA	N.W. Schaad	t	20,7	45,8	3,6	
	ICPB EA 176		USA	N.W. Schaad	t	21,9	23,0	4,8	
	ICPB EA 188		USA	N.W. Schaad	t	15,1	28,3	3,4	
	ICPB EA 210		USA	N.W. Schaad	t	23,2	42,7	4,8	
	ICPB EA 208		USA	N.W. Schaad		16,7	36,4	4,8	
E. amylovora pv. *rubi*	ICPB ER 124		USA	N.W. Schaad	14,4	16,1	24,9	4,0	
	ICPB ER 131		USA	N.W. Schaad	t	16,7	42,9	8,1	
E. tracheiphila (Smith 1895) Bergey, Harrison, Breed, Hammer & Huntoon 1923	LMG 2906^T = LMG 2707^T = ICMP 5845 = NCPPB 2452	*Cucumis melo*	USA, 1972	J. De Ley	6.5	8.0	2.7	2.1	0.4
E. mallotivora Goto 1976	LMG 2708^T = NCPPB 2851	*Mallotus japonicus*	Japan, 1975	J. De Ley	6.8	11.5	0.6	2.9	0.5
E. rhapontici (Millard 1924) Burkholder 1948c	LMG 2688^T = NCPPB 1578	*Rheum rhaponticum*	UK	J. De Ley	12.5	25.0	2.8	3.3	
	572			R. S. Dickey	2.9	25,6	1.7	1.8	0.3
E. toxica	CPPB IMV 8416	*Cucumis sativus*	Ukraine, 1967	A. P. Korobko	8,3	54,0		t	
	CPPB IMV 8417	*Cucumis sativus*	Ukraine, 1967	A. P. Korobko	6,3	58,6		t	
	CPPB IMV 8692	*Cucumis sativus*	Ukraine, 1967	A. P. Korobko	11,7	66,2		t	
Pectobacterium:									
P. carotovorum subsp. *carotovorum* [*Erwinia carotovora* subsp. *carotovora* (Jones 1901) Bergey, Harrison, Breed, Hammer& Huntoon 1923]	LMG 2404^T = CCM 1008^T = ATCC 15713 = CPPB IMV 8982	*Solanum tuberosum*	Denmark	M. Kocur	7,1	43,8		2,1	
	CPPB IMV 8989 = CCM 1012 = NCPPB 468	*Hyacihthus orientalis*		M. Kocur	8,9	45,1		2,4	
	CPPB IMV 8987 = CCM1009 = NCCPB 392	*Cucumis sativus*		M. Kocur	9,2	51,5			
	CPPB IMV 8985 = CCM 96 = NCPPB 547	*Persea americana*		M. Kocur	9,0	51,5		2,3	
	CPPB IMV 9026 = J2 = NCPPB 1744	*Daucus carota v. sativa*		Yu. Fomichov	t	71,0		t	

Table 1. (Continued).

Species name	Strain	Host	Origin	Received from	Polyamines ((mol/g dry weight)				
					DAP	PUT	CAD	SPD	SPM
	CPPB IMV 9028 = EC 153			Ag. Kaght	9,7	51,0		t	
	CPPB IMV 9031 = G 274			Yu. Fomichov	8,1	39,0	t	t	
	CPPB IMV 9032 = H2 = NCPPB 550	*Nicotiana tabacum*	USA	Yu. Fomichov	4,8	35,3		t	
	21 = NCPPB 3387= H. Maas Geesteranus (139), (serogroup 2)	*Solanum tuberosum*	Netherlands	S. H. De Boer	12,7	72,1	t	t	t
	197 =NCPPB 3495 = R. J. Copeman (E295), (serogroup 21)	*Solanum tuberosum*	British Columbia	S. H. De Boer	9,9	53,1	t	t	
	ICPB EC 208 =ATCC 495				t	33,6	t	t	t
	NB 2046		France	S. Priou	12.0	50.0	1.7	0.6	
	NB 87.25		France, 1987	S. Priou	11.0	51.0		2.0	
	NB CIP 009		Peru, 1977	S. Priou	15.0	50.0		2.0	
	NB H 1		Spain, 1989	S .Priou	18.0	87.0		1.4	
	NB 88.29		France, 1988	S. Priou	32.0	111.0		1.4	
	NB PM 2		Malawi, 1986	S. Priou	34.0	96.0		0.8	
P. carotovorum subsp. *atrosepticum* [*Erwinia carotovora* subsp. *atroseptica* (van Hall 1902) Dye 1969]	LMG 2386^T = NCPPB 549	*Solanum tuberosum*	UK, 1957	J. De Ley	37.7	69.9		3.1	
	36A	*Solanum tuberosum*	Byelorussia, 1974	A.N. Evtushenkov	3.8	38.8		1.0	0.3
	NB 1526	*Solanum tuberosum*	Schottland, 1957	S. Priou	4.0	118.0	1.0	4.0	
	31 = NCPPB 3390 = A.Kelman (SR8), (serogroup 1)	*Solanum tuberosum*	Wisconsin	S. H. De Boer		31,3	15,5	t	
	6 = NCPPB 3386 = R.J.Coreman (E17), (serogroup 18)	*Solanum tuberosum*	British Columbia	S. H. De Boer	t	54,7		3,1	
	198 = NCPPB 3406 = R.J.Coreman (E555), (serogroup 22)	*Solanum tuberosum*	British Columbia	S. H. De Boer	5,5	66,5		t	
P. carotovorum subsp. *betavasculorum* [*Erwinia carotovora* subsp. *betavasculorum* Thomson, Hildebrand & Schroth 1984]	LMG 2466^T = ICMP 4226^T = NCPPB 2795	*Beta vulgaris*	USA, 1971	ICMP	16.4	41.0	0.2	1.5	0.6
	NB 2122	*Beta vulgaris*	USA, 1972	S. Priou	30.0	110.0		4.0	
P. carotovorum subsp. *odoriferum* [*Erwinia carotovora* subsp. *odorifera* Gallois, Samson, Ageron & Grimont 1992]	LMG 17566^T = CFBP 1878^T = NCPPB 3839	*Cichorium intubus*	France, 1978	R. Samson	11.9	57.5		2.0	
	CFBP 3259	*Allium porrum*	France, 1980	R. Samson	31.0	69.4		3.2	
	NB 1892		France, 1981	S. Priou	10.0	72.0		4.0	0.5
P. cacticidum [*Erwinia cacticida* Alcorn, Orum, Steigerwalt, Foster & Fogleman 1991]	2N =CPPB IMV 671	*Cactus carnegia*		I. Lazar	10.3	57.9	1.5	2.6	1.1
P. chrysanthemi [*Erwinia chrysanthemi* pv. *chrysanthemi* Burkholder, McFadden & Dimock 1953]	NCPPB 402^T = LMG 2804	*Chrysanthemum morifolium*	USA	J. De Ley		63.0	8.1	2.2	0.8

P. chrysanthemi [E. *chrysanthemi* pv. *zeae* (Sabet 1954) Victoria, Arboleda & Muños 1975]	CFBP 2052 = NCPPB 2538 = LMG 2505	*Zea mays*	USA	R. Samson		62.7	1.4	2.8	
	NCPPB 1065 = CPPB IMV 8447 = LMG 2495	*Zea mays*	Egypt, 1961	I. Lazar		69.5	4.1		0,9
P. chrysanthemi [E. *chrysanthemi pv. dieffenbachiae*]	CPPB IMV 9041 =G 261	*Dieffenbachia picta*	Italy	Yu.K. Fomichov		41,7	31,6	3,5	
	ICPB ED 107		California	N. W. Schaad		44,6	25,0	2,8	
P. chrysanthemi [E. *chrysanthemi pv. dianthicola*]	ICPB EC 258 =NCPPB^T 453 = LMG 2485	*Dianthus caryophyllus*	UK, 1956	N. W. Schaad	t	40,7	16,2	2,4	
	ICPB EC 174	*Dianthus caryophyllus*	Denmark	N. W. Schaad		55,8	21,8	2,3	
P. chrysanthemi [E. *chrysanthemi pv. parthenii*]	CPPB IMV8484=NCPPB^T 516 = LMG 2486	*Parthenium argentatum*	Denmark, 1956	I. Lazar	t	31,4			
P. chrysanthemi [*E.chrysanthemi*]	G 174 = CPPB IMV 9038	*Beta vulgaris*	France	Yu. K. Fomichov		62.7	1.8	1.7	
	NCPPB 533 = EP3 =CPPB IMV 9037 = LMG 2921	*Philodendron sp.*	USA	Yu. K. Fomichov		72.8		7.3	
	G 147 = CPPB IMV 8448	*Zea mays*	Egypt, 1961	I. Lazar	15.0	75.0	0.4	1.3	0.3
	ICPB ED 102		Florida	N. W. Schaad	5,7	58,8		t	
P. chrysanthemi [*E. chrysanthemi* (*syn. E. cytolitica)*]	CPPB IMV 8449			I. Lazar	14.4	62.9	0.5	0.6	2.5
P. cypripedii [*Erwinia cipripedii*]	LMG 2657^T = CPPB IMV 8676 = NCPPB 3004^T	*Cypripedii sp.*	USA		8,9	10,1	23,1	2,9	
Brenneria:									
B. rubrifaciens [*Erwinia rubrifaciens* Wilson, Zeitoun & Fredrickson 1967]	LMG 2709^T = ICPB ER 103 = ATCC^T 29291		USA	N. W. Schaad	t	40,6	15,6	4,1	t
	NCPPB 2021 = LMG 2710	*Juglans regia*	USA, 1965	J. De Ley	0,3	41,0	4,4	2,0	0,5
	ICPB ER 104 =CPPB IMV 8670		USA	N. W. Schaad	t	71,3	9,9	4,7	t
B. nigrifluens [*Erwinia. nigrifluens* Wilson, Starr & Berger 1957]	LMG 2694^T = NCPPB 564^T	*Juglans regia*	USA, 1955	J. De Ley		55.2		3.6	
B. paradisiaca [*Erwinia paradisiaca* (Victoria & Barros 1969) Dickey & Victoria 1980]	LMG 2542^T =ICMP 2349 = NCPPB 2511	*Musa paradisiaca*	Colombia, 1973	ICMP		45.5	1.5	0.6	0.3
B. quercina [*Erwinia quercina* Hildebrand & Schroth 1967]	LMG 2724^T = ICPB EQ 101 = ATCC^T 29281			N.W. Schaad	7,1	28,8	2,1	3,9	
	Ac.1 = CPPBIMV 8452	*Quercus sp.*		I. Lazar	1.5	32.7	2.7	14.2	
	Ac.2 = CPPBIMV 8453	*Quercus sp.*		I. Lazar	4.8	20.9	8.7	1.8	1.0
	ICPB EQ 102 =ATCC 29282			N.W. Schaad		44,9		4,1	
B. salicis [*Erwinia. salicis*]	LMG 2698^T = ICPB ES 102 = ATCC^T 15712	*Salix sp.*	UK	N.W. Schaad	26,4	12,0	11,0	2,2	
	ICPB ES 4 = ATCC 29294	*Salix sp.*	UK	N.W. Schaad	7,1	28,7	t	t	

Table 1. (Continued).

Species name	Strain	Host	Origin	Received from	Polyamines ((mol/g dry weight)				
					DAP	PUT	CAD	SPD	SPM
Pantoea:									
P. agglomerans [*Enterobacter agglomerans, Erwinia herbicola*]	LMG 2660 = ATCC 33261		Belgien		56,0	40,0	3,0	7,0	
	LMG 2571		Belgien		66,0	31,0	3,0	7,0	
P. agglomerans pv. millettiae [*Erwinia milletiae*]	ICPB EM 102		USA	N.W. Schaad	7,1	28,2	8,4	2,9	t
	ICPB EM 116		USA	N.W. Schaad	15,2	6,6	22,6	2,6	
P. ananatis [*Erwinia ananas, Erwinia .uredovora*]	LMG 2665^T = ATCC 33244		Belgien		13,2	37,9	16,3	1,1	
	LMG 2676 = ATCC 19321		Belgien		14,4	28,7	10,7	2,4	
P. stewartii subsp. *stewartii* [*Erwinia stewartii*]	LMG 2715^T = ATCC 8199		Belgien		5,2	22,0	3,0	7,0	2,0
	LMG 2719		Belgien		9,9	10,1	6,8	4,1	
	ICPB ES 104			Corpilin	6,0	25,7	t	1,9	
P. dispersa	LMG 2601		Belgien		72,0	10,0	1,0	5,0	
Enterobacter:									
E. dissolvens [*Erwinia dissolvens*]	LMG 2683^T = NCPPB 1850				27,0	21,3	0,5		
Escherichia:									
E. coli (Migula) Castellani & Chalmers 1919	CCM 5172^T = ATCC 11775= LMG 2092	*Human*, urine		M. Kocur	0.7	46.6	9.1	7.2	0.3

[a] Strains and pathovars are designated according to Hauben L. (1998) and/or Young et al. (1996).
[b] Abbreviations: DAP, diaminopropane; PUT, putrescine; Cad, cadaverine; SPD, spermidine; SPM, spermine; t, trace amount.

of cellular fatty acids (O. ZHEREBILO, unpublished results).

Members of Dye's Herbicola group and further plant pathogenic species, now embraced by the genus *Pantoea*, although heterogeneous, displayed an overall pattern with high amounts of diaminopropane, putrescine and distinct amounts of cadaverine and spermidine (Tab. 1).

Discussion

The results obtained in this study revealed a greater chemotaxonomic heterogeneity of the plant pathogens within the Enterobacteriaceae than previously reported on the basis of paper electrophoresis (cf. Introduction section). The gradient HPLC applied here, allowed to identify diaminopropane and cadaverine as additional major polyamine compounds of the genus besides putrescine. Obviously due to a close electrophoretic mobility diaminopropane and cadaverine escaped detection during paper electrophoresis (ZHEREBILO and VISHTALYUK 1985). Nonetheless, the previous data for *Pectobacterium chrysanthemi* (ZHEREBILO et al., 1984) are still valuable with only minor corrections. The present chemotaxonomic analysis resolved two general polyamine patterns within the emended genus *Pectobacterium*, one typical of *P. carotovorum* and another one characteristic of *P. chrysanthemi* and *P. cypripedii*.

Our observation of a homogeneous polyamine pattern (high content of putrescine and diaminopropane) in the 28 strains of the four subspecies of *P. carotovorum* (Tab. 1) confirms previous reports on high relatedness within this species (GRAHAM, 1964; DYE, 1969a; BRENNER et al.,1973; THOMSON et al., 1981; MERGAERT et al., 1984; DE BOER and SASSER, 1986; VERDONCK et al. 1987; ZHEREBILO and GVOZDYAK 1992; 1994; ZHEREBILO and VISHTALYUK, 1991 a,b; GALLOIS et. al., 1992). Phylogenetic studies may resolve whether the exceptional strains (NCPPB 3390 and NCPPB 3406 received as *E. carotovora* subsp. *atroseptica*, and the isolates ATCC 495 and NCPPB 1744) which displayed a *P. chrysanthemi*-like pattern belong to this species.

The polyamine pattern of *P. chrysanthemi* is characterized by absence of diaminopropane and a high putrescine content. Diaminopropane was also not detected in *E. chrysanthemi* in the course of a larger study with mainly clinical isolates of Enterobacteriaceae (HAMANA, 1996). Thus two different laboratories suggest that absence of diaminopropane in the polyamine pools is characteristic of *P. chrysanthemi*. This conclusion may not be disproved by our finding that two strains of *P. chrysanthemi* had diaminopropane because strains CPPB IMV 8449 and G 147 differ also by cellular fatty acid pattern from *P. chrysanthemi* and are close to *P. carotovorum* (ZHEREBILO et al.1994a). Thus, the homogeneous polyamine pattern of *P. chrysanthemi* allows a rapid chemotaxonomic allocation of putative members to this species known to be heterogeneous with respect to phenotypic properties, plant specialisation (DICKEY, 1979, 1981). This difference is also confirmed by other criteria, i. e., cellular fatty acid patterns (VAN DER ZWET and SASSER, 1985; DE BOER and SASSER, 1986; ZHEREBILO and VISHTALYUK, 1987, 1991a, 1991b; ZHEREBILO and GVOZDYAK, 1992, 1994; ZHEREBILO et al., 1994a) and the type of fatty acids of lipid A (RAY, et al., 1986; BRADSHAW-ROUSE et al, 1988; ZHEREBILO et al., 1990; 1994b; Zherebilo; 1997), and only a moderate or even a low level of DNA-DNA homology (BRENNER et al., 1972, 1973 1974, 1977; GALLOIS et al., 1992). Likewise, a separate position of *P. chrysanthemi* may be indicated by the fact that it is the only species of the family lacking the common enterobacterial antigen (LE MINOR et al., 1972). Finally, the deepest phylogenetic branch (KWON et al., 1997, HAUBEN et al., 1998, SPRÖER et al., 1999) of the soft rot pathogens placed together in the emended genus *Pectobacterium* lies between the two species *P. carotovorum* and *P. chrysanthemi* as reflected by our polyamine data. Furthermore, the 16*S* rRNA based reclassification of *E. cypripedia* by HAUBEN et al., (1998) shows this species as next neighbour of *P. chrysanthemi* in contrast to treeing of KWON et al. (1997) and SPRÖER et al., (1999) who place it into clusters embracing *Erwinia* species. Remarkably, the polyamine pattern of *P. cypripedia* deviates from that of *P. chrysanthemi* and the other species of *Pectobacterium* but resembles those of members of the emended genus *Erwinia.*

Here we have shown that *P. chrysanthemi* differs significantly from *P. carotovorum* and also from *E. amylovora* and other species of the genus by polyamine composition. This clear picture is not abolished by the data of 12 strains from the ICPB received as *E. amylovora* because additional serological data and fatty acids pattern likewise confirmed both their interstrain heterogeneity and separate taxonomic position (unpublished). Above rapid discrimination within the Enterobacteriaceae the polyamine analysis may be of growing interest for practical purposes because this approach likewise allows rapid exclusion from or allocation to other plant-associated genera, namely *Pseudomonas, Xanthomonas,* and *Agrobacterium* (AULING et al., 1991, AULING; 1992, YANG et al., 1993; ZHEREBILO et al., 1997).

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ORIGINAL PAPER

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Genetic analysis of the biosynthesis of non-ribosomal peptide- and polyketide-like antibiotics, iron uptake and biofilm formation by *Bacillus subtilis* A1/3

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Abstract The *Bacillus subtilis* strain A1/3 shows exceptionally diverse antibiotic capacities compared to other *B. subtilis* strains. To analyze this phenomenon, mutants for the putative pantotheinyltransferase gene (*pptS*), and for several genes involved in non-ribosomal peptide synthesis and polyketide synthesis were constructed and characterized, using bioassays with blood cells, bacterial and fungal cells, and mass spectrometry. Among at least nine distinct bioactive compounds, five antibiotics and one siderophore activity were identified. The anti-fungal and hemolytic activities of strain A1/3 could be eliminated by mutation of the *fen* and *srf* genes essential for the synthesis of fengycins and surfactins. Both *pptS* - and *dhb* -type mutants were defective in iron uptake, indicating an inability to produce a 2,3-dihydroxybenzoate-type iron siderophore. Transposon mutants in the malonyl CoA transacylase gene resulted in the loss of hemolytic and anti-fungal activities due to the inhibition of bacillomycin L synthesis, and this led to the discovery of *bmyLD-LA-LB** genes. In mutants bearing disruption mutations in polyketide (*pksM* - and/or *pksR* -like) genes, the biosynthesis of bacillaene and difficidins, respectively, was inactivated and was accompanied by the loss of discrete antibacterial activities. The formation of biofilms (pellicles) was shown to require the production of surfactins, but no other lipopeptides, indicating that surfactins serve specific developmental functions.

Keywords Phosphopantotheine transferase (PPTase) · Lipopeptides · Polyketides · Bioautography · Mass spectrometry

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Introduction

Bacillus strains are known to display various anti-bacterial and anti-fungal activities (with or without hemolytic activities), which are attributed either to bacteriocins of ribosomal origin or to non-ribosomal metabolites, such as lipopeptides and polyketides (Zuber et al. 1993). Lipopeptides are amphiphilic molecules which vary in their peptide or fatty acid moiety (Stachelhaus et al. 2002). Among the lipopeptides produced by *B. subtilis* are the surfactins with seven, fengycins with ten, and the iturins (e.g. mycosubtilin, iturin A, bacillomycins D, L etc.) with seven L and D α-amino acid components. The lipid moiety varies in size from C13 to C17, giving rise to different isomers (*n, iso,* and *anteiso*). In surfactins and fengycins ring closure is achieved by formation of a lactone bond between the C-

terminal amino acid and the hydroxyl group of either the β-hydroxy fatty acid component (surfactins) or an internal tyrosine residue (fengycins). In iturins a peptide bond is formed between the carboxyl group of the C-terminal amino acid and the β-amino group of the β-amino acid residue. Surfactins show weak antibiotic but strong hemolytic and surfactant properties (Kowall et al. 1998), while fengycins are potent anti-fungal agents (Vanittanakom et al. 1986). The greatest molecular diversity is found among the iturins. Consequently, they differ in their hemolytic, anti-bacterial and/or anti-fungal properties (Landy et al. 1948; Peypoux et al. 1984; Eshita et al. 1995).

The biosynthetic apparatus for the production of these bioactive compounds in *B. subtilis* involves non-ribosomal peptide synthetases (NRPS), as well as fatty acid (FAS) and polyketide synthases (PKS) composed of repetitive building blocks called modules comprising 600 to 1300 amino acids. Each module contains highly conserved amino acid sequence motifs which define the catalytic domains for the recognition, activation, processing and condensation of its amino acid substrates in sequential reactions (for a review, see Stachelhaus et al. 2002). The core of each module is the amino acid adenylation domain characterized by the motifs SGTTGxPKG and TGD. These are believed to function in ATP binding and hydrolysis (Elsner et al. 1997; Marahiel et al. 1997). The peptidyl (PCP) and the acyl carrier protein (ACP) domains contain the highly conserved sequence motif [I/L]GG[D/H]SL at their thiolation sites; the invariant serine binds the 4′-phosphopantotheine cofactor (Leenders et al. 1998; Lambalot et al. 1996). This motif is specific for the modular thioester-forming PCPs of NRPS, as well as the ACPs of FAS and PKS enzymes, which catalyze stepwise thiolation reactions (Vater et al. 1997; Stachelhaus et al. 2002). The conserved motifs characteristic of NRPS and PKS modules have been employed to screen gene libraries using oligonucleotides (Borchert et al. 1992; Bernhard et al 1996; Sosio et al. 2000) or to design degenerate oligonucleotide primers for the PCR cloning of NRPS or PKS genes (Turgay and Marahiel 1994; Lee et al. 1998; Neilan et al. 1999; Sokolov et al. 2002) even from uncultured microorganisms (Seow et al. 1997).

All these NRPS and PKS synthetases are converted from the inactive apo-form into the active holo-form by a cognate 4′-phosphopantetheine transferase (PPTase) (Lambalot et al. 1996; Mootz et al. 2001). For these key reactions, bacteria usually employ one PPTase for post-translational modification of fatty acid synthases (FAS), while a different enzyme acts presumably on both NRPS and PKS. In *B. subtilis* these enzymes have been identified as the AcpS- (Mootz et al. 2001) and Sfp-type PPTases (Nakano et al. 1992; Lambalot et al. 1996), respectively. The synthesis of iturin A and mycosubtilin, unlike that of surfactins and fengycins, requires additional, specific, malonyl-CoA transacylases—ItuD and FenF, respectively (Duitman et al. 1999; Tsuge et al. 2001).

Large gene clusters usually encode the enzymes required for the biosynthesis of the lipopeptides produced by *B. subtilis*. The operons for surfactins, fengycins and iturins vary in size and modular composition. The surfactin operon encodes three multifunctional polypeptides (SrfAA, AB, AC) in *B. subtilis* (Menkhaus et al. 1993). In fengycin biosynthesis five such functional units, FenABCDE (Steller et al. 1999; Lin et al. 1999), cooperate, while iturin/mycosubtilin biosynthesis is based upon three synthetase components (Duitman et al. 1999; Tsuge et al. 2001). The dimodular synthetase DhbF and the proteins DhbB and DhbE together comprise the multienzyme system that catalyzes the formation of the iron siderophore bacillibactin, which is a catecholic 2,3-dihydroxybenzoate (DHB)-glycine-threonine (May et al. 2001). A number of polyketide-like antibiotics similar to those produced by *Gluconobacter* sp. (the enacycloxins) or *B. aurantininus* (the aurantinins) have been described for various *B. subtilis* strains (see Patel et al. 1995). Their mode of synthesis and the structures of the corresponding genes are still unknown, but the latter are probably related to the large *pks* gene cluster of *B. subtilis* 168, which is inactive in this organism (Kunst et al. 1997).

The objective of the present study was to explore the exceptional potential of *B. subtilis* A1/3 (Huber et al. 1991; Griesbach and Lattauschke 1991) for the production of peptide and polyketide compounds using genetic methods in combination with bioautographic and mass spectrometric analyses.

Materials and methods

Strains, plasmids and growth conditions

The *B. subtilis* strains and mutants used are listed in Table 1. Plasmids are listed in Table 2. Other strains used were: *Escherichia coli* XL1-Blue [*rec*A1 *end* A1 *gyr* A96 *thi* -1 *hsd* R17 *sup* E44 *rel* A1 *lac* [F′ *proAB lacI* ^{q} *Z*ΔM15 Tn*10* (Tc^{r})], *B. megaterium* PV361 (P. Vary), *Agrobacterium tumefaciens* ATCC 2215, *Clavibacter michiganensis* subsp. *michiganensis* (a streptomycin-resistant mutant; E. Griesbach), *Proteus vulgaris* (from our laboratory collection), *Penicillum chrysogenum* (DSM 844), *Fusarium oxysporum* (DSM 62059), and *Paecilomyces variotii* Tü137 (H. P. Fiedler). Growth media (TBY, SMM) and culture conditions were as described by Chu et al. (2002). For antibiotic production cells were cultured in ACS or in Landy medium (Landy et al. 1948; Vanittanakom et al. 1986) and liquid cultures were grown on a rotary incubator at 32°C for 48 h. The following antibiotics were added to cultures for plasmid selection: for *B. subtilis*, erythromycin (Ery) at 3 μg/ml; chloramphenicol (Chl) at 5 μg/ml; spectinomycin (Spc) at 100 μg/ml, and kanamycin (Kan) at 10 μg/ml; and for *E. coli*, ampicillin (Amp) at 100 μg/ml; Ery at 70 μg/ml; Chl at 10 μg/ml and Kan at 20 μg/ml. The fungal cul-

Table 1 *Bacillus subtilis* **s** trains and mutants used in this study

Strain	Relevant characteristics	Description/proposed mutant gene/reference
ATCC 39320	Wild type, difficidin producer	Zweerink and Edison (1987)
A1/3	Wild type, isolated from tomato hydroponic cultures	Huber et al. (1991)
GB709	Plasmid cured derivative of *B. subtilis* A1/3	Stein et al. (2002)
GSB26	*str* ^{r} mutant of QB1133*amyE aroI906 metB6 sacA321 sfp*^{0}	Steinborn and Hofemeister (1984)
RM125	*r* $^{-}$$_{M}$*m* $^{-}$ $_{M}$*leuA8 arg15*	Uozumi et al. (1977)
GB786	GB709 disruption mutant *pptS*::pME520	*pptS*
BC13	GB709 disruption mutant *srfA*::pMEsd13 (F200r)	*srfA*
BA2444	GB709 transposon mutant *srfA*::Tn24-44	Tn24-44 at codon 3166 of *srfA*
BC7	GB709 disruption mutant *srfB*::pMEsd7 (F229r)	*srfB*
BC51	GB709 disruption mutant *srfC*::pMEsd51 (F7br),	*srfC*
BA3-1	GB709 transposon mutant *srfB*::Tn3-1	Tn3-1 at codon 3513 of *srfB*
BC85	GB709 disruption mutant *fen4*::pMEsd85 (SD85)	*fen4*
BC10	GB709 disruption mutant *fen4*::pMEsd26-5a (FA20u)	*fen4*
BA13-20	GB709 transposon mutant *bmyLD*::Tn13-20	Tn13-20 inserted at codon 85 of *bmyLD*
BA13sd7	BA13–20 transformed with DNA of BC7; *bmyLD*::Tn13-20 *srfB*::pMEsd7	See BA13-20 and BC7, respectively
BC35	GB709 disruption mutant *dhbF*::pMEsdII (F238u)	*dhbF*
BC72	GB709 disruption mutant *dhbF*::pMEsd72 (F18r)	*dhbF*
BC145	GB709 disruption mutant *pksM*::pMES7 (F145r)	*pksM*
AR9	GB709 disruption mutant *pksR*::pKS7a (F44r)	*pksR*

Table 2 Plasmids used in this study

Vector	Characteristics	Description/reference
pMOS *blue*	Amp^{r}	Amersham Pharmacia Biotech
pKS	pBlueskript II KS(+), Amp^{r}	Stratagene
pE194ts	Ery^{r}, integration plasmid	Horionuchi and Weisblum (1982)
pGB354	Chl^{r}	Behnke and Gilmore (1981)
pPS7	Ery^{r}, positive selection vector	Steinborn (1998)
pIC333	Amp^{r}, with Spc^{r} on mini-Tn*10*	Petit et al. (1990)
Super Cos 1	Amp^{r}, Kan^{r}	Stratagene
pPL152	pPS7 with ~22-kb genomic DNA of *B. subtilis* A1/3	This study
pGBA4	pGB354 with ~4.5-kb *srf-pptS* DNA of the pPL152 insert	This study
pMPA4	pMOSblue with ~4.5-kb *srf-pptS* DNA of the pPL152 insert	This study
pTn13-20	pUC18 with excision DNA of transposon mutant BA13-20	This study
pSC9i10	Super Cos 1 with 16.5-kb of genomic DNA of *B. subtilis* A1/3	This study

tures were grown at 30°C on either malt or potato dextrose agar (Difco).

Antibiotic production

The supernatant of Landy cultures was extracted twice with a 1/4 volume of *n* -butanol under vigorous agitation for 16 h at room temperature. The butanol phases were collected by centrifugation, combined and dried in a vacuum centrifuge. The extract was then dissolved in a 1/20 volume of distilled water or methanol. Samples of 10 µl were dispensed into 6-mm wells in indicator plates which were prepared as follows: 40 ml of TBY- or potato dextrose-agar was poured into quadratic 120×120×17 mm dishes (Greiner Labortechnik GmbH) and overlaid with 10 ml of soft agar (0.7%) containing indicator bacteria (~10^{7} cfu/ml) or fungal spores (~10^{6}/ml). The indicator bacteria were from overnight cultures. The fungal mycelium and spores were harvested from malt agar cultures with phosphate buffer. The mycelium was disrupted before further use by vigorous agitation in the presence of glass beads (2 mm diam.).

Hemolysis

Aliquots (10–20 µl) of culture supernatant or dissolved extracts were added to 6-mm-wells containing indicator Columbia sheep blood agar (Oxoid), incubated overnight at 30°C and subsequently stored for clearing of halo zones at 4°C. As a quantitative assay, a modification of the spectrometric method of Vanittanakom et al. (1986) was employed. Undiluted or diluted 1.4-ml samples of culture supernatant were mixed with 0.1 ml of defibrinated sheep blood. The mixture was incubated for 1 h at 37°C and subsequently centrifuged for 10 min at 6.175× *g* and 4°C, and the absorbance of the supernatant at 546 nm was measured. A water sample was used to achieve 100% lysis. This test requires a blood sample (0.1 ml blood in 0.9%-NaCl) with a supernatant absorbance of less than 0.2.

Iron uptake

Growth of strains in TBY medium supplemented with the iron-chelating compound ethylenediamine-N,N′-diacetic acid (EDDA, Sigma) at 12 mM were conducted to test siderophore activities for iron uptake.

Bioautography

The *n* -butanol extracts of Landy cultures were dissolved in 1/20 vol of methanol. Then 1–10 µl aliquots of undiluted or diluted sample were spotted on silica gel 60 F_{254} TLC aluminium sheets (20×20 cm; Merck, Darmstadt) and the chromatograms were developed at room temperature with chloroform-methanol-water (65:25:4, v/v). The sheets were then air-dried and photographed under UV light (254 nm). The lanes were cut into 10–15 mm-wide strips, and these were placed on the surface of Columbia blood agar or bacterial indicator plates (see below) for 2 h at room temperature, or inserted into grooves cut into the top-agar layer of fungal indicator plates and incubated at 30°C (see below). The plates were then inspected for localized zones of growth inhibition, indicating the presence of antibiotic activity associated with the test compounds. The bioautographs were repeated at least three times. The data presented in the Figures are from one representative experiment and the R_f values given in Fig. 1 were within 5–10% of the averages.

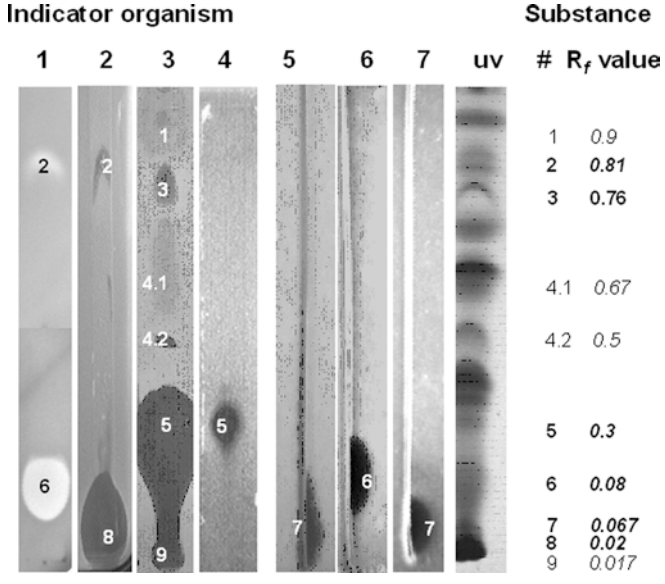

Fig. 1 Bioautographic detection of hemolytic and antibiotic activities of *B. subtilis* A1/3 after thin-layer chromatography. A butanol extract of Landy cultures was dried under vacuum and dissolved in methanol. Aliquots (5 µl) of the extract were spotted on TLC sheets and processed in parallel. The air-dried chromatogram (uv) of the extract was photographed under UV light. Parallel strips were applied to the following indicator plates: Columbia blood agar (1), *C. michiganensis* (2), *B. megaterium* (3), *A. tumefaciens* (4), *Paecilomyces variotii* Tü 137 (5), *Penicillium chrysogenum* (6), and *Fusarium oxysporum* (7). Lane 1 shows the separately incubated top and bottom parts of a strip. The white and black halos seen in the photographs are due to the illumination conditions. The R_f -values of antibiotic spots of putative substances #1 to #9 are indicated

Recombinant DNA techniques

The methods employed were as described by Chu et al. (2002). The TGD/LGG oligonucleotide primers were used according to Turgay and Marahiel (1994). Other oligonucleotide primers used are listed in Table 3. PCRs were run in a ThermoCycler GeneAmp 2400 (Perkin Elmer) with chromosomal DNA from strain GB709. PCR fragments were cloned in pMOS *blue* or Bluescript II pKS(+) vectors as listed in Table 2. Nucleotide sequences were determined on both strands, either in Gatersleben (using an Applied Biosystems 373S sequencer) or by AGOWA Gesellschaft für molekularbiologische Technologie mbH in Berlin. Sequence data were evaluated by online database scanning using the NCBI BLAST software (http://www.ncbi.nlm.nih.gov/) or the SubtiList World-Wide Web Server (http://genolist.pasteur.fr/SubtiList/). The multiple protein alignment was constructed with the Alignment Workspace MegAlign included in the LASERGENE software (DNASTAR Inc.). ORF structures and sequence motifs were analyzed using the Vector NTI v4.01 Deluxe software (InforMax Inc.). Southern blotting and hybridization were performed according to the protocol supplied with the ECL Random Prime Labeling and Detection kit (Amersham Life Science).

Gene disruption

The disruption mutants used are listed in Table 1. They were constructed by protoplast transformation of strain GB709 using integrative (pME/pKSE) plasmids. With the exception of pKSE-7a, the integrating DNAs with homology to NRPS- and PKS-like genes used for construction of disruptants were selected from a pool of DNA fragments which were generated by PCR amplification with the TGD/LGG primer pair, cloned in the pMOS *blue* vector and sequenced. To obtain the plasmid pKSE-7a, a 114-bp *Mbo* I subfragment of an 0.7 kb-DNA segment that was PCR amplified using the oligonucleotide primers CSM/RSM (Table 3) was inserted into the Bluescript II pKS(+) vector. The *E. coli* plasmids were linearized and ligated with *Pst* I- or *Xba* I-digested pE194 (a plasmid which contains a temperature-sensitive *Bacillus* origin), generating the integrative shuttle plasmids listed in Table 4. These were used for protoplast transformation of strain GB709 by selection at 32°C. Integrative recombination was achieved after repeated rounds of cultivation at 50°C and selection on erythromycin-containing medium (Conrad et al. 1992). Since semi-specific recombination due to the modular structure of synthetase genes (Schwartz et al. 1996) could

Table 3 Oligonucleotide primers used

Primer	Sequence (5′ → 3′) [a]	Target
CSM	TGYTCCATGGCIATHGCCGGAGGGGTCAAYCTIAC	F44-7a
RSM	YGGACGIGGYTTNCTYAAHTAGTA	F44-7a
FFA	ATCTCATGAACAATCTTGCC	*bmyLD* DNA
FFS	ACCGGTGTAATACAGTCTG	*bmyLD* DNA
PKM3	GCGCCTGCATCCCTTCCGCCCG	F145
PKM5	GATATGATAGCTGAATGCCTTG	F145
SFP1	AARGARGAYGCYCAYCGIACYCTSCTSGGIGAC	M520 *sfp* DNA
SFP6	GAARTCSGGRTGIGCIGCRCASACIGCCAT	M520 *sfp* DNA

[a]The IUPAC-code is used to designate alternative nucleotides

Table 4 PCR DNA and integrative plasmids used for mutant constructions

Plasmid	Integrative plasmid	Insert size (homology) [a]	Reference[b], GenBank Accession No.	Mutant[c]
pM520	pME520, Amp^r, Ery^r	~500 bp (codons 49–107 of PptS)	AF233756	GB786
pMOS-sd13	pMEsd13, Amp^r, Ery^r	~471 bp (codons 842–999 of SrfA)	F200r, AF520858	BC13
pMOS-sd7	pMEsd7, Amp^r, Ery^r	~480 bp (codons 840–1000 of SrfB)	F229u, AF520861	BC7
pMOS-sd51	pMEsd51, Amp^r, Ery^r	~510 bp (codons 839–1009 of SrfC)	F7br, AF520864	BC51
pMOS-sd85	pMEsd85, Amp^r, Ery^r	~485 bp (codons 846–1007 of Fen4)	FA23r, AF520855	BC85
pMOS-sd26	pMEsd265a, Amp^r, Ery	~146 bp (codons 3548–3596 of Fen4)	FA20r, AF520856 [d]	BC10
pMOS-sdII	pMEsdII, Amp^r, Ery^r	~469 bp (codons 838–994 of DhbF)	F238u, AF520839	BC35
pMOS-sd72	pMEsd72, Amp^r, Ery^r	~292 bp (codons 1998–2095 of DhbF)	F18r, AF520840	BC72
pMOS-s7	pMEs7, Amp^r, Ery^r	~3.5 kb (codons 679–1736 of PksM)	F145, AF520841	BC145
pKS-7a	pKSE7a, Amp^r, Ery^r	114 bp (codons 797–835 of PksR)	F44, AF520843	AR9

[a]The size of the DNA and the homology of the deduced protein is indicated
[b]Refer to the DNA sequence in Fig. 2
[c] see Table 1
[d]The target DNA sd26 represents a 5′-terminal 146-bp subfragment of FA20r

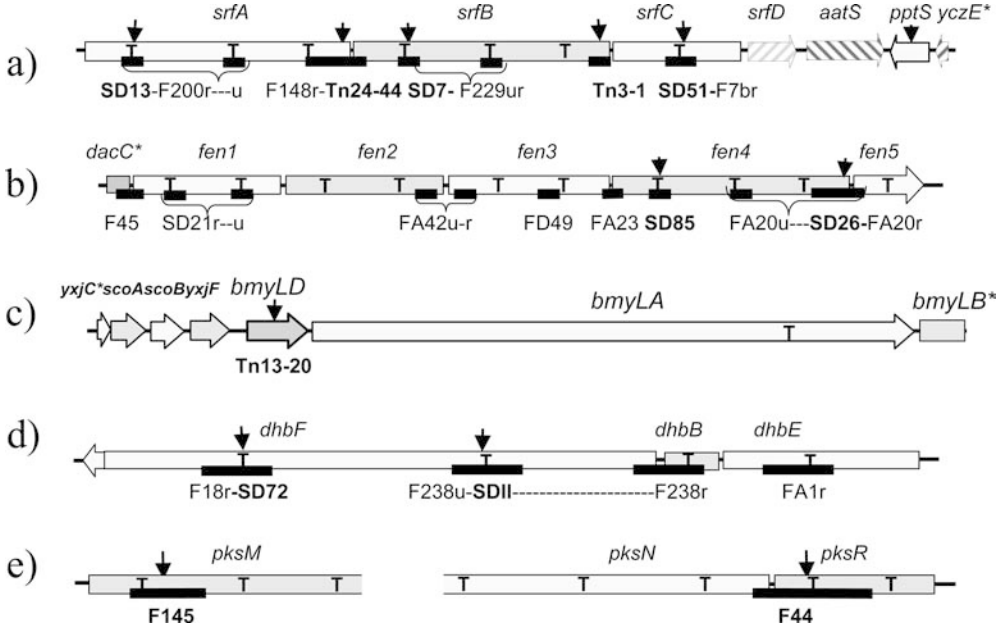

Fig. 2a–e Schematic diagrams showing the genomic organization of putative biosynthetic gene clusters of *B. subtilis* A1/3. **a** The surfactin locus *srf*. **b** The fengycin locus *fen*. **c** Organization of the 16.539 kb-DNA insert in cosmid pSC9i10. **d** The DHB iron siderophore locus *dhb*. **e** Polyketide (*pks* -like) gene clusters. The *black bars* indicate the position and extents of cloned single-domain (SD) and inter-domain (F) DNA sequences relative to the putative ORF. The *vertical arrows* indicate the sites of disrupting insertions or transposons in mutants (see Tables 1 and 4). Thiolation domains (T) of master NRPS or PKS genes are indicated according to the SubtiList database (Kunst et al. 1997)

not be excluded, the specificity of integration was tested by Southern hybridization of chromosomal DNA from the mutants, using the target DNA as probe. The PCR fragments were sequenced and the data were submitted to GenBank. The Accession Nos. of the relevant DNA species used in the mutant studies are listed in Table 4. Additional accessions used to construct the maps shown in Fig. 2 were: AF520837, AF520838, AF520844, AF 520845, AF520846, AF520847, AF520848, AF520849, AF520850, AF520851, AF520852, AF520853, AF520854, AF520856, AF520857, AF520859 and AF520860.

Construction of a plasmid library and cloning of a PPTase gene

Genomic DNA of *B. subtilis* A1/3 was digested with *Not* I, and DNA fragments larger than 15 kb were isolated by electroelution after fractionation by agarose gel electrophoresis. The vector pPS7, which exploits the principle of palindrome non-viability (Steinborn 1998), was used for cloning. The stuffer DNA of the vector was removed after digestion with *Not* I. The vector backbone and the genomic DNA (1 μg each) were ligated using T4 DNA ligase. The ligation mix was then used to transform protoplasts of *B. subtilis* RM125, selecting for erythromycin resistance. In all, 237 transformant colonies were scored. About 95% contained plasmids with 15–30-kb inserts. Clone #152 was found to hybridize with a putative PPTase sequence, obtained from strain A1/3 by PCR amplification with the primers SFP1/SFP6 (see Table 3). The clone #152 contained plasmid pLP152 which harbors 22 kb of *B. subtilis* A1/3 genomic DNA. This plasmid was used to transform *B. subtilis* GSB26. The hemolytic properties of transformant colonies on Columbia sheep blood agar (Oxoid) indicated the expression of an active PPTase gene which complements the *sfp* deficiency of the host (Nakano et al. 1992). The insert DNA was cleaved with *Sma* I, and ligated with pGB354. The ligation product was then used to transform GSB26. The plasmid pGBA4 also conferred hemolytic activity on its host, like its progenitor pLP152. It contained 4.5 kb of the original 22-kb insert DNA. This fragment was sub-cloned into plasmid pMPA4, and sequenced. The sequence has been submitted to GenBank under the Accession No. AF233756.

Transposon mutagenesis and excision cloning

Transposon mutagenesis with the transposon mini-Tn*10* on plasmid pIC333 was performed as described by Petit et al. (1990). After transformation of strain GB709, transposition was achieved by shifting the culture to the non-permissive temperature (37°C) under spectinomycin selection. About 4500 putative mutant colonies were screened for alteration or loss of hemolytic activity by replica plating onto Columbia sheep blood agar (Oxoid). The plasmid pUC origin was used for excision cloning of transposon-tagged DNA, screening for spectinomycin resistance (Steinmetz and Richter 1994). The excised DNAs (Tn3-1, Tn24-44 and Tn13-20) obtained from the transposon mutants BA3-1, BA24-44 and BA13-20, respectively, was sequenced and submitted to GenBank under the Accession Nos. AF520863, AF520865, and AF499447 (see below).

Construction of a cosmid library and cloning of bacillomycin L genes

Genomic DNA of *B. subtilis* A1/3 strain GB709 was partially digested with *Mbo* I, and DNA fragments in the size range 20–30 kb were isolated after agarose gel electrophoresis. The *Xba* I-cleaved, dephosphorylated and *Bam* HI-digested vector Super Cos1 was used according to the protocol supplied by the manufacturer (Stratagene). Recombinant colonies were replicated from 384-well microtiter plates onto Nylon N+ membranes and hybridized with a labeled 660-bp DNA probe, which was PCR amplified from plasmid pTn13-20 using the primers FFA and FFS (Table 3). The cosmid pSC9i10 hybridized with the probe, and was found to contain a 14.5-kb insert of *B. subtilis* A1/3 genomic DNA. The DNA was sequenced and submitted to GenBank under Accession No. AF499447.

Mass spectrometry

The lipopeptide products of *B. subtilis* A1/3 and mutant strains were identified using matrix-assisted laser desorption ionization/time of flight mass spectrometry (MALDI-TOF-MS) in delayed extraction mode on whole cells and surface extracts according to Vater et al. (2002, 2003). Between 100 and 200 single scans were accumulated for each spectrum. Lipopeptide fractions for post source decay MALDI-TOF-MS were purified by successive rounds of reversed-phase HPLC. Crude lipopeptide products were applied to an ODS Hypersil C18 column and eluted at a flow rate of 400 μl/min with a linear gradient from eluent A (10 mM Na_2HPO_4 pH 2.5) to 80% eluent B over a period of 45 min. Eluent B consists of 40% eluent A and 60% acetonitrile (v/v). Final purification was achieved by Smart HPLC, using a μRPC 2.1/10 C2/C18 column (Amersham Biosciences Europe, Germany) at a flow rate of 100 μl/min and a linear gradient from 5% eluent B to 45% eluent B over 40 min, where eluent A was 0.1% trifluoric acid (TFA) in water (v/v) and eluent B 0.08% TFA in acetonitrile. α-Cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid was employed as the matrix for MALDI-TOF-MS of either lipopeptide- or polyketide-like compounds. LC-Electrospray mass spectrometry (LC-ESI-MS) of the polyketide compounds produced by *B. subtilis* was performed with an Esquire 3000^+ ion trap mass spectrometer (Bruker Daltonik, Leipzig, Germany) equipped with an API-ESI interface in combination with an Agilent HP 1100 HPLC System (Waldbronn, Germany). Aliquots (5–10 μl) of butanol extracts of the culture filtrates of wild-type and mutant strains were fractionated by reversed-phase HPLC on a Phenomenex Luna C18 column 3 μ (2×100 mm) at a flow rate of 300 μl/min using an acetonitrile gradient from 10–100% in 30 min. Gradient solutions were 0.05% formic acid in water and 0.05% formic acid in acetonitrile. The eluent was fed on-line into the spray chamber of the Esquire instrument at a flow rate of 300 μl/min. Using ESI-MS in positive mode, mass spectra were acquired in the range of m/z = 150–1000 at a scan rate of 13,000 Da/s. For spectrum acquisition, a total of 15 scans were summarized. The potentials of the capillary exit and skimmer 1 were set to

3500 and 40 V, respectively. Nitrogen was used both as the nebulizing gas (at 35 psi) and the drying gas (10 l/min at 350°C).

Results

Bioautography of antibiotic substances

The antibiotic and/or hemolytic activities of Landy-cultures of *B. subtilis* A1/3 shown in Fig. 1 were detected by bioassays after thin-layer chromatography (TLC) of butanol extracts of culture filtrates from at least three independent extraction and separation experiments, and are characterized by the indicator organisms affected and by the R_f values of the compound(s). The blood assay (lane 1) revealed two hemolytic substances #2 and #6 with R_f values of about 0.81 and 0.08, respectively. The latter was predominant and also showed anti-fungal activity against *Penicillium chrysogenum* (lane 6). The assay with *Clavibacter michiganensis* (lane 2) detected two anti-bacterial activities, a minor one, which apparently correlated with substance #2 identified in the blood agar test, and a major one, which was attributed to substance #8. The bioautographs with *Bacillus megaterium* (lane 3) discriminated several antibiotics, substances #3 and #5 being the dominant agents. The latter, unlike substance #3, was active even after 10^4-fold dilution of the extract (data not shown) and was less active in the bioautographs with *Agrobacterium tumefaciens* (lane 4) and *Proteus vulgaris* (see below). It was also active against other Gram-positive as well as Gram-negative bacteria, e.g. *Paenibacillus polymyxa*, *B. sphaericus*, *Serratia marcescens*, *Corynebacterium glutamicum*, *Proteus mirabilis*, and *E. coli* (data not shown). The anti-fungal tests with *Paecilomyces variotii* Tü137 and *Fusarium oxysporum* (lanes 5 and 7) allowed us to discriminate substances #7 and #6, the latter being active in the test with *P. chrysogenum* (lane 6) as well as on blood agar (lane 1), while the former was not. The compounds responsible for these activities were identified by MALDI-TOF-MS of whole cells and surface extracts in the context of our mutant studies (see Tables 5 and 6).

Localization of a PPTase gene and characterization of a *pptS* mutant

The DNA sequence of plasmid pGBA4 (see Materials and methods) revealed the presence of five putative ORFs in the *sfp* -gene region of the chromosome of *B. subtilis* A1/3, which showed 71, 78, 58, 99 and 65% identity to surfactin synthetase subunit SrfAC and the surfactin thioesterase enzyme SrfAD of *B. subtilis* 168, to an aspartate amino transferase (AspTR) of *B. circulans* (Battchikova et al. 1996), to the Lpa-14 protein of *B. subtilis* RB14 (Huang et al. 1993) and related PPTases, as well as to the YczE protein of *B. subtilis* 168, respectively (Fig. 2). The plasmid pME520 was used to construct mutant GB786, in which the PPTase-homolog is disrupted. The bioautographs of mutant cultures indicated the loss of all the hemolytic and antibiotic activities described in the previous section, with the exception of substance #8. Moreover, mutant GB786 had lost the ability to grow under conditions of iron limitation (data not shown). MALDI-TOF MS analyses of whole cells and surface extracts showed that the mutation was correlated with the loss of peptides with mass peaks in the ranges $m/z = 1021$–1087 and $m/z = 1450$–1556, respectively (Table 5). The production of metabolites with mass numbers $m/z = 2900$ and 3350, previously shown to be the subtilin-like peptides ericinA and ericinS (Stein et al. 2002), was not affected. This correlates with the continued production of substance #8 (data not shown).

PCR-based DNA cloning and mutant construction

Due to the DNA sequence divergence relative to *B. subtilis* 168, the use of degenerate TGD/LGG oligonucleotide primers according to Turgay and Marahiel (1994) was required to amplify a series of DNA fragments of homologous NRPS and PKS domain regions from genomic DNA of strain GB709 by PCR. About one-third of the ~160 DNA species recovered, with sizes of between 0.5 and 5 kb, were found to display homology to either a single or neighboring domain regions of known NRPS and PKS synthetases. These were cloned and at least partly sequenced from both ends. The genomic organization of single domain (SD-) and inter-domain (F-) DNA sequences related to surfactin (*srf*)-, peptide (*pps*)-, fengycin (*fen*)-, bacillibactin (*dhb*)- and polyketide (*pks*)-like genes is shown in Fig. 2. The allocation of DNA fragments to particular genes was based on BLASTX results and additional sequence features, i.e. homology of inter-domain regions and the organization of domains and genes in the cloned DNA. Selected PCR-DNA species, cloned in integrative shuttle plasmids as listed in Table 4, were then used to construct gene disruption mutants (Table 1). The mutant cultures were subsequently characterized by biological methods, e.g. bioautography, growth tests and screens for biofilm formation, as well as by MALDI-TOF and LC-ESI mass spectrometry.

Identification and mutation of *fen* genes, and mass spectrometric detection of fengycins and substance #7

BLASTX analysis of the DNA fragments F45, SD21, FA42, FD49, FA23, SD85, and FA20 (with domain SD26) disclosed similarity in the range of 56–76% and 45–65% to 10 distinct regions of the five putative peptide (Pps) and fengycin (Fen) synthetases of *B. subtilis* 168 and *B. subtilis* F29-3, respectively (Fig. 2). Moreover, the DNA fragments F45, FA42, FA23 and FA20 displayed homology to the apparently adjacent genes *dacC(pbp)-ppsA*, *ppsB-C*, *ppsC - D*, and *ppsD-E* of *B.*

Table 5 Lipopeptides of *B. subtilis* A1/3 wild type and mutants, based on MALDI-TOF-MS data

Strain[a]	Characteristic *m*/*z* peaks		
	Surfactins	Bacillomycin L	Fengycins[b]
GB709	1030.7 (C13, Na^+); 1044.7 (C14, Na^+); 1058.7 (C15, Na^+); 1060.8 (C14, K^+); 1074.9 (C15, K^+)	1021.5 (C14, H^+); 1043.5 (C14, Na^+); 1057.6 (C15, Na^+); 1073.6 (C15, K^+)	1449.8 (C15, H^+, Ala); 1463.8 (C16, H^+, Ala); 1477.8 (C17, H^+; Ala); 1487.9 (C15, K^+, Ala); 1501.8 (C16, K^+, Ala); 1515.8 (C17, K^+, Ala); 1529.8 (C16, K^+, Val)
GB786	Not found	Not found	Not found
BC145	1022.6 (C14, H^+); 1044.3 (C14, Na^+); 1046.6 (C13, K^+); 1060.7 (C14, K^+); 1074:8 (C15, K^+)	1035.6 (C15, H^+); 1043.6 (C14, Na^+); 1057.6 (C15, Na^+); 1059,6 (C14, K^+); 1071.6 (C16, Na^+); 1073.6 (C15, K^+)	1449.8 (C15, H^+, Ala); 1471.8 (C15, Na^+, Ala); 1485,5 (C16, Na^+, Ala); 1501.8 (C16, K^+, Ala); 1515.8 (C17, K^+, Ala); 1529.8 (C16, K^+, Val)
BC85, BC10	1044.8 (C14, Na^+); 1046.6 (C13, K^+); 1060.7 (C14, K^+); 1074.8 (C15, K^+); 1088.5 (C16, K^+)	1035.6 (C15, H^+); 1043.6 (C14, Na^+); 1057.6 (C15, Na^+); 1059,6 (C14, K^+); 1071.6 (C16, Na^+); 1073.6 (C15, K^+)	Not found
BC13, BA2444, BC7, BC51	Not found	1021.4 (C14, H^+); 1043.6 (C14, Na^+); 1057.6 (C15, Na); 1059.5 (C14, K^+); 1073.6 (C15, K^+)	1463.7 (C16, H^+, Ala) ; 1485.7 (C16, Na^+, Ala); 1487.7 (C15, K^+, Ala); 1501.7 (C16, K^+, Ala); 1515.8 (C17, K^+, Ala); 1529.7 (C16, K^+, Val)
BA13-20, BA23-17	1030,6 (C13, Na^+); 1044,6 (C14, Na^+); 1046.6 (C13, K^+); 1058.6 (C15, Na^+); 1060.6 (C14, K^+); 1074.6 (C15, K^+)	Not found	1447.8 (C15, H^+, Ala)[c]; 1463.8 (C16, H^+, Ala); 1487.7 (C15, K^+, Ala); 1501.7 (C16, K^+, Ala); 1503,7 (C17, H^+, Val)[c]; 1515.8 (C17, K^+, Ala); 1529.7 (C16, K^+, Val)
BA13sd7	Not found	Not found	1487.8 (C15, K^+, Ala); 1501.8 (C16, K^+, Ala); 1515.8 (C17, K^+, Ala); 1529.8 (C16, K^+, Val)

[a]The mass data represent monoisotopic mass numbers from series of independently analyzed samples. The main ions in the area of *m* / *z* 800 to 1900 of the MALDI-TOF mass spectrometric investigation of whole cells and culture supernatants of *B. subtilis* wild type and mutants are summarized and referred to the bioautographs shown in Fig. 1

[b]Note that the amino acid in position 6 is indicated

[c]Fengycin species with one double bond. wt, wild type

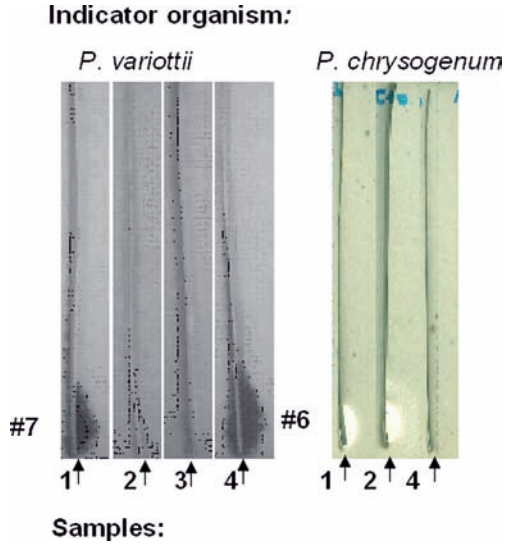

Fig. 3 Bioautographs with *Paecilomyces variotii* Tü 137 or *Penicillium chrysogenum* of wild type (1), *fen4* disruption mutants BC10 (2) and BC85 (3) in contrast to transposon mutant BA13-20 (4). The arrows label the groove of inserted strips, which were alongside graved into the lawn of the indicator plate. The black and white halos of antibiotic substances #7 (R_f = 0.067) and #6 (R_f = 0.08) are due to the contrast under illumination from above or transillumination

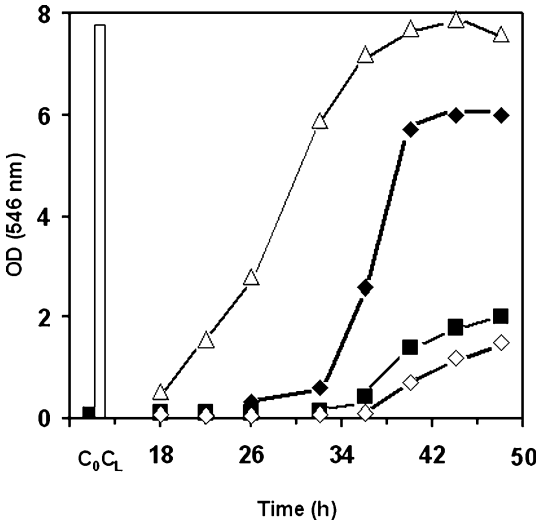

Fig. 4 Lysis of blood cell suspensions (per h and sample at 37°C) by cell-free supernatants of 18- to 48-h-old-Landy cultures of the wild type (*open triangles*), the disruption mutant BC13 (*srfA* $^-$; *filled diamonds*), the transposon mutant BA13-20 (*bmyLD* $^-$; *filled squares*) or the PPTase mutant GB786 (*pptS*$^-$; *open diamonds*). The bars labelled C_0 and C_L indicate the OD values of control blood and water samples, respectively

subtilis 168. The DNAs SD85 and SD26-5, which show homology to a PCP domain T7 and the 3′-terminus of the putative *fen4* gene, respectively, were used to construct the mutants BC85 and BC10. These two mutants had lost the ability to produce anti-fungal substance #7 (Fig. 3), corresponding to lipopeptides with mass numbers in the range m/z = 1447.8–1529.8 attributed to fengycins (Table 5).

Identification and mutation of *srf* genes, and mass spectrometric detection of surfactins and substance #2

The deduced translation products of the F-DNA fragments F200, F148 and F229, as well as F7br, showed 69 to 86% identity to distinct regions of surfactin synthetases SrfAA, -AB, and -AC of *B. subtilis* 168 (Fig. 2). Fragment F148r was assigned to the 3′-end of *srfAA*, as were the excision DNAs Tn24-44 and Tn3-1 from transposon mutants BA24-44 and BA3-1. In Southern hybridization experiments, however, Tn24-44, but not Tn3-1, DNA cross-hybridized with F148r DNA (data not shown). These data suggest that the DNAs Tn24-44 and Tn3-1 originated from different but quite similar sites, i.e. the ends of genes *srfA* and *srfB*, respectively (Fig. 2). F7br cross-hybridized with the 4.5-kb DNA-insert in plasmid pGBA4 (data not shown). This implies that the PPTase gene *pptS* of *B. subtilis* A1/3 lies downstream of the surfactin (*srf*) genes. The integrative plasmids pMEsd13, pMEsd7 and pMEsd51, carrying the *srf* -type DNA species SD13, SD7, and SD51, were used to construct the disruption mutants BC13, BC7 and BC51, respectively (Table 4). The *srf* -type mutants, like transposon mutants BA24-44 and BA3-1, displayed reduced, but residual hemolytic capacities (Fig. 4). This phenotype correlated with the loss of substance #2 (Fig. 5) and in the MALDI-TOF-mass spectra with the disappearance of mass peaks in the range between m/z = 1022.6 and 1088.5, which were attributed to surfactin isoforms

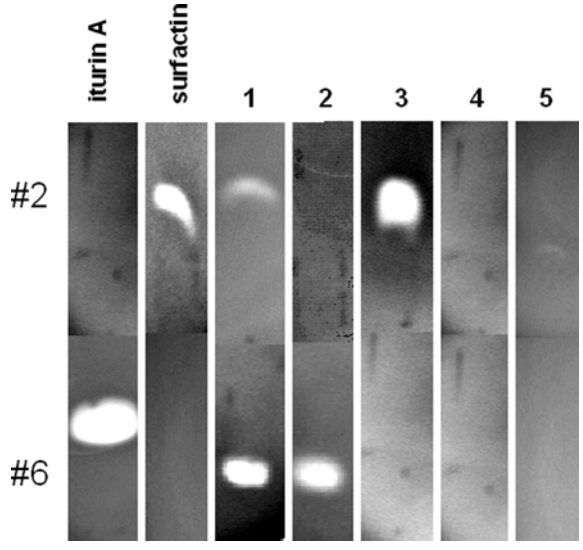

Fig. 5 Bioautographic detection of hemolytic activities in the butanol extracts of Landy cultures of (1) wild type, (2) BC7 (*srfB*::pMEsd13), (3) BA13-20 (*bmyD*::Tn13-20), (4) BA13sd7 (*bmyLD*::Tn13-20, *srfB*::pMEsd13), and (5) GB786 (*pptS*:: pME520). Proposed substances #2 and 6 are defined according to Fig. 1. Control samples of iturin A and surfactin (about 1 µg) were also tested. The separately incubated top and bottom parts of a strip were combined for this illustration

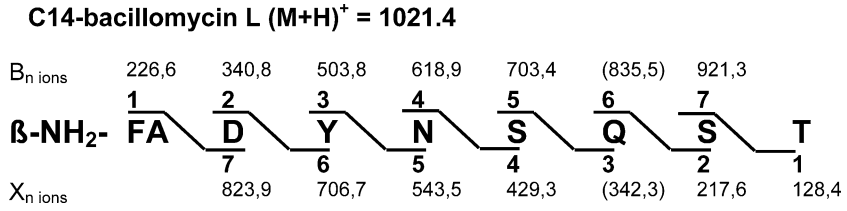

Fig. 6 Structural analysis of the lipopeptide product (m / z = 1021.4) isolated from the *srf* -type mutant BC13 of *B. subtilis* A1/3 by PSD-MALDI-TOF mass spectrometry after purification by reverse phase HPLC and SMART chromatography using an ODS-Hypersil column. The structure was calculated from series of N- and C-terminal fragments (B_n and X_n ions). β-NH_2-FA, β-amino fatty acid. The masses given in *parentheses* are of lower significance than the others

(Table 5). The mutants, however, retained hemolytic substance #6 and mass ions indicating the presence of another lipopeptide (Table 5), which was not iturin A (Fig. 5).

Transposon mutant BA13-20, identification of *bmyL* genes, and mass spectrometric detection of bacillomycin L and substance #6

Mutant BA13-20 showed even less hemolytic activity (Fig. 4) than the *srf* -type transposon mutants BA24-44 and BA3-1 (see above). In BA13-20, the transposon is present at codon 85 of a gene which shows similarity to the iturin A and mycosubtilin biosynthetic genes *ituD* and *fenF* (Duitman et al. 1999; Tsuge et al. 2001). This correlated with the loss of hemolytic substance #6 (see above) and of a set of mass peaks with mass numbers in the range of m/z = 1021.5–1073.6 (Table 5). Because these mass peaks overlap with the mass peaks of surfactins, we used the *srf* -type mutant BC7 for structural analysis of the lipopeptide after fractionation by successive rounds of reversed-phase HPLC. The parent ion m/z = 1021.4 was subjected to post source decay MALDI-TOF-MS analysis. Its structure was corroborated by the detection of immonium ions at m/z = 87 (N), 101 (Q), and 136 (Y), as well as the appearance of B_n-ions of dipeptide fragments with mass numbers of m / z = 278.0 (N-Y), 198.1 [S-Q (-H_2O)] and 171.3 [S-T (-H_2O)]. Proline was not found in this lipopeptide. The series of N- and C-terminal fragments (B_n- and Y_n-ions) shown in Fig. 6 identified the lipopeptide as the protonated form of a bacillomycin L (Peypoux et al. 1984; Eshita et al. 1995).

Screening of a cosmid library of *B. subtilis* A1/3 with a 660-bp-core DNA of the mutated gene by Southern hybridization led to the identification of cosmid pSC9i10. It contained a 16.5-kb-DNA insert, which was sequenced. Downstream of the *fenF*/*ituD* -like gene mutated in the strain BA13-20, ORFs showing greatest similarity to the bacillomycin D genes *bmyAB* and the iturin A genes *ituAB* of strains FZB42 and RB14 (Tsuge et al. 2001; Koumoutsi et al. 2004), as well as genes *yxjC-scoA-scoB* - *yxjF* of *B. subtilis* 168 (Kunst et al. 1997), were identified. The double mutant BA13sd7, derived by transferring the disruption allele *srfB*::pMEsd7 of mutant BC7 into the transposon mutant BA13-20, had completely lost its hemolytic capacity and the ability to produce hemolytic substances #2 and #6 (Fig. 5). This correlated with the disappearance of the mass peaks of surfactins and bacillomycins L (Table 5) in the MALDI-TOF mass spectra, indicating that the *bmyLD* gene is necessary for the production of bacillomycin L.

dhbF gene mutants and iron siderophore activities

The inter-domain DNA F238 had a size of about 3.5 kb. Its predicted product showed ~85% identity to the PCP domain T1 of DhbF, as well as ~73% identity to amino acids 1–238 of DhbB of *B. subtilis* 168 (Fig. 2). The DNA fragments F18r and FA1r encode a predicted product that could be aligned with amino acids 112–277 of DhbE, the third component of the multienzyme system responsible for the production of the 2,3-dihydroxybenzoate (DHB) containing siderophore bacillibactin (May et al. 2001). The PCR products SDII and SD72, with closest similarity to PCP domains T1 and T2 of DhbF, were used to create the disruption mutants BC72 and BC35. These retained their spectrum of hemolytic and antibiotic activities, but showed slow growth in the presence of the iron chelator EDDA, and growth could be markedly enhanced by the addition of an iron salt (data not shown).

Identification and mutation of *pks* genes

Three 500-bp-PCR products exhibited 54–77% identity to putative PKS synthases of *B. subtilis* 168 (data not shown). The primary PCR fragments were used to generate the larger products F145 and F44, which are about 3.2 and 5.6 kb long, respectively. Their translation products revealed similarity throughout their sequences to ACP modules of the polyketide synthases PksM and PksNR, respectively, from *B. subtilis* 168 (Figs. 2 and 7). The predicted product of F145 could be aligned with PksM from amino acid 679 to 1736 and contained an extra-sequence of 342 bp compared to the corresponding ORF of *B. subtilis* 168. The protein sequence derived from F44 could be aligned with PksN from position 5296 to its end and with PksR from its translation start to amino acid 1928. F145 and a 114-bp-DNA subfrag-

Fig. 7a, b Specificity of PCR-amplified *pks* - like DNA. **a** PCR DNA F145. Alignment of the N-terminal region of the translated sequence (*bold letters*) with the first ACP domain of the polyketide synthase PksM (P40872/BG10931) of *B. subtilis* 168. The domain and its thiolation (T) site with the essential serine (S) at position 367 are *shaded*. **b** PCR DNA F44-7a. Homology of the translated sequence to the polyketide synthase PksR (F69679/BG12654) of *B. subtilis* 168 and the consensus sequence of ACP domains of polyketide synthases. Domain and consensus data are from NCBI conserved domain search (http://www.ncbi.nlm.nih.gov)

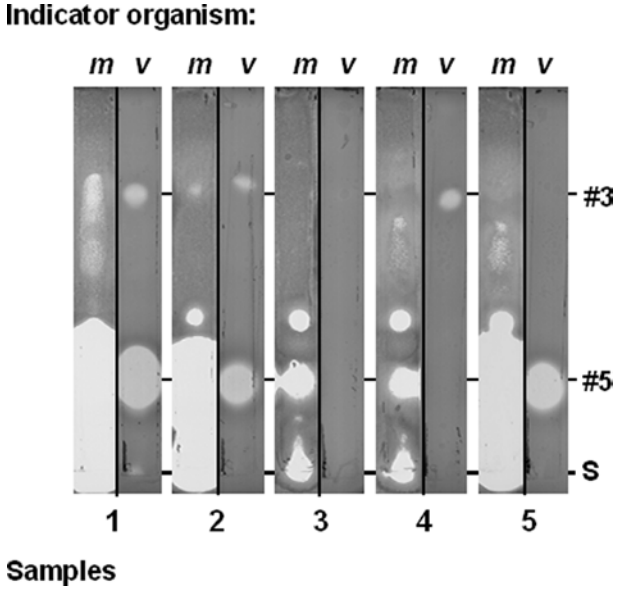

Fig. 8 Bioautographs of wild type *B. subtilis* and *pks* mutants. Aliquots (5 µl) of butanol extracts of cultures of the natural *B. subtilis* strains (1) ATCC 39320, (2) and A1/3, and the *B. subtilis* A1/3 mutants (3) GB786 (*pptS* $^-$), (4) BC145 (*pksM* $^-$) and (5) AR9 (*pksR* $^-$) were spotted and processed by thin layer chromatography. The chromatograms were incubated on a lawn of *B. megaterium* (***m***) or *P. vulgaris* (***v***). The origin (S) and the final location of substances #3 and #5 are indicated

ment (F44-7a) of F44 (Fig. 7) were used to construct the disruption mutants BC145 and AR9, which were found to have lost the antibiotic activities correlating with substances #5 and #3, respectively. The former predominated in the bioautographs with *B. megaterium*, *A. tumefaciens* and *P. vulgaris*, while substance #3 was most active against *P. vulgaris* (Figs. 1 and 8). The character of the cloned DNA and the antibacterial spectrum of substances #3 and #5 indicate that these antibiotics are polyketide in nature.

Mass spectrometric investigation of bacillaene and difficidin

The polyketide compounds of *B. subtilis* A1/3 were analyzed in comparison to those of strain ATCC 39320, a difficidin/oxydifficidin producer (Zimmerman et al. 1987), by LC-electrospray (ESI)- and MALDI-TOF-MS. The results obtained are summarized in Table 6. In both strains essentially the same two types of polyketides were found, which appeared with different retention times (R_f) at several positions on the total ion current (TIC)-chromatograms of the LC-ESI-MS experiments. At $R_f = 12.4$–12.5 min, ions with mass numbers of $m/z = 561.1$ (A) and 579.1 (B), with a minor peak at $m/z = 545.1$ (C), were detected. According to Wilson et al. (1987), compounds C and A can be identified with the protonated forms of difficidin and oxydifficidin, while B is the hydrated form with an opened lactone ring $[M + H + H_2O]^+$ of A. At slightly higher R_f (12.6 min) another pair of mass peaks appeared, i.e. $m/z = 563.1$ (D) and 581.1 (E), with similar intensities, each two mass units larger than compounds A and B.

The mass peaks at $R_f = 18.2$–18.4 min with mass numbers of $m/z = 581.1$ (F) and 563.1 (G) indicate the formation of bacillaenes (Patel et al. (1995), F being the protonated $[M + H]^+$ form and G the dehydrated $[M + H–H_2O]^+$ species. As in the case of the difficidins, at a slightly higher R_f (18.6 min) a pair of mass peaks was found at $m/z = 583.1$ (H) and 565.1 (I), which differ from compounds F and G, respectively, by two mass units. In the case of *B. subtilis* A1/3 their intensity was higher than those of compounds F and G. Species H and I, as well as D and E, are presumably biosynthetic variants of bacillaenes and difficidins in which one of the C-C double bonds is saturated.

The PPTase mutants of both strains had lost all these mass peaks A–I. The effects of the *pksM* and *pksR* gene disruptions in mutants BC145 and AR9 on

the production of these polyketide compounds were investigated by MALDI-TOF-MS in comparison to the wild type. For *B. subtilis* A1/3 this method revealed peaks with mass numbers at m/z = 583.3/599.3 and m/z = 603.3/619.3, which can be attributed to the alkali adducts of oxydifficidin (A) and bacillaene (F), respectively. In agreement with the bioautographic data, mutant BC145 lost the peaks at m/z = 583.3 and 599.3, correlating with the compound #5, while in the case of mutant AR9 the peaks at m/z = 603.3 and 619.3 disappeared, correlating with compound #3. The spots corresponding to the two substances were scraped off the thin-layer plate, and the silica gel was extracted with *n* -butanol. As expected, MALDI-TOF-MS analysis of compounds #5 and #3 showed peaks at m/z = 583.3 and 599.3 and at m/z = 603.3 and 619.3, respectively.

Biofilm formation by lipopeptide-deficient mutants of *B. subtilis* A1/3

It was recently reported that natural *B. subtilis* strains require surfactin to form thin structureless pellicles (biofilm) on the surface of standing liquid cultures (Branda et al. 2001). Here we addressed the question whether *B. subtilis* A1/3 requires other lipopeptides in addition to surfactins for biofilm formation. To test for biofilm formation the wild type as well as cultures of the mutant strains BC13 and BA3-1, BC10, BA13-20, BC145 and GB786 were grown in yeast extract (0.01%)-enriched Spizizen's minimal medium (SMM) at room temperature (Fig. 9). The wild type as well as mutants BC10, BC145 and BA13-20 completed pellicle formation after 4 days. In contrast, the *srf* mutants BC13 and BA3-1 and the *pptS* mutant GB786 formed loose aggregates that remained floating in suspension. Bioautographs of the cultures indicated the production of surfactin, bacillomycin L and fengycin, depending on the mutant status of the strain considered. Moreover, the addition of surfactin (Fig. 9), but not of hemolytically equivalent concentrations of iturin A or bacillomycin L, restored pellicle formation by the mutants BC13 and BA3-1 (data not shown). For this effect, a threshold value of about 100 μg of surfactin per ml was necessary.

Discussion

The aim of this study was to explore the manifold antibiotic and iron siderophore activities produced by A1/3, a natural strain of *B. subtilis* (Griesbach and Lattauschke 1991; Huber et al. 1991) in the context of its potential as a biocontrol agent (Sinclair 1989; Shoda 2000). Based upon a bioautographic comparison with other *B. subtilis* strains, we suspected the presence and/or activity of known as well as novel species of NRPS and PKS genes. Using a combination of transposon

Table 6 Detection of polyketide compounds from wild type and *pks* mutant *B. subtilis* strains by LC-ESI-MS and MALDI-TOF-MS

Strain/sample	Retention time R_f (min)	Mass ion (m/z)	Assignment
ATCC 39320[a]	12.4–12.5	561.1	Oxydifficidin; [M + H]+
		579.1	Oxydifficidin; [M + H + 18]+
	12.6	563.1	Oxydifficidin; [M + H + 2]+
		581.1	Oxydifficidin; [M + H + 2 + 18]+
	18.2	581.1	Bacillaene; [M + H]+
		563.1	Bacillaene; [M + H-18]+
A1/3[a]	12.4–12.5	561.1	Oxydifficidin; [M + H]+
		579.1	Oxydifficidin; [M + H + 18]+
		545.1	Difficidin; [M + H]+
	18.2–18.4	581.1	Bacillaene; [M + H]+
		563.1	Bacillaene; [M + H-18]+
	18.6	583.1	Bacillaene; [M + H + 2]+
		565.1	Bacillaene; [M + H + 2-18]+
PPTase mutants[a]			
#3_ATCC 39320	No polyketide products		
GB786_A1/3	No polyketide products		
A1/3[b]		583.3	Oxydifficidin; [M + Na]+
		599.3	Oxydifficidin; [M + K]+
		581.3	Bacillaene; [M + H]+
		603.3	Bacillaene; [M + Na]+
		619.3	Bacillaene; [M + K]+
pksM mutant BC 145[b]		581.3	Bacillaene; [M + H]+
		603.3	Bacillaene; [M + Na]+
		619.3	Bacillaene; [M + K]+
pksR mutant AR 9[b]		583.3	Oxydifficidin; [M + Na]+
		599.3	Oxydifficidin; [M + K]+
Substance #3 from BC145[b]		603.3	Bacillaene; [M + Na]+
		619.3	Bacillaene; [M + K]+
Substance #5 from AR 9[b]		583.3	Oxydifficidin; [M + Na]+
		599.3	Oxydifficidin; [M + K]+

[a]Data for wild type and *pptS* mutants of *B. subtilis*ATCC 3-9320 and A1/3 were obtained by LC-ESI-MS

[b]These data were obtained by MALDI-TOF-MS

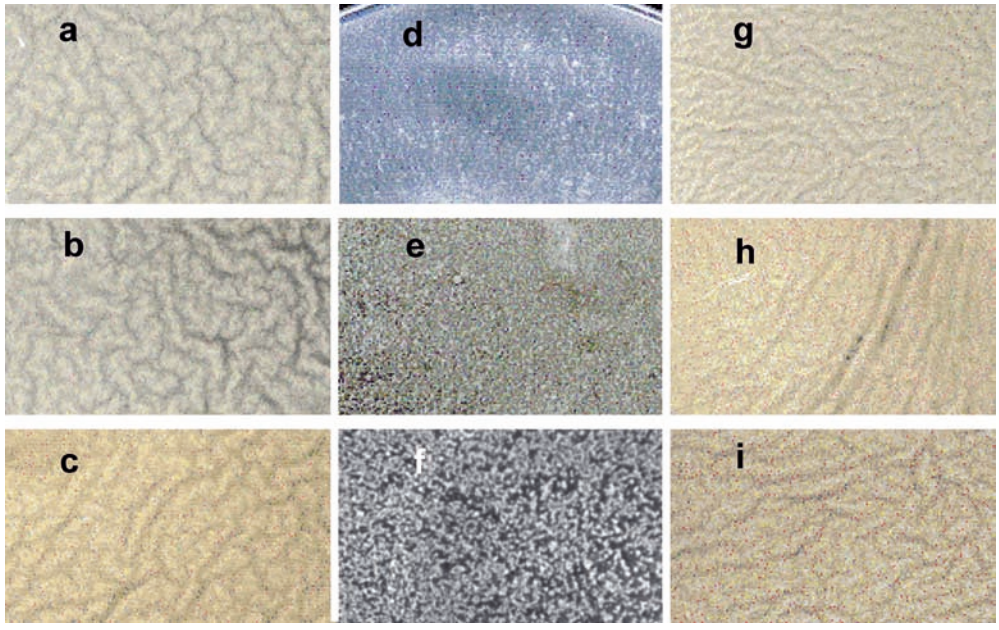

Fig. 9a–i Pellicle formation on liquid cultures of wild type and mutant bacteria left standing at room temperature for 4 days. **a** Wild type. **b** BC10 (*fen* $^{-}$). **c** BA13-20 (*bmyLD* $^{-}$). **d**) GB786 (*pptS* $^{-}$). **e** BA3-1 (*srfB* $^{-}$). **f** BC13 (*srfA* $^{-}$). **g** BC145 (*pksM* $^{-}$). **h** BA3-1 + surfactin (170 µg/ml). **i** BC13 + surfactin (170 µg/ml)

tagging and PCR cloning, we intended to identify the relevant members of the gene inventory and determine their homologies, rather than providing a detailed characterization of the gene clusters themselves. About 160 PCR and excision DNA fragments were sequenced and examined by BLASTX homology analyses. The results indicated the presence of known as well as new NRPS and PKS genes with similarities to corresponding homologs in *B. subtilis* 168 in the range of 50–96% (Fig. 2). The gene activities, as well as their non-ribosomal, i.e. PPTase-dependent, mode of synthesis, were subsequently tested by the construction and phenotypic characterization of mutants. To facilitate this, the PPTase gene *pptS* was cloned, and although it is also localized downstream of the surfactin *srf* genes, it was found to be most closely related to the *lpa -14* gene of *B. subtilis* RB14 (Huang et al. 1993). Its chromosomal context differs from that of its counterpart in the laboratory strain 168, i.e. the unknown genes *ycxAB* were not found and *ycxD* was replaced by an aspartate aminotransferase gene (*aatS*), which showed the highest homology to AspTR of *B. circulans* (Battchikova et al. 1996), as found in *B. subtilis* B3 (Yao et al. 2003). The bioautographs of the PPTase mutant revealed the loss of five out of nine antibiotics and an iron siderophore, indicating that the biosynthesis of all these compounds involves the thiotemplate mechanism. In a second step we investigated specific *srf*, *fen*, *dhb* and *pks* gene disruptions, as well as transposon mutants, primarily in order to identify the antibiotic and siderophore compounds concerned.

The PCR approach revealed the presence of single, or pairs of neighboring, thiolation (T) domains with sequence identity in the range of 45 to 89% to known surfactin, fengycin and DHB synthetases from the laboratory strain *B. subtilis* 168. All putative *srf* gene disruptions, as well as several transposon mutants, were characterized by the loss of hemolytic substance #2, which the MALDI-TOF-MS data indicated to be surfactin (Kakinuma et al. 1969; Kowall et al. 1998). A second hemolytic substance, #6, which dominated in the hemolysis tests, was bacillomycin L (Besson et al. 1978; Eshita et al. 1995). It also displays anti-fungal activity, which, in contrast to another anti-fungal substance (#7), was most active against *Penicillium chrysogenum*, and less or not at all active against *Paecilomyces variotii* Tü137 and *Fusarium oxysporum*. As expected from earlier data (Steller et al. 1999), this antibiotic was fengycin (Vanittanakom et al. 1986) and was not produced in cultures inoculated with either of the two *fen4* mutants. Moreover, irrespective of the fact that it shows 10% less overall similarity to the fengycin (Fen) synthetases of *B. subtilis* F29-3, the proximity of *dacC* and *fen1* genes indicated that this gene cluster is located in a similar position in the genome (Lin et al. 1999; Kunst et al. 1997). Mutants BC35 and BC72, which were constructed by targeting with *dhbF* -like DNA, proved that the DhbF synthetase participates in the biosynthesis of a bacillibactin-like siderophore, as has been found in several other bacilli (May et al. 2001).

Although the inspection of homologous T domains of mycosubtilin/iturin synthetases indicated the presence of homologous TGD/LGG consensus motifs (data not shown), the TGD/LGG primer approach failed to tag any domain DNA of putative bacillomycin L (BmyL) synthetase genes. This was apparently due to insufficient degeneracy of the TGD/LGG primer pair used. The *bmyL* gene cluster has since been tagged in transposon mutants that show reduced hemolysis due to the loss of substance #6 and bacillomycin L production. The transposition site lacked any homology to the genome sequence of *B. subtilis* 168, but revealed a close relationship with the genes *fenF*

and *ituD* of strains ATCC 6633 and RB14, which encode malonyl-CoA transacylases that are involved in the synthesis of the β-amino fatty acid component of iturin-like lipopeptides (Duitman et al. 1999; Tsuge et al. 2001). Hence, we identified the gene disrupted by the transposon as *bmyLD*, which lies upstream of the initiator bacillomycin L synthetase *bmyLA* gene. The sequences are nearly identical to the recently discovered bacillomycin D orthologs of strain FZB42 (Koumoutsi et al. 2004) and show less (66–87%) similarity to the mycosubtilin and iturin A synthetases of strains ATCC6633 and RB14 (Duitman et al. 1999; Tsuge et al. 2001).

PCR DNA fragments F145 and F44 proved to possess sequence homology to genes for various polyketide and fatty acid synthases, in particular PksM, N and R of *B. subtilis* 168 (Kunst et al. 1997). The product of F145, however, in distinction to the product of F44, carried an extra sequence of 116 amino acids and ∽20% less identity to its putative homologue PksM, which strongly indicated that these two DNA fragments from *B. subtilis* A1/3 originate from different rather than only one *pks* gene locus as in the case of *B. subtilis* 168. This is also compatible with the finding that, in the two disruption mutants targeted by either F145 or a 114-bp *Mbo* I subfragment of F44, the synthesis of different antibiotics was affected. Indeed, the genome of a *B. amyloliquefaciens* strain, FZB42, was recently shown to contain not one, but three *pks* loci, i.e. *pks1*, *pks2* and *pks3*, with the *pks1* locus being most closely related to the *pks* genes of *B. subtilis* 168 (Koumoutsi et al. 2004). The detection of substances #3 and #5 in our mutant studies thus implies the production of at least two different polyketide-like antibiotics. The LC-ESI- and MALDI-TOF-MS data for *B. subtilis* A1/3, as well as of the difficidin producer ATCC 39320 as a reference strain, proved that both strains produce difficidins and bacillaenes, which have previously been isolated and identified from strains ATCC 39320 and ATCC 39374 (Zimmermann et al. 1987; Wilson et al. 1987; Zweerink and Edison 1987) and ATCC 55422 (Patel et al. 1995). The disruption of a *pksM* - or *pksR* -like gene led to the loss of difficidins or bacillaenes, respectively, and the antibiotic spectrum of these compounds, including Gram-positive as well as Gram-negative organisms, was in a good agreement with the antibiotic activities of substances #5 and #3. The genomic location of the two proposed *pks* gene clusters in *B. subtilis* A1/3, however, awaits further investigation.

In an earlier study (Stein et al. 2002), the gene cluster *eri*, which is slightly different from the *spa* -genes of strain ATCC 6633, was shown to control the production of two subtilin-like peptides, ericinA and ericinS in *B. subtilis* A1/3. The latter corresponds to substance #8 (Fig. 1). Moreover, the genes *bac* related to the production of the antibiotic dipeptide bacilysin were detected in this strain (Steinborn and Hofemeister 1998/2000). Its antibacterial spectrum overlaps with that of the polyketide products difficidin and bacillaene.

Surfactins and bacillomycin L display surfactant properties, as indicated by their hemolytic activities. Based on recent findings reported by Branda et al. (2001), surfactins have been proposed to initiate biofilm formation in standing liquid cultures by lowering the surface tension of water. In our study, surfactin-deficient, but not bacillomycin L-deficient, mutants were found to have lost the ability to form biofilms (Fig. 9). This indicates that the surfactins, and not the bacillomycins L, enable motile *B. subtilis* cells to form biofilms, presumably because of specific surface- and membrane-active properties of the surfactins. These results in turn emphasize that surfactins perform developmental rather than defense functions. It might also explain why surfactins are produced by most *B. subtilis* strains (Leenders et al. 1999; Vater et al. 2002; Pabel et al. 2003), whilst the spectrum of antibiotic lipopeptides, polyketides and other products with likely environmental functions, as demonstrated in the present study, varies widely among different *B. subtilis* strains.

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Two novel *Venturia inaequalis* genes induced upon morphogenetic differentiation during infection and *in vitro* growth on cellophane

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ABSTRACT

Venturia inaequalis is a hemibiotrophic ascomycete that causes apple scab. Germ tubes, from conidia or ascospores, penetrate the leaf or fruit surface directly via appressoria-like swellings; subsequently the hyphae divide laterally to form a stroma between the cuticle and the outer wall of the epidermal cells. This morphological switch can be mimicked by growing the fungus *in vitro* on cellophane discs. The aim of this work was to identify genes upregulated *in planta* using growth on cellophane as a model. Four cDNA clones were found to be induced by growth on cellophane, and qRT-PCR showed two of these genes were up-regulated over a thousand fold in infected apple leaves compared to liquid culture. The predicted proteins for both genes possess putative signal peptides for secretion but have no similarity to sequences in publicly available databases. Both genes encode proteins with novel, imperfect repeat domain structures, the number of which vary in an isolate-specific fashion. Cin1 has seven or eight repeats of about 60 amino acids with four conserved cysteine residues per repeat, while Cin3 has four or five repeats of 32 amino acids with no cysteines. Both proteins appear to have evolved through internal duplication. Cin3, in particular, shows considerable between-strain variation in domain structure, indicating a high degree of recombination at this locus and revealing that the repeat structure has most likely arisen by unequal crossing-over. Results of this study support the hypothesis that cellophane-grown *V. inaequalis* mimics aspects of biotrophic infection and provide the first insights into novel fungal genes expressed during apple scab infection and their mechanisms of evolution.

1. Introduction

Fungal and oomycete pathogens have developed a range of specialized morphological structures to facilitate colonization and the extraction of nutrients from their plant hosts during the biotrophic phase of growth. These include the formation of appressoria, penetration pegs, infection hyphae, stromata and haustoria (Mendgen and Deising, 1993; Mendgen and Hahn, 2002; Mendgen et al., 1996). This infection-related morphological differentiation is usually specifically triggered by either chemical signals or the physical properties of the plant surface (Andrews et al., 2004; Deising et al., 1995; Gniwotta et al., 2005; Hahn and Mendgen, 1997; Kahmann and Basse, 2001). In addition to the expression of those genes required for morphogenesis, pathogens secrete an array of effector proteins which are designed to facilitate infection, primarily by suppressing host defense responses (Jones and Dangl, 2006). A subset of these effector proteins, termed avirulence gene products, are directly or indirectly recognized by host proteins encoded by resistance genes (*R*-genes) and trigger a hypersensitive response (Chisholm et al., 2006). Effector genes are often only expressed *in planta*, thus discovery and isolation of their cDNAs or proteins is hindered by the presence of high levels of host products. The ability to induce morphological changes *in vitro* that mimic those that occur *in planta* would greatly facilitate the isolation of these genes.

Several *in vitro* conditions have been established that induce morphogenetic differentiation of various plant pathogenic fungi similar to those observed during infection (Collins et al., 2001; Mendgen et al., 2006). In the rust fungus *Uromyces appendiculatus* early stages of pathogenic development can be induced on synthetic membranes containing ridges that simulate stomata (Hoch and Staples, 1991). Appressorium formation in *Magnaporthe grisea*

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and *Colletotrichum* spp. occurs on natural or artificial hydrophobic surfaces (Howard and Valent, 1996; Perfect et al., 1999). It was found that the response to host signals in *C. gloeosporioides*, such as wax and ethylene, requires contact of conidia with a hard surface (Flaishman et al., 1995). Hard surface contact induces Ca^{2+} calmodulin signaling in this fungus (Kim et al., 1998) and the expression of a number of *Colletotrichum* hard-surface-induced (CHIP) genes (Kim et al., 2000; Kolattukudy et al., 1995; Liu and Kolattukudy, 1998). Nutrient starvation is also known to regulate morphogenesis and the expression of some pathogenicity genes in *Cladosporium fulvum, M. grisea* (Lau and Hamer, 1996; van den Ackerveken et al., 1994) and the oomycete *Phytophthora infestans* (Lauge and De Wit, 1998; van West et al., 1998).

Venturia inaequalis (Cooke) Wint. causes black spot or scab of apple (*Malus* X *domestica* also referred to as *M. pumila* (Mill.) Henry). The life cycle of this hemibiotrophic ascomycete includes a parasitic phase as a sub-cuticular biotroph and a saprophytic sexual phase on infected fallen leaves (MacHardy et al., 2001). After spore germination and the formation of an appressorium-like swelling, penetration occurs directly through the cuticle, followed by the establishment of infection hyphae and sub-cuticular stromata (which do not penetrate the epidermal cell walls). The stromata are made up of single or multiple layers of pseudoparenchyma, a laterally dividing cell-type, distinct from the usual tubular mode of hyphal growth. Finally, conidiophores are produced from the stromata through the perforated cuticle and are associated with the development of scab lesions. Delamination of the cuticular membrane from the epidermal cell wall during proliferation of the sub-cuticular stroma then occurs (Yepes and Aldwinckle, 1993).

Apple pathogenesis-related (PR) but not *V. inaequalis* proteins have been identified in the apoplastic fluid of infected leaves (Gau et al., 2004). We are interested in identifying pathogenicity determinants from *V. inaequalis* during the biotrophic phase of growth. We have focused particularly on infection-specific morphological differentiation and sub-cuticular stroma formation that is critical for establishing infection. During growth on cellophane *V. inaequalis* exhibits morphological differentiation; both infection-related mycelial development and the formation of structures similar to the sub-cuticular stromata produced during apple leaf infection. Here we propose that growth on cellophane is a model for sub-cuticular growth and morphogenetic differentiation of *V. inaequalis* during infection of apple leaves, and we have used this system for the isolation of fungal genes up-regulated on cellophane. The expression of *c*ellophane *in*duced (*cin*) genes during apple leaf infection was analyzed. Two *cin* genes were shown to be up-regulated over a thousand fold during infection of apple leaves. Full-length cDNA copies of these genes were identified in an EST library made from infected apple leaves. A haplotype-based sequence analysis of one of these genes in a number of isolates reveals evidence that the novel repeat structure in this gene may have been actively evolving by a mechanism such as unequal crossing-over. Thus, isolation of cellophane-induced genes may provide a rapid means of identifying novel fungal pathogenicity determinants, including effector proteins, and a better understanding of the pathogenicity of *V. inaequalis*.

2. Materials and methods

2.1. Strains and growth conditions

Isolates of *V. inaequalis* and their origin are given in Table 1. *V. inaequalis* was grown on either 2% (w/v) malt or potato dextrose agar (PDA) covered with a single layer of cellophane membrane (Parker et al., 1995) and plates were incubated at 18 °C for 10–21 days in continuous darkness or under near-UV light (12 h light period per day). Cellophane membranes were either processed for total RNA extraction or for spore suspension production. For the latter, membranes were rinsed with water for 5 min to remove the conidia, filtered through a 20 μm nylon filter and centrifuged at 3000*g* for 10 min. Liquid cultures containing 180 ml of 2% (w/v) malt extract broth were inoculated with 20 ml of the conidial suspension (10^7 conidia ml^{-1}) and incubated at 18 °C under continuous shaking at 150 rpm for 10–21 days. The mycelium was removed by centrifugation and processed for total RNA extraction. *Escherichia coli* cultures were propagated in Luria Broth at 37 °C. Recombinant phages and plasmids were amplified in *E. coli* XL1-blue MRF' (Stratagene, La Jolla, CA). The cDNA library clones were stored as aliquots of the primary and amplified library in 7% (v/v) DMSO at −80 °C.

Table 1
The origin and race status of the *V. inaequalis* isolates used in this study

Isolate	Race status	New race name*	Country of origin
ZH1	1	(1)	Switzerland
EUB04	1	(1)	Belgium
EUB05	1	(1)	Belgium
EUNL19	1	(1)	Netherlands
MNH120	1	(1)	New Zealand
MNH135	1	(1)	New Zealand
1639	2	(1, 2, 8)	France
1770-3	2	(1, 2)	United States of America
1774-1	3	(1, 3)	United States of America
1638M	4	(1, 4)	France
USR4	4	(1, 4)	United States of America
163	5	(1, 5)	France
USR5	5	(1, 5)	United States of America
EUD42	6	(1, 6)	Germany
EUNL24	6+7	(1, 6, 7)	Netherlands
1066	7	(6, 7)	France
EUNL05	7	(6, 7)	Netherlands
188	8	(1, 8)	New Zealand
TR2	Unknown		United Kingdom

* A new system for classifying race status has recently been proposed (Bus et al., in press). The race status of all isolates was not confirmed in this study.

2.2. Plant material, maintenance and inoculation

Seedlings originating from *Malus* X *domestica* cvs. 'Golden Delicious' or 'Royal Gala' were used for generating infected leaves. The seeds were held in sand saturated with water for a stratification period of 6–8 weeks at 4 °C. To produce material for microscopy two month old apple seedlings were incubated in a small plastic growth chamber at 100% relative humidity for 24 h before inoculation. The seedlings were sprayed with a suspension of 10^5 conidia ml^{-1} in water to saturation and incubated for 48 h in a plastic mist chamber with 100% relative humidity at 18 °C. The seedlings were then moved to a growth room and incubated for 3–4 weeks under optimal conditions for infection (18 °C, 70% relative humidity, 16 h photoperiod). To produce material for real time quantitative RT-PCR (qRT-PCR) analysis detached leaves were inoculated by the method of Win et al. (2003).

2.3. Microscopy

Two types of material were prepared for microscopy: apple leaf material infected with *V. inaequalis* and *V. inaequalis* forming stromata in cellophane (both two weeks post-inoculation). Tissue from both material types (1 × 5 mm) was fixed in 2% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 under vacuum for 1 h. The material was washed three times in buffer, dehydrated in an ethanol series and embedded in LR White resin (London Resin, Reading, UK).

Structural observations were carried out on 1 μm sections of resin embedded material stained in 0.05% (w/v) solution of toluidine blue in borate buffer (pH 4.4), dried and mounted in Surmount (Triangle Biomedical Sciences, Durham, NC). Sections were viewed using an Olympus Vanox AHT3 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) and images collected with a CoolSnap color digital camera (Roper Scientific Ltd., Tucson, AZ). Immunolabeling for fluorescence light microscopy was performed as described previously (Johnston et al., 2006).

For Low-Temperature Scanning Electron Microscopy (LTSEM) *V. inaequalis* samples growing on cellophane were mounted on sample holders using Tissue-tek (Sakura, Tokyo, Japan) and plunge-frozen in nitrogen slush. Ice was sublimated from sample surfaces in an Alto 2500 (Gatan, Oxford, UK) vacuum preparation chamber (pressure $< 2 \times 10^{-4}$ Pa). For sublimation the temperature was raised from 93 K to 176 K for 30 s. Samples were sputter-coated with a 5 nm platinum layer prior to transfer on the SEM cryostage built into an S-4700 field emission scanning electron microscope (Hitachi, Tokyo, Japan). SEM micrographs were digitally recorded after samples were stabilized at 148 K at an acceleration voltage of 2 kV.

2.4. RNA extraction

For the construction of the cDNA library from cellophane-grown mycelia, 21-day-old cellophane discs were frozen in liquid nitrogen and ground with a pre-cooled mortar and pestle to a fine frozen powder. Isolation of total RNA from frozen cellophane-fungal powder was based on a method of physical disruption of cells by glass beads under rapid agitation and peqGold RNAPure™ solvent extraction as described by the manufacturer (Peqlab, Erlangen, Germany). Poly(A) mRNA was purified from total RNA preparations with a mRNA isolation kit (Roche, Basel, Switzerland) using biotin-labeled oligo(dT) and streptavidin-coated magnetic particles according to the manufacturer's instructions.

For qRT-PCR total RNA was extracted by the method of Chang et al. (1993) and concentration quantified using a Nanodrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE). RNA was extracted from 10 day old PDB shake culture *in vitro*-grown *V. inaequalis* mycelium and cellophane-grown *V. inaequalis*, and from detached leaves inoculated with *V. inaequalis* or SDW harvested at 5 and 10 days post-inoculation (dpi).

2.5. Construction of the cellophane cDNA library

Poly(A) mRNA was used for the construction of a cDNA library in lambda vector ZAP Express (Stratagene) according to the manufacturer's instructions. EcoRI adapters were ligated to the blunt ends of double-stranded cDNA. After digestion with EcoRI and size-fractionation by gel filtration on a Sepharose CL-2B column, two fractions of cDNA fragments were separated and ligated into the EcoRI site of the lambda ZAP Express vector (Stratagene). Fraction 1 contained inserts between 1–2 kb while fraction 2 had inserts of between 0.5 and 1.0 kb. The ligation mixture was packaged into phages using the Gigapack III Gold packaging extract (Stratagene) and transfected into *Escherichia coli* XL1-blue MRF' strain. The cloning efficiency, titer and fraction of phage containing inserts were determined by plaque assay with blue-white color selection. Part of the primary lambda library was amplified according to the manufacturer's instructions and stored at −80 °C. Primary titer of the first library containing the smaller cDNA inserts was 1×10^6, and the titer of the second library with larger insert size was 6×10^6 pfu ml^{-1}. The percentage of plaques containing cDNA inserts was approximately 93% and 91% in the first and second library, respectively. The library clones were plated, and replicate filters were hybridized with DIG-labeled probes derived from cultures grown either in cellophane or in liquid medium. Aliquots of 0.5 μg poly(A) mRNA for the amplified cDNA probes were used for differential hybridization. Hybridization signals of different intensity were detected, indicating strong or weak expression respectively, of the corresponding cDNAs. Most plaques yielded signals of similar intensity, while some showed marked differences. For further confirmation of the hybridization results, individual cDNAs representing these clones were amplified directly by PCR from plaques with vector-specific primers flanking the insert. Similar amounts of the PCR products were applied as duplicate dots to a nylon membrane and hybridized with DIG-labeled cDNA probes derived from either cellophane- or liquid-grown cultures.

2.6. Screening of the cellophane cDNA library for differentially expressed genes

The recombinant phages from the cDNA library were screened for differentially expressed genes by plating plaques (about 50,000 pfu/plate) on NZY top agar plates. Plaques from each plate were transferred to Hybond-N nylon membranes (GE Healthcare, Giles, Buckinghamshire, UK), the hybridization and detection of DIG-labeled probes were carried out with the DIG chemiluminescent labeling and detection kit (using 0.5 μg mRNA) following the protocols of the supplier (Roche). Putative differentially expressed clones, displaying signals of different intensity (strong or weak expression) after hybridization, were selected, purified at least three times, then plated and screened by a second round of differential hybridization as described above. Inserts from the verified cDNA clones were PCR-amplified with Taq polymerase directly from plaques with vector-specific primers flanking the insert. For dot blot hybridization, the PCR products were denatured with 0.5 M NaOH for 5 min and 1 μl aliquots spotted onto nylon membrane before hybridization. DNA from positive clones was transferred to the plasmid vector by *in vivo* excision using the ExAssist helper phage and the *E. coli* XLOLR strain (Stratagene). Then, cDNA fragments of *cin* genes were isolated by PCR-amplification, purified with a PCR purification kit (Qiagen) and sequenced from both strands using commercial sequencing services.

2.7. qRT-PCR

RNA was treated with DNAse I (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions to remove any contaminating genomic DNA prior to cDNA synthesis and absence of genomic DNA checked by PCR using primers specific for *V. inaequalis* glyceraldehyde 3-phosphate dehydrogenase (*gapdh*; EU873167) and apple *gapdh* (Table 2). cDNA was synthesised from this RNA using the Transcriptor first strand cDNA synthesis kit (Roche) according to the manufacturer's instructions; cDNA from two separate syntheses were pooled to control for cDNA synthesis bias. One in 10 dilutions of the cDNA in SDW were used for qRT-PCR.

qRT-PCRs were carried out on a Lightcycler® 480 instrument (Roche) using a SYBR Green detection system. The default cycling programme was used with the following modifications: 40 cycles, with 62 °C annealing for 10 s and a 6 s extension. Primers used for qRT-PCR are shown in Table 2. Data were gathered from two independent infection/growth assays, with an additional assay including only cellophane-grown material. Each treatment within the assays was represented by three biological samples and the PCR for each biological sample was conducted in triplicate. PCR efficiency for each set of primers was calculated using the Lightcycler® 480 internal software. A gene expression normalization factor was calculated for each sample based on the geometric mean of two

Table 2
List of primers used

Gene	Name	Sequence(5′–3′)	Purpose
cin1	CIN1-1F	TAT-CAA-AAC-ATC-GGC-CAC-GA	qPCR
cin1	CIN1-1R	CTC-GAA-CCG-TTT-TTC-TTC-CA	qPCR
cin3	CIN3-2F	AGC-GCC-AGA-GTG-ATG-CTC-CC	qPCR
cin3	CIN3-2R	CCT-GAG-CGC-CCG-CCT-CAT	qPCR
β-tubulin	Vi-TUB-F	CGT-CGT-GAG-GCT-GAA-GGT	qPCR
β-tubulin	Vi-TUB-R	CGA-TGG-GAC-AAC-AGA-GAA-TG	qPCR
ribo L12	Vi-L12-F	GTT-GTC-CCA-TCT-GCC-TCT-TC	qPCR
ribo L12	Vi-L12-R	GTC-CTT-GGC-CAT-TGA-CTT-GT	qPCR
Vi-gapdh	Vi-GAPDH-F	GGC-AAG-ACC-ATC-CGT-TTC-TA	qPCR
Vi-gapdh	Vi-GAPDH-R	GAC-ACC-CAT-GAC-GAA-CAT-TG	qPCR
Md-gapdh	Md-GAPDH-F	TGG-AAA-ATT-GAC-CGG-AAT-GT	qPCR
Md-gapdh	Md-GAPDH-R	GAC-CTG-CTG-TCA-CCA-ATG-AA	qPCR
cin1	CIN1-F	GGG-AAT-TCA-AAA-TGC-AGT-ATT-CCA-GCC	Sequencing
cin1	CIN1-R	AAA-AGG-AAA-AAA-GCG-GCC-GCT-CTAGTA- TCC-TCC-TGG-ACC	Sequencing
cin1	CIN1-InF	GTC-GTC-GAC-CCT-AAC-CCA-CCT-GCC-T	Sequencing
cin1	CIN1-InR	TAG-GCA-GGT-GGG-TTA-GGG-TCC-TAC-GAC	Sequencing
cin3	CIN3-F	TTC-ACT-GTT-CCG-TCC-AAC	Sequencing
cin3	CIN3-R	CTA-GGC-CTT-TTG-AGC-ATC-CG	Sequencing

stably expressed reference genes, β-tubulin (EU853839) and 60s ribosomal protein L12 (EU853840) (W. Cui, unpublished) with geNorm software v3.4 (Vandesompele et al., 2002) and used for the calculation of relative expression of each targeted gene in each sample. The mock-inoculated leaves did not amplify any bands with the fungal-specific primers.

2.8. Amplification and sequence analysis of cin1 and cin3 alleles

Genomic DNA was extracted from three-week-old cultures of *V. inaequalis* grown in 100 ml liquid PDB cultures using a modification of the Yoder method (Yoder, 1988). Freeze-dried mycelia were ground in liquid nitrogen and extracted in 15 ml of extraction buffer (150 mM EDTA, 50 mM Tris/HCl pH 8.0, and 1% (v/v) Sarkosyl). RNAse A (Sigma–Aldrich, Milwalkee, WI) was added to a final concentration of 4 U ml^{-1} and the solution was incubated at 37 °C for 30 min. Proteinase K was added to a final concentration of 1.5 U ml^{-1} and the solution was incubated at 50 °C for 2 h. Following a low-speed centrifugation the supernatant was extracted with phenol/chloroform then twice by chloroform. Genomic DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate and two volumes of 95% ethanol. The pellet was washed with 75% ethanol, dried and resuspended in 10 mM Tris/HCl, pH 8.0, and 1 mM EDTA. DNA was quantified using a Nanodrop® ND-1000 Spectrophotometer. A stock of 20 ng μl^{-1} was used for PCR.

PCR primers were designed to amplify the entire predicted coding sequence of each gene (Table 2). PCR conditions in 20 μl reactions were as follows: 1 U Expand Long Template Taq Tgo (Roche), dNTPs (350 nM), primers (300 nM of each) and 1× PCR buffer. PCR amplification was carried out for 35 cycles of denaturation at 94 °C for 10 s, annealing at 60 °C for 1 min, and elongation at 72 °C for 3 min. Before cycling, samples were incubated at 94 °C for 5 min, a final extension was performed at 72 °C for 10 min. Amplicons were resolved on 1% (w/v) agarose gels, and either cloned into pGEMT-Easy and sequenced or sequenced directly using an ABI PRISM 3100 DNA sequencer (Applera, Norwalk, CT). Sequence annotation and alignment was performed using VectorNTI Version 9 (Invitrogen). BLAST analysis was performed against NCBI (http://www.ncbi.nlm.nih.gov) and the Broad fungal genome (http://www.broad.mit.edu) databases. A haplotype based sequence analysis of the repeats was performed with the aid of the dot plot mode in VectorNTI and the differences mode in Genedoc Version 2.6.003 (http://www.psc.edu/biomed/genedoc/).

3. Results

3.1. Differentiation of V. inaequalis on apple leaves and cellophane

Spores of *V. inaequalis* germinated and formed appressoria-like swellings on the apple leaf surface within two days (Fig. 1A). After penetrating the host cuticle, *V. inaequalis* grew between the cuticle and the top of the epidermal cells, before undergoing a dramatic morphological change to produce sub-cuticular stromata (Fig. 1B). The stromata began developing 5–7 dpi, were between 50 and 100 μm in diameter and had a distinct boundary. Within the stromata, the cells differentiated into pseudoparenchyma and were larger and more irregular in size and shape than hyphal filaments and appeared to be dividing laterally (Fig. 1B). Conidiophores and conidia were produced from stromata after 10–14 dpi (Fig. 1C). In addition to stromata, *V. inaequalis* produced sub-cuticular runner hyphae which were significantly wider, with diameters of over 5 μm, than typical tubular, surface hyphae (Fig. 1D). Transverse sections of infected leaves showed the sub-cuticular hyphae to be somewhat flattened in cross-section (Fig. 1E–F). The cuticle surrounding the sub-cuticular hyphae appeared to be significantly thickened compared to uninfected areas. With β-(1–3)-glucan antibodies we observed specific staining of fungal cells walls, clearly showing fungal location, and of callose, a defense response in plants (Fig. 1F). Callose formation under the sub-cuticular hyphae was often observed, suggesting that there is some recognition of *V. inaequalis* even in a compatible interaction.

On cellophane discs placed on PDA, conidia of *V. inaequalis* germinated and formed appressoria-like swellings (Fig. 2A). Similarly to the effect observed in infected leaves, the penetrating hyphae were larger than surface hyphae with diameters of over 5 μm (Fig. 2A). The swollen, runner-like hyphae within the cellophane are obviously different to the tubular hyphae found on the surface of the cellophane. This is demonstrated dramatically when hyphae grow out of the cellophane and growth reverts to the tubular hyphal form, with reduced hyphal diameter (Fig. 2B). Within the cellophane after a week these hyphae differentiate further into structures resembling stromata that are formed under the cuticle in leaves (Fig. 2C). Transverse sections confirmed that the stroma-like structures were growing within the cellophane discs (Fig. 2D–E). The stroma-like structures consisted of single or multiple layers of cells resembling pseudoparenchyma. These cells had irregular shapes and dimensions similar to the stromal cells found *in planta*, with diameters of 10–15 μm. Macroscopic observations of *V. inaequalis* grown on cellophane discs after three weeks re-

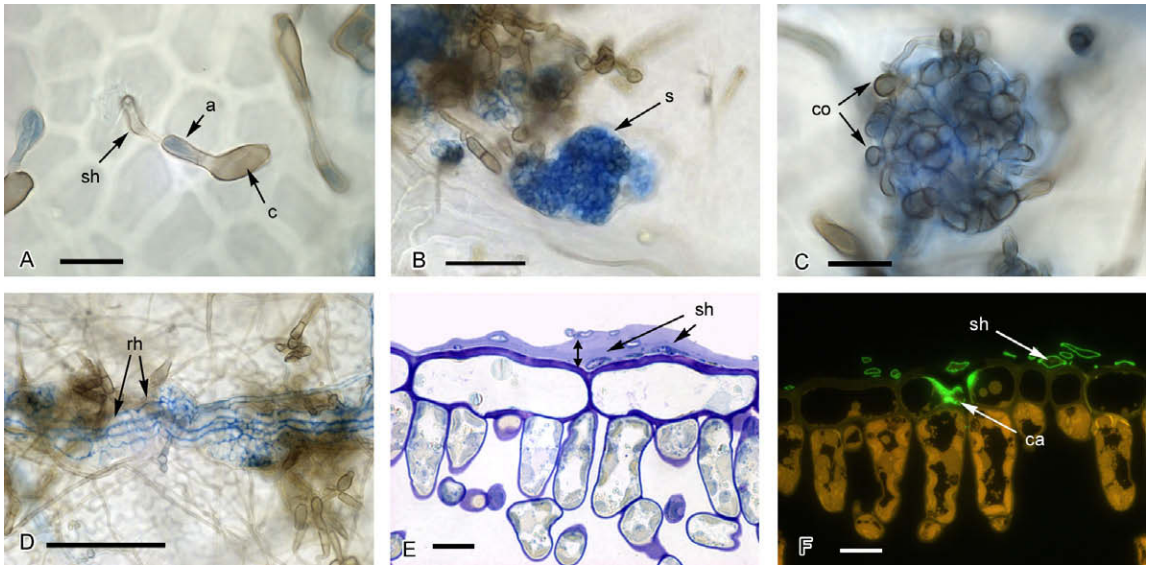


Fig. 1. Cytology of *V. inaequalis* apple leaf infection. (A) *V. inaequalis* conidium germinating and forming an appressorium on the surface of the leaf at 2 dpi. Bar = 10 μm; (B) *V. inaequalis* sub-cuticular stroma formed inside the leaf at 10 dpi. Bar = 100 μm; (C) *V. inaequalis* conidiophores growing out from the stroma at 14 dpi. Bar = 10 μm; (D) Sub-cuticular *V. inaequalis* hyphal at 14 dpi. Bar = 50 μm; (E) Cross section of an infected leaf at 14 dpi. Note the increased thickness of the cuticle (double arrow). Bar = 10 μm; (F) *V. inaequalis* stroma formed inside an apple leaf at 14 dpi immuno-stained with anti-(1→3)-β-glucan antibodies. Bar = 10 μm. (A–E) Light microscopy of infected tissues stained with aniline blue (Bruzzese and Hasan, 1983). (F) Fluorescence microscopy under UV excitation. Key: dpi = days post-inoculation; a = appressorium; c = conidium; ca = callose; co = conidiophores; rh = runner hyphae; sh = sub-cuticular hyphae; s = stroma.

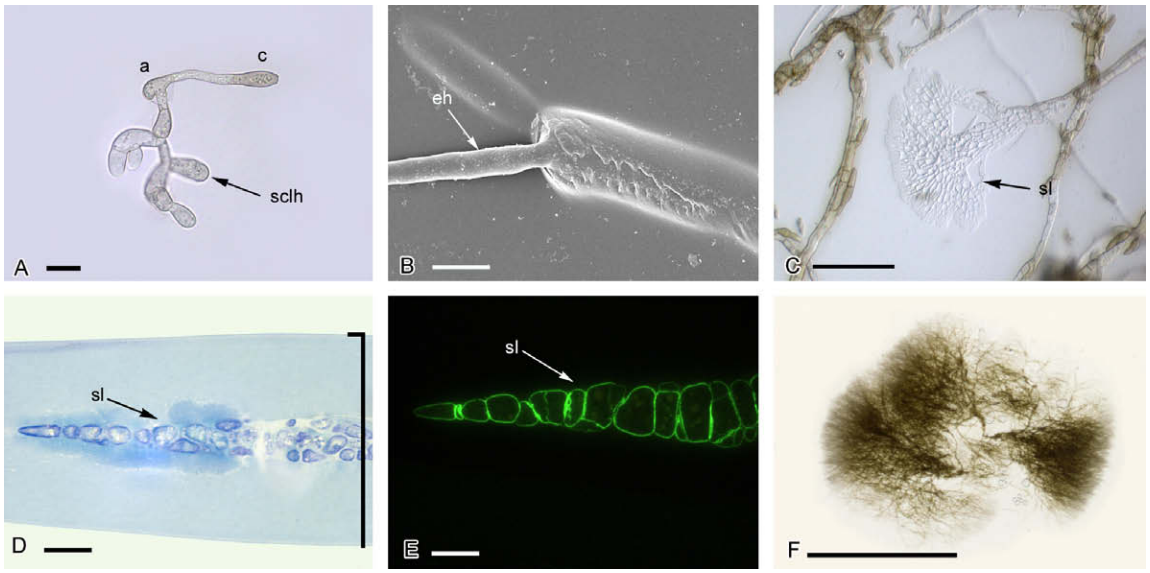


Fig. 2. Cytology of *V. inaequalis* inside a cellophane membrane. (A) *V. inaequalis* conidium germinating, forming an appressorium at the surface of the cellophane and differentiating subcuticular-like hyphae inside the membrane at 2 dpi. Bar = 10 μm. (B) Scanning electron micrograph of hypha emerging from cellophane. Note reduction of hyphal diameter. Bar = 10 μm. (C) Stroma-like structure formed inside the cellophane at 10 dpi. Bar = 100 μm. (D) Stroma-like structure formed inside the cellophane stained with aniline blue 14 dpi. Bar = 10 μm. Bracket indicates the width of cellophane. (E) Stroma-like structure formed inside the cellophane at 14 dpi immuno-stained with anti-(1→3)-β-glucan antibody. Bar = 10 μm. (F) Stroma-like structures formed inside cellophane at 21 dpi, surface mycelia were scraped off. Bar = 5 mm. (A, C, D, and F) Light microscopy, (E) Fluorescence microscopy under UV excitation. Key: a = appressorium; c = conidium; eh = emerging hypha; sclh = sub-cuticular-like hyphae; sl = stroma-like.

vealed a fan-like morphology of the resulting colonies (Fig. 2F). Interestingly the hyphal mass grew in the same linear orientation along the disc (Fig. 2F), perhaps following microscopic grooves in the cellophane. These structures were induced by solid cellophane sheets on nutrient agar, but not directly on 0.5% carboxymethylcellulose (Sigma–Aldrich) malt extract medium.

3.2. Construction of cDNA library from V. inaequalis grown on cellophane, and screening for differentially expressed genes

The growth of *V. inaequalis* within cellophane, as an *in vitro* model for sub-cuticular growth and morphogenetic differentiation, was used for the cloning of genes that might be involved in infection-specific differentiation. To search for genes specifically expressed by *V. inaequalis* on cellophane, a differential screening of the cellophane-cDNA library was performed. Single plaques showing stronger hybridization with the cellophane-cDNA probe were isolated, re-plated, and replicate filters were hybridized again with DIG-labeled cellophane- and liquid-derived probes. In total, five differentially expressed clones were isolated. Re-screening confirmed that all of these clones were differentially expressed. Fig. 3 shows replicate filters of a constitutively expressed gene (*cbh1*) and the five differentially expressed clones (*cin1* to *cin5*) hybridized to these probes.

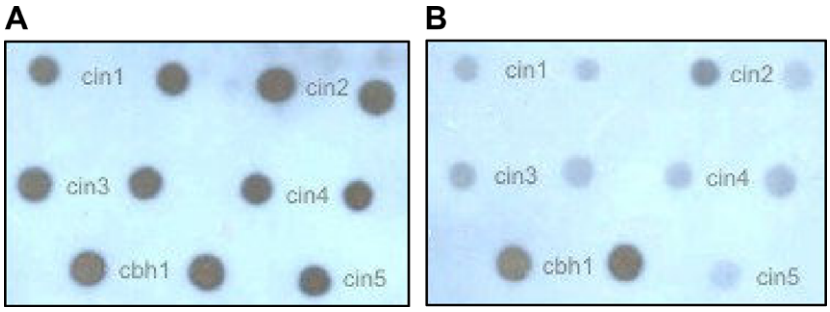


Fig. 3. Differential hybridization of cDNA library clones. cDNAs amplified by PCR were applied as duplicate dots (except *cin5* which is the same as *cin4*) to a nylon membrane and hybridized with DIG-labeled single-stranded cDNA probes derived from cellophane-grown mycelium (A) and liquid-grown culture (B). *cbh1* encoding a cellobiohydrolase gene that shows similar expression in liquid culture and cellophane and up-regulated cellophane-*in*duced genes *cin1*, *cin2*, *cin3*, *cin4*, and *cin5* are shown.

3.3. Identification and sequence analysis of the V. inaequalis cin genes induced during growth on cellophane

Recombinant plasmids were excised as pBK-CMV phagemids from all verified cDNA bacteriophage clones. Sequence analysis revealed that *cin4* and *cin5* cDNAs corresponded to the same gene. Thus we obtained four independent clones, named *cin1* to *cin4*, whose expression was potentially up-regulated during growth on cellophane (Table 3). Full-length cDNAs corresponding to *cin1* and *cin3* were identified in an EST library produced from apple seedling leaves infected with *V. inaequalis* isolate MNH135 (Newcomb et al., 2006). The sequence analysis of *cin1* and *cin3* showed no similarity to any sequences in publicly available databases. The predicted Cin1 protein (Fig. 4A) is 523 amino acids in length, has a putative signal peptide (25 amino acids), and eight imperfect amino acid repeats of about 60 amino acids each. Each repeat has four conserved cysteine residues, and the predicted protein has a preponderance of charged residues (40%) with a slightly greater proportion of basic groups. The predicted Cin3 protein has 228 amino acids with five repeats of 32 amino acids (Fig. 4B). It also has a high proportion of charged residues (40%), but no cysteines. In contrast, *cin2* has a strong similarity (76% identity) to the 39 kDa subunit of the *Aspergillus nidulans* NADH-ubiquinone oxidoreductase (E.C.1.6.5.3) (Table 3). *cin4* is not full-length but has significant similarity (65% identity) to proteins from the cupin-2 superfamily from *Botrytis cinerea* (BC1G_07827.1) and *Sclerotinia sclerotiorum* (SS1G_03393.1) that have no assigned function. The analysis of *cin2* and *cin4* was not pursued any further.

3.4. Expression analysis

The expression of *cin1* and *cin3* during infection (5 and 10 dpi), and *in vitro* growth on cellophane and PDB liquid culture was analyzed using qRT-PCR. Both genes were expressed to very high levels in infected tissue at both time points investigated compared to liquid culture (Fig. 5). The infection time points sampled correlate

Table 3
Characterization of cellophane-induced cDNAs

cDNA	Strain	Full-length	ORF size (bp)	Protein size (kDa)	Blastp $<e^{-15}$	Genbank accession Nos.	Amino acid repeats	SigP-HMM (probability)[a]
cin1	MNH135	Yes[b]	1569	58.2	No	EU189192	8 (~60 aa)	Yes (1.000)
cin2	ZH1	Yes	1029	38.7	Yes		None	No
cin3	MNH135	Yes[b]	684	22.5	No	EU189193	5 (32 aa)	Yes (0.924)
cin4	ZH1	No	561	20.4	Yes		None	No

[a] The SigP-HMM probability was derived from the following website: http://www.cbs.dtu.dk/services/SignalP/.
[b] Partial cDNA clones were found in the cDNA library from isolate ZH1, but full length ESTs were only cloned from isolate MNH135.

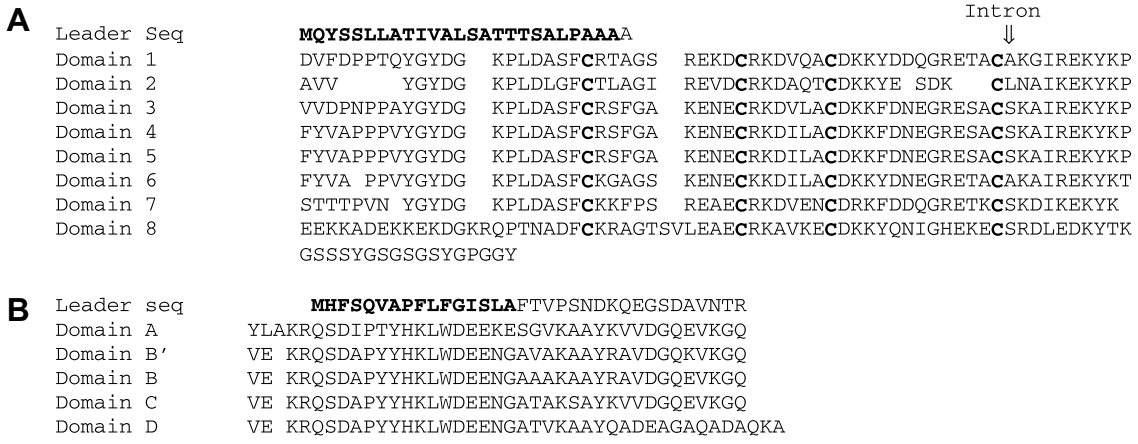


Fig. 4. (A) Alignment of the eight repeated domains from the predicted amino acid sequence of Cin1 from *V. inaequalis* (MNH135). The predicted signal peptide and the cysteine residues are highlighted in bold. The arrow indicates the position of the introns in the gene sequence. (B) Alignment of the 5 repeated domains from the predicted amino acid sequence of Cin3 from *V. inaequalis* (MNH135). The predicted signal peptide is highlighted in bold.

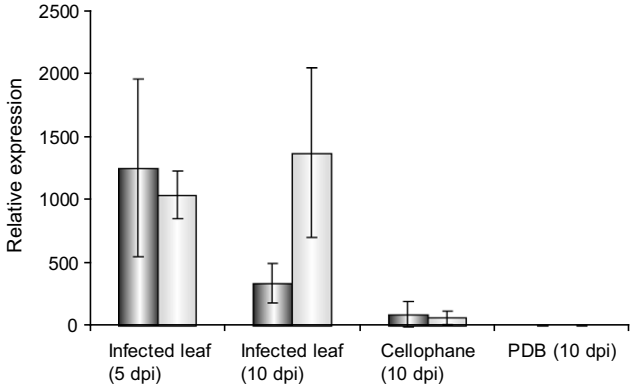


Fig. 5. Expression of *cin1* and *cin3* during differentiation of *V. inaequalis in vitro* and infection *in planta*. qRT-PCR was performed as described in methods. *cin1* data are represented by dark grey bars and *cin3* by light grey.

with the appearance of stroma (5 dpi) and the initiation of conidiogenesis from stromata (10 dpi). The expression of *cin1* is significantly higher in the early stages of infection ($P = 0.02$). In contrast *cin3* is strongly induced at both infection time points. During growth on cellophane both genes are also significantly upregulated when compared to growth on PDB, but to a lower extent than observed in infected leaves (Fig. 5).

3.5. Allelic variation of cin1 and cin3 across V. inaequalis races

Nineteen isolates of *V. inaequalis*, including representatives of all eight characterized physiological races, were collected from a variety of geographical locations (Table 1) (Bénaouf and Parisi, 2000; Bus et al., 2005; Lespinasse, 1994; Parisi and Lespinasse, 1996; Parisi et al., 1993; Roberts and Crute, 1994; Shay and Williams, 1956; Williams and Shay, 1957). Both *cin1* and *cin3* were amplified from genomic DNA from 18 isolates. *cin1* genomic sequences have a complex structure comprising nine exons and eight introns, except in isolate MNH135 which has ten exons and nine introns (Fig. 6A). The introns are located at the same position of each repeat and are highly conserved, but they are not inserted at the predicted domain boundaries (Fig. 4A). This structure suggests that the gene has evolved by internal duplications. This is corroborated by the observation that isolate MNH135 has eight repeated domains whereas all the others have only seven (Fig. 6B). Two domains in MNH135 (protein domains 4 and 5, including their introns) are identical, suggesting that they are the result of a recent duplication event. Further comparisons of the sequences of different isolates indicated there are other synonymous, and more infrequently non-synonymous, single-nucleotide polymorphisms (SNPs) but none of these differences appear to correlate with the known race status of the isolates (data not shown).

The *cin3* gene from MNH135 has no introns and encodes a protein with five repeated domains (Fig. 4B). *cin3* is more polymorphic than *cin1* as we identified five alleles that vary in number and sequence of repeats and two isolates (1066 and USR4) are missing one of these repeats (Fig 6B). This DNA sequence variation allowed us to perform a haplotype-based sequence analysis of the repeats to look for any underlying evidence that might point towards the mechanism(s) that gave rise to the different repeat structures within the isolates. A phylogenetic analysis of the nucleotide sequences of the *cin3* alleles from the 18 *V. inaequalis* isolates revealed five clades (data not shown) corresponding to five different alleles (Fig. 6B). Although most *cin3* alleles have five repeats, sequence analysis revealed that there are four different domains (A–D) and that most isolates have duplicate domains. The domain structure from members of each clade is shown in Fig. 6B.

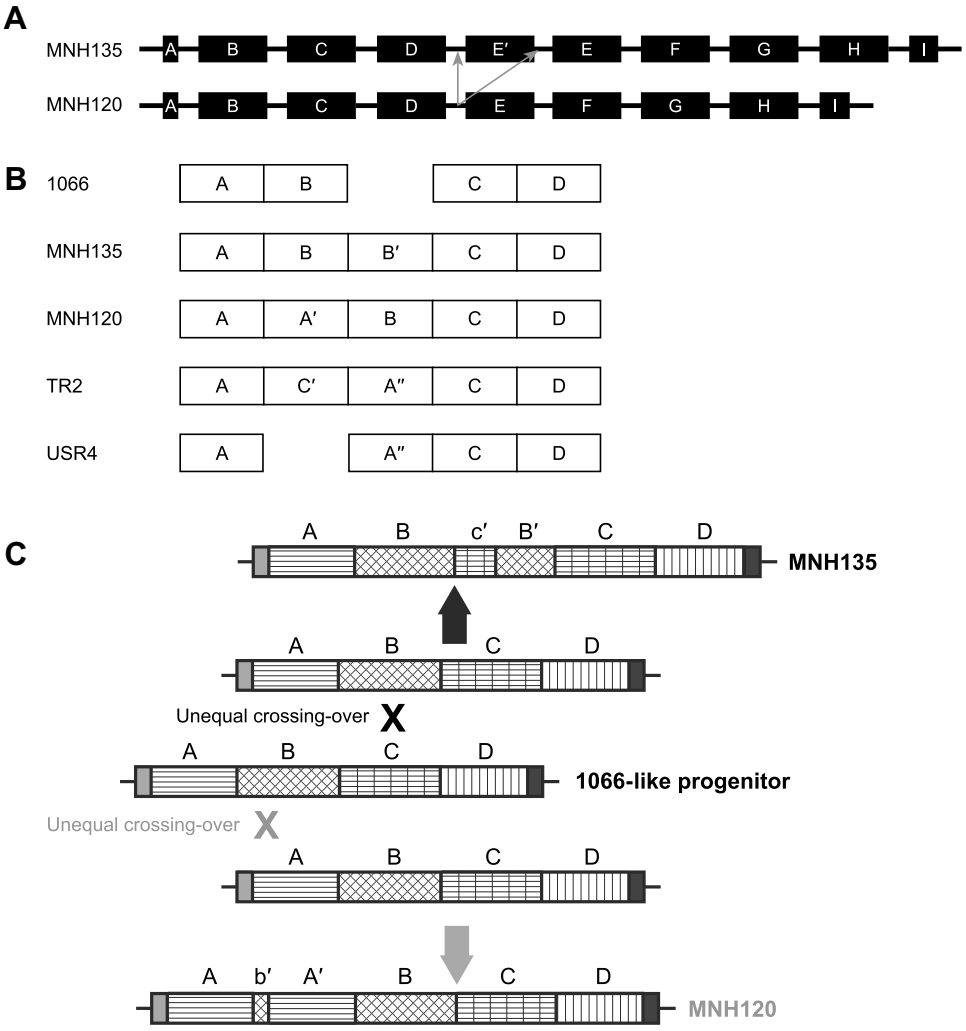
The haplotype analysis revealed strong evidence that the second (A′-type) repeat domain in the MNH120 group has arisen by an unequal inter-allelic recombination event between the first (A-type) and third (B-type) domains (Fig. 6C). In fact, the probable position of the cross-over can be accurately predicted to lie at the beginning of the A-type repeat (between bases 15 and 24 of the A-type and B-type repeats, respectively). A likely origin of this isolate group is a progenitor with the simple 4 domain (ABCD) structure of isolate 1066. By analogy, a slightly different origin can be postulated for the MNH135 isolate group, with an independent recombination event in the middle of the B-type repeat being the most likely scenario. Based on the sequence variation between the B-type and C-type domains (the most likely position is between bases 61 and 67 of the B-type and C-type repeats, respectively). Notably unique haplotype structures were found at the first 10 bases of the A-type repeat and the last 30 bases of the D-type repeats, respectively (indicated by a darker shading in Fig. 6C). Consistent with these deductions the domains with putative recombination events ended up outside of the core clusters of A, B, C, and D domains in a phylogenetic tree of all the different domains (Supplementary Fig 1). As for *cin1* this variation and other SNPs did not correlate with race-specificity or geographical location.

4. Discussion

We have observed that growth of *V. inaequalis* on cellophane is morphologically similar to sub-cuticular biotrophic growth observed *in planta*. It is not uncommon for spores of plant pathogenic fungi to germinate and form appressoria on hard or hydrophobic artificial surfaces, similar to their behavior on plant surfaces (Howard and Valent, 1996; Perfect et al., 1999). It is, however, less common for subsequent infection structures to be induced *in vitro*, i.e., in rust fungi (Deising et al., 1995). Spores of *V. inaequalis* can germinate, form appressoria, penetrate and colonize cellophane. This is clearly due to physical recognition as opposed to a merely chemical response since soluble cellulose did not trigger this differentiation. The hyphae differentiate and form stromata within the cellulose sheet but do not appear to form them on the cellophane surface or in the agar below. This suggests a delicate sensing mechanism that recognizes texture and/or pressure of the surrounding medium. If the hyphae emerge from the cellophane they immediately revert to the tubular hyphal growth observed on agar (Fig. 2B), suggesting that this mechanism is reversible and dynamic.

The sub-cuticular hyphae and stromal cells have morphology distinct from typical tubular growth observed on the leaf surface or *in vitro*. The cells appear to be larger and do not appear to rapidly melanize in the same way as surface, tubular hyphae (Fig. 1D). Some of the increase in width of sub-cuticular *in planta* hyphae may be attributable to their flattened nature (Fig. 1E–F). However, in cellophane these cells do not appear to be compressed and grow up to 15 μm in diameter. Little is known about the genes involved in such morphological switches and the importance of these structures in the pathology of *V. inaequalis*. This is due primarily to the difficulty in identifying *V. inaequalis* genes or proteins specifically up-regulated in infected apple tissue. Growth of *V. inaequalis* on cellophane is a potential *in vitro* model for biotrophic growth and we have exploited this system to identify genes specifically induced during this morphological switch.

We have successfully used differential screening of a cDNA library prepared from mycelia grown within cellophane to identify four genes from *V. inaequalis* up-regulated during growth on cellophane compared to liquid nutrient media. Using qRT-PCR two of these genes, *cin1*, and *cin3*, were confirmed to be up-regulated *in*



Alignment of MNH120 polymorphic bases (haplotype)

```
                 6 9 15 30 42 54 62 66 69 76 85 89 96 101 106 112 116
                4 8 12 26 32 51 60 64 68 71 84 87 93 99 103 110 114
MNH120_D1A : TACCACCACATAACGACGAGAGTTGGAGTGACAAGTTATGCAAGAGAAAGGCCAG
MNH120_D2bA: ---GCAGGTATAACGACGAGAGTTGGAGTGACAAGTTATGCAAGAGAAAGGCCAG
MNH120_D3B : ---GCAGGTGCATAGATGGCGTGTGTCGCGACCGGCTCCGTAGGAGAAAGGCCAG
X-over position bp 15-X-24 bp
```

Alignment of MNH135 polymorphic bases (haplotype)

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                 6 9 15 30 42 54 62 66 69 76 85 89 96 101 106 112 116
                4 8 12 26 32 51 60 64 68 71 84 87 93 99 103 110 114
MNH135_D2B : ---GCAGGTGCATAGATGGCGTGTGTCGCGACAGGCTCCGTAGAAGAAAGGCCAG
MNH135_D3cB: ---GTAGGTGCATAGATGGTGTGTGCCGCGACAGGCTCCGTAGGAGAAAGGCCAG
MNH135_D4C : ---GTAGGTGCATAGATGGTGTGTACCGCTACAAATTCCGTAGGAGGAAGGCCAG
Likely X-over position       bp 61-X-67 bp
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Fig. 6. Variations in domain organization among Cin1 and Cin3. (A) Structure of *cin1* from MNH135 and MNH120. Note that the letters represent exons but because of the position of the introns within each domain these only partially match the numbered domains in Fig. 4. The arrows represent the location of a putative unequal crossing-over event leading to the insertion of an additional domain (E′) in MNH135. (B) Schematic of the domain structures of five of the *cin3* alleles in *V. inaequalis* strains from different groups. A′, A″, B′, and C′ represent significant sequence and position variants of A, B, and C, respectively. (C) Model for the evolution of *cin3* domain structure. Note bp positions given above the sequences are from domain 1 (A) and give the positions of the polymorphic bases in the repeat. Lower case b′, and c′ signify a minor portion of a repeat rather than a complete repeat and indicate the likely relationship of this portion of the repeat with respect to the neighboring repeat. Note that these lower case letters are not shown in the other figures. Upper case A′ and B′ indicate the remaining (major proportion) of these "hybrid" repeat domains. D1, D2, D3, and D4 refer to the position of the repeats in the protein. The A and C domain haplotype sequences of MNH120 and MNH135 respectively are shown in grey while the B domain is shown in black. The A′ and B′ domains are shown in both shades to emphasize the likely positions of the recombination events in MNH120 and MNH135 or their ancestors. The arrows indicate the consequence of the postulated unequal crossing over events leading to the formation of the MNH135 and MNH120 groups, respectively.

planta during infection of apple seedlings compared with expression in liquid medium. In addition, *cin1* and *cin3* were independently found in an EST library from infected leaf tissue, from which full-length clones were obtained (Newcomb et al., 2006). The expression patterns of these two genes suggest an involvement in *V. inaequalis* pathogenesis during fungal sub-cuticular invasion of host tissues (Fig. 5). We expected to discover more cellophane-induced genes, however, the differential screening of the li-

brary was not performed exhaustively. To determine whether *in vitro* growth on cellophane is a model for pathogenesis, more clones would need to be tested, perhaps using a different approach such as suppression subtractive hybridization.

Neither *cin1* nor *cin3* have significant matches to known genes, including those present in the 25 whole fungal genome sequences now available (http://www.broad.mit.edu/). Genes encoding secreted proteins with internal sequence repeats have been identified in the genomes of many fungi however (Levdansky et al., 2007; Puyesky et al., 1999; Verstrepen et al., 2005). Many of these proteins have been shown to be coating the outer cell wall, and to be participating in cell-to-cell or cell-to-substrate adherence, for instance during mating, flocculation or biofilm formation (Dranginis et al., 2007; Hung et al., 2002; Linder and Gustafsson, 2008; Wang and St. Leger, 2007; Li and Palecek, 2008; Verstrepen et al., 2005). Other roles of such proteins include sexual differentiation (Kim and Nelson, 2005), cell wall organization (Ruiz-Herrera et al., 2008), or rendering cell walls hydrophobic (Teertstra et al., 2006). While no homologs of Cin1 have been identified in other fungi, fungal proteins exist with similar overall characteristics (leader peptide, repeated domain structure, cysteine-rich, high proportion of charged residues). An interesting example is Qid74, a cell wall protein from *Trichoderma harzianum qid74* deletion mutants exhibit increased sensitivity to cell wall degradation by different lytic enzymes and reduced capacity for adherence (Rey et al., 1998; Rosado et al., 2007). The repetitive structure of Cin3 is comparable to several proteins identified in the secretome of *Ustilago maydis* (Mueller et al., 2008; Müller et al., 2008; Teertstra et al., 2006; Wösten et al., 1996). Of the genes that encode these proteins, *rsp1* has been shown to play an essential role in the pathogenicity of this fungus when simultaneously deleted with a second gene from this group, *hum3*, whereas single *rsp1* or *hum3* gene deletions have no effect on pathogenicity (Müller et al., 2008; Teertstra et al., 2006). In common with Cin1 and Cin3 some of these internal repeat proteins vary in the number and sequence diversity of their domains between isolates (Fidalgo et al., 2006; Hoyer, 2001; Hung et al., 2002; Levdansky et al., 2007; Verstrepen et al., 2005; Zhang et al., 2003). Repeated proteins are not exclusively involved in adhesion and cell wall function however. Several secreted effector proteins of phytopathogenic microbes also possess repeat domains (Allen et al., 2004; Gürlebeck et al., 2006; Nissan et al., 2006; Tian and Day, 2006), and in some cases, variation in domain structure has been implicated in mediating host specificity (Herbers et al., 1992; Yang et al., 1994).

The repeat domain structures of both *cin1* and *cin3* are likely to have arisen from ancestral genes by duplication through unequal crossing over. There are five alleles for *cin3* amongst the 18 isolates including two (1066 and USR4) which have whole domain deletions, that indicates considerable variation at this locus. The variation is most pronounced in the middle domains, whereas domains A and D, are highly conserved between different isolates (although the A and D domains are the most different from each other) (Fig. 6B). The evidence for a recent unequal crossing-over event that gives rise to the second domain in the MNH120 group is strong. While there is evidence for a similar event giving rise to the MNH135 group the most likely origin of both groups is derivation from a progenitor that has a structure similar to the 1066 isolate. This evidence also suggests that unequal crossing-over events, like the one that probably gave rise to the MNH120 group, are the most likely mechanism that gave rise to the repeat structure of the Cin3 protein. Such unequal cross-over events could occur either at meiosis between paired homologues or between sister chromatids. Gene conversion is another possible explanation that has been postulated for minisatellite expansion in fungi (Pâques et al., 2001), but there is no clear evidence of the ‘‘random’ pattern of origin of polymorphisms from the two putative recombining molecules typical of gene conversion. Intra-allelic recombination events caused by replication slippage can also generate duplications in fungi (Richard and Dujon, 2006). However a SNP shared the domain B’ of MNH135 and the B domain from MNH120 (position 103 in Fig. 6C) suggests inter-allelic recombination as a more likely route than intra-allelic replication slippage. Existing evidence from mammalian systems (Buard et al., 1998) also suggests that intra-allelic recombination becomes predominant when repeat array lengths are considerably greater (over 18 repeats) than the number of repeats in *cin1* and *cin3* (four to eight). With a more extensive haplotype-based sequence analysis it may be possible to deduce the origin of both the Cin3 repeat structure and the likely origin of the remaining two isolates (TR2 and USR4) that possess different repeat structures.

There is less between-strain variation of *cin1* with only two significant alleles, one of which has an additional internal domain that appears to have arisen from a recent duplication. In contrast to *cin3* the variation within the *cin1* gene between isolates is greatest in the domains at the termini of the proteins. The central domains have the greatest sequence conservation when comparing their sequence between isolates. Whether this variation is related to function is unknown.

While variation in the domain structure of both *cin1* and *cin3* was observed across 18 *V. inaequalis* isolates, there were no obvious correlations to geographic location or race. Correlations of obvious differences between isolates of the same race would suggest they may function as avirulence effectors and be associated with the recognition specificity of a complementary resistance gene in the host but these differences are not necessarily always obvious. Whether these genes play a role in pathogenicity and/or specificity will need to be determined using gene replacement or knock-down experiments using RNAi (Fitzgerald et al., 2004). These experiments are currently being performed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2008.07.010.

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1971 – 1982 Allgemeinbildende Schule Nr. 149 mit vertieftem Deutschunterricht, Kiew, Ukraine. Abschluss der oberen Sekundarstufe mit dem Zeugnis über die vollständige mittlere Bildung (mit dem Abitur vergleichbar)

1982 – 1983 Laborantin am Institut für Probleme der Kanzerogenese, Kiew, Ukraine, Abteilung Biophysik der Kanzerogenese

1983 – 1984 Laborantin am Institut für Gerontologie, Kiew, Ukraine, Abteilung Physiologie und Hygiene der Arbeit

1984 – 1989 Studium der Biologie an der Nationalen Taras-Schewtschenko-Universität Kiew, Ukraine, Fachrichtung Physiologie mit Abschluss Diplom-Biologin, Pflanzenphysiologin, Lehrerin für Biologie und Chemie

Diplomarbeit am Institut für Hydrobiologie, Kiew, Ukraine

- Thema „Einfluss von Salzwasser auf den physiologischen Zustand der Wasserpflanzen und deren Rolle in der Selbstreinigung von Wasserbecken“

1989 – 1996 Aspirantin/Ingenieurin am Institut für Mikrobiologie und Virologie, Kiew, Ukraine, Abteilung Pflanzenpathogene Bakterien

- Analyse der Lektine, Polyamine, Exo- und Lipopolysachcharide von pflanzenpathogenen Bakterien

1996 – 1998 DAAD-Stipendiatin am Institut für Mikrobiologie der Universität Hannover

- Suche und Entwicklung von mikrobiellen Antagonisten gegen den Apfelschorferreger *Venturia inaequalis*
- Polyamine als Biomarker der pflanzenassoziierten und pflanzenpathogenen Enterobakterien

1998 – 2000 Wissenschaftliche Mitarbeiterin am Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Abteilung Molekulare Genetik, AG Bakteriengenetik

- Diversität der mikrobiellen Gemeinschaft im Boden, Identifizieren neuer Gene für Enzyme und Wirkstoffe mittels Metagenomik mithilfe der Genexpression-Screening der angelegten Metagenombanken
- Konstruktion von *E. coli-Bacillus*-Shuttle-Cosmidvektor, Anlegen der Genbank von einem Wirkstoffproduzenten *Bacillus subtilis* A1/3, Identifizieren der Gencluster für Wirkstoffbiosynthese mittels des automatisierten Hochdurchsatzscreenings

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- Pathogenese-relevante Differenzierung und Genexpression von Apfelschorferreger *Venturia inaequalis*, Identifizierung der Pathogenitätsgene

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PUBLIKATIONSLISTE

Kucheryava, N., Bowen, J.K., Sutherland, P.W., Conolly, J.J., Mesarich, C.H., Rikkerink, E.H.A., Kemen, E., Plummer, K.M., Hahn, M., Templeton, M.D. 2008. Two novel *Venturia inaequalis* genes induced upon morphogenetic differentiation during infection and *in vitro* growth on cellophane. *Fungal Genetetics and Biology,* **45**: 1329–1339.

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Votselko, S.K., Iutinskaya, G.A., Kovalenko, E.A., Kucheryava, N.S. 2000. The molecular-weight characteristics of the bacterial lectins and humus components in soil. *Mikrobiolohichnyi Zhurnal,* **62**: 17-25 (Russisch).

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ERKLÄRUNG ZU DEM EIGENEN ANTEIL AN DEN PUBLIKATIONEN UND MANUSKRIPTEN

Die Promotionsarbeit wurde als kumulative Dissertation eingereicht und umfasst 2 Manuskripte und 5 Publikationen. In der vorliegenden Arbeit wurde der Eigenanteil an den Publikationen in der ausführlichen Zusammenfassung der Ergebnisse verdeutlicht.
Hiermit erkläre ich, dass ich im Rahmen dieser Promotionsarbeit eigene Beiträge zu folgenden wissenschaftlichen Publikationen und Manuskripten erstellt habe:

1. Kucheryava, N., Bäumlein, H., and Hofemeister, J. Metagenomic expression libraries from soil DNA for accessing the diversity of soil microbial community and new genes related to antibiotic and hydrolytic activities.

Diese Arbeit wurde von mir unter der Betreuung von Herrn Dr. Bäumlein und Herrn PD Dr. Hofemeister durchgeführt.

2. Kucheryava, N., Steinborn, G., Adler, B., and Hofemeister, J. Construction and use of a genomic cosmid library of an environmental *Bacillus subtilis* A1/3 strain to explore its genetic capacity.

Die Arbeit wurde zum großen Teil von mir unter der Betreuung von Herrn PD Dr. Hofemeister durchgeführt. Die Konstruktion des *E. coli-B. subtilis*-Shuttle-Cosmidvektors pSB 681 erfolgte gemeinsam mit Herrn Dr. Steinborn. Die Sequenzierung und Charakterisierung des Genclusters für die Biosynthese von Bacillomycin L erfolgte durch Frau Dr. Adler.

3. Kucheryava, N., Fiss, M., Auling, G., and Kroppenstedt, R.M. 1999. Isolation and characterization of epiphytic bacteria from the phyllosphere of apple, antagonistic *in vitro* to *Venturia inaequalis*, the causal agent of apple scab. *System. Appl. Microbiol*. 22: 472-478.

Die Arbeit wurde zum überwiegenden Teil von mir unter der Betreuung von Herrn Prof. Dr. Auling durchgeführt. Die Isolierung der Bakterien aus der Phyllosphäre von Apfelbäumen, die Substratnutzungsanalyse von antagonistischen Bakterien und die HPLC-Analysen von Polyaminen wurden von Herrn Dr. Fiss durchgeführt. Die Infektionsversuche mit *V. inaequalis* in Anwesenheit der Antagonisten wurden gemeinsam mit Herrn Dr. Fiss durchgeführt. Die Identifizierung der antagonistischen Bakterienisolate durch Fettsäure-

Analyse erfolgte in der Arbeitsgruppe von Herrn Prof. Dr. Kroppenstedt (DSMZ, Braunschweig).

4. Fiss, M., Kucheryava, N., Schönherr, J., Kollar, A., Arnold, G., and Auling, G. 2000. Isolation and characterization of epiphytic fungi from the phyllosphere of apple as potential biocontrol agents against apple scab (*Venturia inaequalis*). *J. Plant Dis. Protection.* 107: 1-11.

Bei der Publikation beteiligte ich mich bei der Charakterisierung der Pilzisolate aus der Apfelphyllosphäre. Gemeinsam mit Herrn Dr. Fiss untersuchte ich die antagonistische Wirkung der Pilzisolate gegen *V. inaequalis in vitro* sowie *in planta* bei den Infektionsversuchen an Apfelsämlingen. Die Isolierung von Pilzen aus der Phyllosphäre von Apfelbäumen wurden von Herrn Dr. Fiss durchgeführt. Die virulenten Einzelsporenisolate von *V. inaequalis* wurden von mir aus den Schorfläsionen von Blättern und Früchten verschiedener Apfelsorten isoliert, die für die Infektionsversuche und Tests auf antagonistische Wirkung von epiphytischen Mikroorganismen an Apfelsämlingen sowie *in vitro* bzgl. der Hemmung der Konidienkeimung verwendet wurden. Die Identifizierung von antagonistischen Pilzisolaten wurde von Herrn Dr. Fiss und Herrn Dr. Arnold durchgeführt

5. Zherebilo, O.E., Kucheryava, N., Gvozdyak, R.I., Ziegler, D., Scheibner, M., and Auling, G. 2001. Diversity of polyamine patterns in soft rot pathogens and other plant-associated members of the *Enterobacteriaceae. System. Appl. Microbiol*. 24: 54-62.

Mein Anteil an dieser Arbeit bestand in der Extraktion von Polyaminen aus den untersuchten Bakterienstämmen der Sammlung von phytopathogenen Bakterien des Instituts für Mikrobiologie und Virologie (Kiew, Ukraine) sowie Auswertung der erhaltenen Polyaminprofile.

6. Hofemeister, J., Conrad, B., Adler, B., Hofemeister, B., Feesche, J., Kucheryava, N., Steinborn, G., Franke, P., Gramme, N., Zwintscher, A., Leenders, F., Hitzeroth, G., and Vater, J. 2004. Genetic analysis of the biosynthesis of non-ribosomal peptide- and polyketide-like antibiotics, iron uptake and biofilm formation by *Bacillus subtilis* A1/3. *Mol. Genet. Genomics*. 272: 363-378.

Meine Beteiligung an dieser Publikation bestand in der Konstruktion des *E. coli-B. subtilis* Shuttle-Cosmidvektors pSB 681 (gemeinsam mit Herrn Dr. Steinborn), in dem die genomische Genbank von *B. subtilis* A1/3 angelegt wurde und durch das Screening der Genbank auf Gene für Wirkstoffbiosynthesen der komplette Gencluster für die Biosynthese

von Bacillomycin L identifiziert wurde. Die Sequenzierung und Charakterisierung des Genclusters für die Biosynthese von Bacillomycin L erfolgte durch Frau Dr. Adler.

7. Kucheryava, N., Bowen, J.K., Sutherland, P.W., Conolly, J.J., Mesarich, C.H., Rikkerink, E.H.A., Kemen, E., Plummer, K.M., Hahn, M., and Templeton, M.D. 2008. Two novel *Venturia inaequalis* genes induced upon morphogenetic differentiation during infection and *in vitro* growth on cellophane. *Fungal Genetetics and Biology*. 45: 1329-1339.

Diese Arbeit wurde zum großen Teil von mir unter der Betreuung von Herrn Prof. Dr. Hahn durchgeführt. Die Identifizierung der full-length *cin1*- und *cin3*-cDNA, Sequenzanalyse der Domänenstruktur dieser Gene in verschiedenen physiologischen Rassen von *V. inaequalis* sowie die Immunfluoreszenz-Markierung und -Mikroskopie erfolgte in den Arbeitsgruppen von Herrn Dr. Templeton (University of Auckland, New Zealand) und Frau Dr. Plummer (LaTrobe University, Australien).

Halle, 20.12.2006

Nataliya Kucheryava

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, dass die vorliegende Arbeit von mir selbstständig und ohne fremde Hilfe verfasst wurde. Es wurden keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht.

Diese Arbeit wurde keiner anderen Einrichtung zur Begutachtung vorgelegt.

Halle, 20.12.2006

Nataliya Kucheryava