Zur Homepage der Dissertation

Untersuchungen auf Sekundärstoffproduktion und physiologische Charakterisierung von marinen heterotrophen Bakterien aus dem deutschen Wattenmeer

Secondary metabolite production and physiological characterisation of marine heterotrophic bacteria from the german Wadden Sea

Von der Fakultät für Mathematik und Naturwissenschaften
der Carl-von-Ossietzky-Universität Oldenburg zur
Erlangung des Grades und Titels eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

angenommene Dissertation von

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Tag der Disputation: **22.09.2005**

Erklärung

Teilergebnisse dieser Arbeit sind als Beiträge bei den genannten Fachzeitschriften erschienen (Kapitel V) oder als Manuskripte eingereicht (Kapitel II, III, VI). Mein Beitrag an der Erstellung der Arbeiten wird im folgenden erläutert:

Martens, T., Steven, H., Simon, M., Brinkhoff, T. (2005) PCR-based screening of marine gram-positive bacteria for genes involved in secondary metabolite production. Submitted to Archives of Microbiology.

Durchführung der praktischen Arbeiten durch T. M.; Baumberechnung von H. S.; Erstellung des Manuskriptes durch T. M. und T.B., Überarbeitung durch T. M., M. S. und T. B.

Martens, T., Kessler, D., Gram, L., Grossart, H.-P., Simon, M., Brinkhoff, T. (2005) Bacteria of the *Roseobacter* clade show high potential for secondary metabolite production. Submitted to Applied and Environmental Microbiology.

Primer-Entwicklung, Sequenzierung, phylogenetische Analysen durch T. M.; PCR und Inhibitionstest von D. K. und T. M.; AHL Screening durch L. G.; Bereitstellung von Bakterien H.-P. G.; Erstellung des Manuskriptes durch T. M., Überarbeitung durch T. M., M. S., L. G.und T. B.

Dickschat, J. S., Martens, T., Brinkhoff, T., Simon, M., Schulz, S. (2005) Volatiles Released by a *Streptomyces* Species Isolated from the North Sea. *Journal of Natural Products*, in press.

Durchführung der chemischen Analysen durch J. D. und S. S.; Kultivierung von Bakterien, Untersuchungen zur Bioaktivität und konstruktive Lösung bei der Entwicklung des Versuchsaufbaus durch T.M.; Erstellung von Teilen des Manuskriptes durch T. M.,Überarbeitung des Manuskriptes durch T. M., M. S. und T. B.

Martens, T., Heidorn, T., Pukall, R., Simon, M., Brinkhoff, T. (2005) Reclassification of *Roseobacter gallaeciensis* Ruiz-Ponte *et al.* 1998 as *Phaeobacter gallaeciensis* gen. nov., comb. nov., and description of *Phaeobacter inhibens* sp. nov., antibiotic-producing members of the *Roseobacter* clade. Submitted to International Journal of Systematic and Evolutionary Microbiology.

Durchführung der praktischen Arbeiten durch T. M. und T. H.; Phylogenetische Analysen durch T. M.; Erstellung des Manuskriptes durch T. M. und T. B., Überarbeitung durch T. M., M. S. und T. B.

Weitere Veröffentlichungen:

Grossart, H. P., Brinkhoff, T., Martens, T., Duerselen, C., Liebezeit, G., Simon, M. (2004) Tidal dynamics of dissolved and particulate matter and bacteria in a tidal flat ecosystem in spring and fall. *Limnol Oceanogr* **49:** 2212-2222.

Rink, B., Seeberger, S., Martens, T., Duerselen, C., Simon, M., Brinkhoff, T. (2005) A spring phytoplankton bloom affects the composition of the bacterial communities in a coastal ecosystem. To be submitted to Environmental Microbiology.

Rink, B., Martens, T., Grossart, H. P., Simon, M., Brinkhoff, T. (2005) Tidal effects on coastal bacterial plankton in spring and fall. To be submitted to Limnology and Oceanography.

Tagungsbeiträge:

Martens, T., Stevens, H., Grossart, H.-P., Selje, N., Simon, M., Brinkhoff, T. (2002) PCR based screening for novel secondary metabolites from marine bacteria. Jahrestagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM), 24.-27.03.02, Göttingen.

Martens, T., Stevens, H., Grossart, H.-P., Selje, N., Simon, M., Brinkhoff, T. (2002) PCR based screening for novel secondary metabolites from marine bacteria. 3rd European Conference on Marine Natural Products, 15.-20.09.02., Elmau (Bayern)

Martens, T., Stevens, H., Grossart, H.-P., Selje, N., Simon, M., Brinkhoff, T. (2003) Screening of Marine Bacteria for new Secondary Metabolites. Jahrestagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM), 23.-26.03.03, Berlin

Martens, T.; Kessler, D.; Stevens, H.; Brinkhoff, T.; Simon, M. (2004) High Potential for Secondary Metabolite Production within the *Roseobacter*-Clade. 104th ASM General Meeting, 23.-27.05.04, New Orleans

Abkürzungsverzeichnis

AHL	acylated homoserine lactone
ASW	artificial seawater
Bchl *a*	Bacteriochlorphyll *a*
CLSA technique	closed-loop-stripping-apparatus technique
bp.	base pairs
DNA	desoxy-ribonucleic-acid
dNTP	didesoxy-ribonucleic-acid
et al.	et alii
FAD	flavin-adenine-dinucleotide
GC-MS	gas chromatography –mass spectronomy
gen. nov.	genus novum
Gram +	gram-positive
ISS	inorganic salt starch agar
LB	Luria-Bertani medium
MB	marine broth
NRPS	non ribosomal peptide synthetase
OD_{600}	optical density at 600 nm
OMA	oatmeal agar
PCR	polymerase chain reaction
PKS	polyketide synthase
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	rotations per minute
RT-PCR	reverse trancriptase - polymerase chain reaction
sp. nov.	species nova
v/v	volume / volume
w/v	weight / volume
μ_{max}	maximum growth rate
z. B.	zum Beispiel
z. T.	zum Teil

Zusammenfassung

Im Rahmen des niedersächsischen Forschungsschwerpunktes „Marine Biotechnologie“, dessen Kernaufgabe die Erforschung neuer mariner Naturstoffe aus neuen Bakterien bildete, wurden über 90 verschiedenen Isolate auf ihr Potential zur Sekundärstoffproduktion hin untersucht. Nach 16S rRNA Gen-Analyse gehörten 56 Stämme zur Gruppe der gram-positiven (42 Actinonacteria, 14 Firmicutes) und 36 Stämme zur *Roseobacter*-Gruppe, einer Untergruppe der alpha-*Proteobacteria*. Der überwiegende Teil dieser Bakterien wurde aus Wasser- oder Sedimentproben des deutschen Wattenmeeres isoliert. Das Wattenmeer der südlichen Nordsee ist ein durch die Tide beeinflusste besonderes Ökosystem, dessen biologische und chemische Eigenschaften sich von anderen marinen Habitaten unterscheiden, und gleichzeitig im Hinblick auf die Exploration von bakteriellen Sekundärstoffen weitgehend unerforscht. Auf Grundlage dieser Idee wurde ein interdisziplinärer Forschungsverbund, aus Chemikern, Biologen, Biotechnologen und Pharmazeuten gegründet, um gezielt neue Organismen aus diesem Habitat nach Naturstoffen zu untersuchen. Kultivierungsabhängige Screening-Verfahren werfen oftmals Probleme im Hinblick auf die Expression von Sekundärmetaboliten auf, z.B. abhängig vom Medium und den Inkubationsbedingungen. Um diese Problem zu umgehen wurde ein molekularbiologisches, PCR-basiertes Verfahren angewendet, dass die Bakterien auf Gene der Polyketidsynthase, der $FADH_2$-Halogenase und der nicht-ribosomalen Peptidsynthetase untersucht. Um die antagonistische Aktivität der Stämme zu erforschen wurden ergänzende Agar-Diffusionstest durchgeführt und ein Teil der Bakterien auf die Bildung von Acetyl-Homoserin-Lactonen (AHL) hin untersucht.

Zusätzlich wurden insgesamt drei Isolate in Zusammenarbeit mit Dr. Jeroen Dickschat und Prof. Stefan Schulz (Institut für Organische Chemie, Technische Universität Braunschweig) auf die Synthese von flüchtigen Sekundärmetaboliten gescreent, die entsprechenden Verbindungen isoliert und ihre Struktur aufgeklärt.

Die wichtigsten Ergebnisse dieser Arbeit können wie folgt zusammengefasst werden:

- Insgesamt ergaben die Screeningverfahren Innerhalb der Gruppe der gram-positiven Isolate, dass es bei mehr als 37 % aller Stämme Hinweise auf Sekundärstoffproduktion gibt. Gene für NRPS konnten in 11 und eine Hemmwirkung bei 10 Isolaten entdeckt werden. Ein Vergleich dieser Testergebnis mit der Phylogenie der untersuchten

Stämme ergab, dass 52 % dieser Stämme zu den Gattungen *Kocuria*, *Nocardioides*, *Mycobacterium* und *Bacillus* gehörten. PKS-Gene konnte nicht entdeckt werden.

- Die Bakterien der *Roseobacter*-Gruppe wiesen insgesamt ein höheres Potential für die Sekundärstoffbildung auf. Zwar wurden nur bei drei Vertretern eine inhibitorische Wirkung nachgewiesen, aber dafür wurden Gene die eine Sequenzähnlichkeit mit bekannten NRPS oder PKS Sequenzen aufwiesen in 7 (19,4 %) bzw. 5 (14 %) Stämmen detektiert.
- Das gleichzeitige Auftreten von PKS und NRPS Sequenzen in einigen *Roseobacter*-Stämmen, sowie eine phylogentische Analyse der detektierten PKS-Sequenzen indizieren, dass diese Gene für Hybrid-Multienzymkomplexe kodieren, welche sowohl PKS- als auch NRPS-Module enthalten.
- Durch Kultivierung mit Flüssigmedium konnten aus dem *Streptomyces* spp. Stamm GWS-BW-H5 acht bisher unbekannte γ- and δ-Lactone isoliert werden, die auf Agarplatten mit dem gleichen Substrat nicht produziert wurden. Eines dieser Lactone besaß eine schwache antibakterielle Wirkung gegen 4 von 12 getesteten Isolaten aus dem Wattenmeer.
- Durch detaillierte Charakterisierung von *R. gallaeciensis* und „*Phaeobacter inhibens*“ und dem Vergleich mit phylogenetisch verwandten Spezies, darunter die phototrophen Vertreter *R. denitrificans* und *R. litoralis*, konnte gezeigt werden, dass das hohe Maß an Übereinstimmung des 16S rRNA Genes nicht mit der gesamtgenetischen und damit auch der physiologischen Ähnlichkeit korespendierte. Die Überbetonung des 16S rRNA Genes als phylogentischen Marker, führte in der Vergangenheit zu einer fragwürdigen taxonomischen Einordnung von *R. gallaeciensis*, welche durch eine neu zu schaffende Gattung korrigiert werden könnte.

Die Untersuchungen der Wattenmeer-Isolate ergab, dass 20 bis 37 % der Stämme potentielle Sekundärstoffproduzenten sind. Organismen der *Roseobacter-Gruppe* enthalten mehr PKS–Gene, als die untersuchten Isolate der Ordnung *Actinomycetales*. Vertreter der *Roseobacter*-Gruppe kommen in hohen Abundanzen vor und sind, im Vergleich zu anderen phylogenetischen Gruppen der alpha-*Proteobacteria*, leicht zu kultivieren. Da die Anzahl der gescreenten Stämme innerhalb der *Roseobacter*-Gruppe, und damit verbunden die der redundant isolierten Naturstoffe, noch gering ist, sind Vertreter dieser Gruppe interessante Kandidaten für weitere Untersuchungen bezüglich neuer Sekundärstoffe.

Summary

Within the research network “marine biotechnology” in Lower Saxony, which focused on the investigation of novel marine natural products from new bacteria, over 90 isolates were screened for their potential for secondary metabolite production. 16 S rRNA gene analysis revealed, that 56 strains affiliated with gram-positive bacteria (42 *Actinonacteria*, 14 *Firmicutes*) and 36 strains with the *Roseobacter*-clade, a subgroup of alpha-*Proteobacteria*.
The majority of these bacteria was isolated from water- and sediment-samples from the German Wadden Sea. Influenced by the tides, the Wadden Sea in the southern region of the North Sea is a special ecosystem, which chemical and biological properties are distinct from other marine habitats, and which is simultaneously unexplored with respect to bacterial secondary metabolites. Based on this idea an interdisciplinary research network, consisting of chemists, biologist, biotechnologists and pharmacists was founded, in order to explore well directed new organisms from this habitat for novel natural compounds. With culture dependent screening procedures the expression of secondary metabolites often cause problems, e.g. depending on broth and culture conditions. To avoid these problems a PCR-based approach was applied, in which bacteria were screened for polyketide synthase-, $FADH_2$-halogenase- and non-ribosomal peptide synthetase genes. Additionally agar diffusion assays were applied to investigate the antagonistic activity of the strains, and some bacteria were tested for acylated homoserine lactones (AHL) production.
In a cooperation with Dr. Jeroen Dickschat und Prof. Stefan Schulz (Institut für Organische Chemie, Technische Universität Braunschweig) three isolates were additionally screened for synthesis of volatile metabolites, the corresponding compounds were isolated and their structure elucidated.

The major findings of these thesis can be summarized as follows:

- In total the screening procedures revealed for the gram-positive isolates, that there hints for secondary metabolite production for more than 37 % of all strains. Genes for NRPS were detected in 11 and Inhibitory effects in 10 isolates. The comparison of this results with the phylogeny of the tested strains revealed, that 52 % of these strains affiliated with geni *Kocuria*, *Nocardioides*, *Mycobacterium* und *Bacillus.* No PKS genes were detected.

- All in all bacteria of the *Roseobacter*-clade feature a higher potential for secondary metabolite production. In fact an inhibitory effect was only tested in three organisms, but genes with a high sequence similarity with known NRPS or PKS sequences were detected in 7 (19,4 %) and 5 strains (14 %), respectively.
- The simultaneously appearance of PKS and NRPS sequences in some *Roseobacter*-strains, as well as the phylogenetic analysis of the detected PKS sequences indicate, that this genes code for hybrid multienzyme complexes, which contain both PKS and NRPS modules.
- Culturing with culture broth induced production of 8, former unknown, γ- and δ-lactones, in *Streptomyces* spp. strain GWS-BW-H5, which were not synthesized on agar plates with the same medium. One of these lactones shows weak antibacterial activity against 4 out of 12 tested isolates from the German Wadden Sea.
- Detailed characterization of *R. gallaeciensis* and "*Phaeobacter inhibens*" and the comparison with phylogeneticly related species, e.g. the phototrophic representatives *R. denitrificans* and *R. litoralis*, revealed strong accordance of the 16S rRNA genes, which was not reflected by the total genetic an therefore physiological similarity. Overemphasis of the 16S rRNA gene as a phylogenetic marker caused a questionable taxonomical classification, which could be emended with a new genus.

The investigations with Isolates from the German Wadden Sea revealed, that 20 to 37 % of the strains are potentially producers of secondary metabolites. Organisms of the *Roseobacter*-clade exhibit more PKS-genes compared with screened Isolates of the order *Actinomycetales*. Representatives of the *Roseobacter*-clade are high abundant and, compared with other phylogenetic groups of the alpha-*Proteobacteria*, easy to cultivate. Because the number of screened strains within the *Roseobacter*-clade, and therefore the number of redundant isolated natural compounds, is still low, representatives of this cluster are interesting candidates for further investigations for novel secondary metabolites.

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I

Einleitung

Einleitung

Unter Naturstoffen versteht man chemische Verbindungen, die aus lebenden Tieren, Pflanzen oder auch Mikroorganismen gewonnen werden. Gewöhnlich wird hierbei zwischen Produkten des Primär- und des Sekundärstoffwechsels, unterschieden. Die erste Gruppe umfasst weit verbreitete Verbindungen, die essentiell für die Lebensprozesse des entsprechenden Organismus sind, während Sekundärmetabolite im Prinzip nicht essentiell für das Überleben sind, aber oftmals einen wichtigen Beitrag zum Überleben der Spezies leisten. Deshalb sind sie oft charakteristisch für bestimmte biologische Gruppen (Trossell, 1993).

Wie frühe Quellen belegen, machten sich Menschen im Asiatischen Raum schon seit Jahrtausenden die Sekundärstoffe insbesondere aus Pflanzen medizinisch nutzbar (Barton & Nakanishi, 1999). Aber im erst im Jahre 1806 gelang durch E. Merck in Darmstadt die Isolierung des ersten chemisch reinen Naturstoffes aus der Opiumpflanze, Morphium, welches bis heute eine große Bedeutung in der Schmerztherapie besitzt (Grabley & Thiericke, 1999a). Mit der Entdeckung des Penicillins (Flemming, 1929) und des bakteriell gewonnenen Streptomycins (Waksman, et al., 1944) begann die Ära der Antibiotika, die aus der modernen Medizin nicht mehr wegzudenken sind. In unserer heutigen Zeit gehören bioaktive Sekundärnetabolite aus Mikroorganismen oder Pflanzen, genauso wie die daraus gewonnenen Derivate zu den am häufigsten eingesetzten Therapeutika in Human- und Veterinär-Medizin (Scrip, 1993).

Naturstoffe aus dem Meer

Bis Anfang der 60er Jahre des letzten Jahrhunderts wurden vornehmlich terrestrische Habitate und Organismen auf Naturstoffproduktion untersucht. Die Häufigkeit isolierter „neuer“ Substanzen schwand rapide und „nicht-terrestrische Habitate“ wurden zunehmend untersucht. Nachdem 1966 das erste von einem marinen Bakterium produzierte Antibiotikum beschrieben wurde (Burkholder 1966), stieg die Anzahl der Verbindungen von Jahr zu Jahr.

In der noch relativ kurzen Phase von etwa 50 Jahren, in der nach marinen Naturstoffen gesucht wurde, konnten bis heute ca. 7000 Verbindungen neu beschrieben werden (Cannell, 1998; Laatsch, 2003). Viele besitzen einzigartige funktionelle Gruppen oder Strukturen, die ihnen eine biologische Aktivität verleihen, und zwischen 1969 und 1999 wurden mehr als 300 Patente über marine Naturstoffe erteilt (Kerr & Kerr, 1999). Es ist bemerkenswert, dass gerade die in jüngster Zeit gefundenen neuen Strukturen allesamt aus marinen Organismen stammen. Einige befinden sich bereits in der klinischen Erprobungsphase und könnten zu

neuen Medikamenten reifen (Kerr & Kerr,1999). Insgesamt basierten 34 % aller zwischen 1992 und 1995 eingeführten Medikamente auf marinen Naturstoffen (Zeeck, 1997), so dass die Erschließung mariner Ressourcen auch in naher Zukunft einen wichtigen Beitrag zur Entwicklung pharmazeutisch interessanter Stoffe sein wird. Die Zahl der Antibiotika-Resistenzen bei Bakterien erhöht sich und die Globalisierung führt zu einer zunehmenden Verbreitung dieser resistenten Krankheitserreger. Dieser Gefahr kann durch Naturstoffe gemildert werden, die mit ihrer strukturellen Diversität eine Möglichkeit besitzen, die doch einfachen Produkte der kombinatorischen Chemie zu ergänzen (Zeeck, 1997). Einige der wichtigsten Antibiotika wurden aus Bakterien oder Pilzen isoliert, so dass die Chance aus dieser Organismen-Gruppe, weitere Medikamente zu erhalten relativ, hoch ist. Die größte Veränderung in der marinen Naturstoffchemie seit 1993 ist der stark wachsende Anteil an Berichten über neue Metabolite aus marinen Mikroorganismen. Nichts desto trotz ist die Anzahl von ca. 600 beschriebenen Produkten noch relativ gering, im Vergleich zu den über 28000 Verbindungen, die aus terrestrischen Mikroorganismen stammen (Bakterien und Pilze). Ein neuerer jährlich erscheinenden Bericht (Blunt, et al., 2003), beobachtete allerdings zwischen 1996 und 2001 erstmals einen Rückgang an neu beschriebenen Verbindungen aus marinen Organismen. Diese Entwicklung ist möglicherweise ein Hinweis auf eine Abnahme in der Entdeckungsrate durch redundante Isolierung identischer Verbindungen, wie sie aus terrestrische Habitaten bereits bekannt ist. Da aber die Kultivierungsrate der marinen Mikroorganismen nach wie vor deutlich hinter ihren terrestrischen Verwandten bleibt, kann durch eine Verbesserung der Kultivierung noch neues Potential erschlossen werden, zumal gerade zu Beginn der 90er Jahre die Studien über Naturstoffe aus Schwämmen, Algen und Muscheln überwogen (Fenical, 1993) und bis heute dominieren (Kelecom, 2002).

Die Rolle mariner Bakterien als Sekundärstoffproduzenten

Die intensive Untersuchung von terrestrischen Actinomyceten über den Zeitraum der letzten 60 Jahre hat zur Entdeckung vieler pharmazeutisch interessanter Stoffe geführt. Aber die Effektivität, mit der Sekundärstoff-produzierende Taxa (z.B. *Streptomyces*) untersucht wurden, führte zu einer Situation, in der neue Verbindungen immer schwerer zu entdecken sind. Dieser Rückgang hat größtenteils mit der wiederholten Isolierung bestimmter Spezies zu tun und führt in Folge zu einer ineffizient hohen Wiederfindungsrate bereits beschriebener Substanzen. Eine Möglichkeit diesem Prozess zu begegnen liegt in der Fokussierung auf neue Habitate und neuen oder in geringen Abundanzen vorkommenden Organismen.

Im Unterschied z.B. zu marinen Invertebraten, die klar definiert werden können, ist eine genaue Abgrenzung mariner Bakterien von ihren terrestrischen oder limnischen Verwandten schwieriger. Im allgemeinen werden marine Bakterien durch ihr Bedürfnis nach Meerwasser, oder spezifischer nach NaCl bestimmt (Mcleod, 1965). Dieses Problem einer Einordnung verschärft sich noch im Fall von küstennahen Standorten oder Ästuaren, wo meist eine große Anzahl mariner Bakterien an variable Salzkonzentrationen angepasst ist. Zudem wurden eine ganze Reihe von Mikroorganismen aus dem Meer isoliert, die physiologisch und phylogenetisch ähnlich oder identisch waren mit gut untersuchten terrestrische Spezies (Jensen & Fenical, 2000; Kelecom, 2002). In pragmatischer Lesart werden marine Bakterien als diejenigen definiert, die aus marinen Habitaten isoliert wurden und mit Meersalz-Medien wachsen (Faulkner, 1999).

Obwohl viele Bakterien als Sekundärstoffproduzenten bekannt sind oder bakterielle Symbionten (z.B.von Schwämmen) als Produzenten vermutet werden, wurden in vielen Untersuchungen keine phylogenetische Einordnung der Bakterien vorgenommen (Kelecom, 2002). Zu den bekannten Bakterien-Gattungen, aus denen am häufigsten bioaktive Verbindungen isoliert wurden zählen: *Pseudoalteromonas*, *Cytophaga*, *Alteromonas*, *Micrococcus*, *Bacillus*, *Acinetobacter*, *Agrobacterium*, *Pseudomonas* und insbesondere *Streptomyces* (Wagner-Döbler, et al., 2002). Das Ziel dieser Arbeit war insbesondere zwei Gruppen zu untersuchen: marine Vertreter der Ordnung *Actinomycetales*, die zu den potentesten Sekundärstoffproduzenten gehören und die *Roseobacter*-Gruppe (Untergruppe der alpha-*Proteobacteria*), deren Sekundärstoffpotential trotz stetig wachsenden Isolierungserfolgen noch relativ unbekannt ist.

Marine Actinomyceten

Seit mehr als 50 Jahren ist bekannt, dass gram-positive Bakterien im Meer vorkommen. Lange Zeit ging man jedoch davon aus, sie wären dort nur aus terrestrischen Habitaten eingetragen (Cross & Ellis, 1981; Jensen & Fenical, 1995; Moran, et al., 1995; Zobell & Upham, 1944). So nimmt zum Beispiel die Anzahl an Streptomyceten ab, je größer die Entfernung zur Küste wird. Andererseits nimmt die Anzahl von *Actinoplanates* deutlich zu (Jensen, et al., 1991). Erst seit Mitte der 1990er Jahre jedoch konnte durch Untersuchungen sowohl mit kulturvierungs-unabhängigen Methoden als auch durch Kultivierung gezeigt werden, dass gram-positive Bakterien eine unerwartet hohe Diversität in marinen Bakterioplanktongemeinschaften aufweisen (Fuhrman, et al., 1993; Rappé, et al., 1997;

Rappé, et al., 1999; Suzuki, et al., 1997) und auch in hohen Abundanzen vorkommen (Jensen & Fenical, 1995; Mincer, et al., 2002; Moran, et al., 1995; Urakawa, et al., 1999).
Die beste marine Quelle für Actinomyceten ist das Sediment. Für dieses Habitat ist die Isolierung durch verschiedene Untersuchungen vielfach belegt (Goodfellow & Haynes, 1984; Barcina, et al., 1987; Pisano, et al., 1989; Jensen, et al., 1991). Wie Dot-Blot-Untersuchungen und Fluoreszenz *in situ* Hybridisierungen zeigten, machten *Actinobacteria* bis zu 5 % aller Bakterien in flachen marinen Sedimenten aus und über 1,4 % in einem arktischen Tiefsee-Sediment (Moran, et al., 1995; Llobet-Brossa, et al., 1998; Ravenschlag, et al., 1999).

Durch phylogenetische Analyse von Sequenzen unkultivierter Bakterien und von Isolaten aller Untersuchungen wurden bestimmte marine Sequenzgruppen (Cluster) entdeckt, die nur geringe Ähnlichkeiten mit Clustern mit terrestrischen oder limnischen gram-positiven Bakterien aufweisen. Dazu gehört der „Marine Actinobacteria Clade“ (Rappé, et al., 1999) und das MAR 1 Cluster (Mincer, et al., 2002), das Sequenzen von Isolaten aus tropischen und subtropischen Sedimenten enthält. Aus dem letztgenannten Cluster konnten über 2500 Stämme isoliert werden, die bereits erfolgreich auf die Produktion bioaktiver Sekundärmetabolite getestet wurden (Feling, et al., 2003). Dabei zeigte sich innerhalb der Gattung „Salinospora“, dass es eine Korrelation zwischen den Spezies und der Stoffklasse von produzierten Verbindungen gibt (Jensen, et al., 2005). Zusammenfassend weisen diese Studien Argumente auf, die Suche nach neuen marinen Taxa als Strategie für die Entdeckung neuer Sekundärmetabolite zu intensivieren und auszuweiten.

Verschiedene Screening Verfahren für Naturstoffe

Die Entdeckung von Streptomycin und Actinomycin begründete das Feld des sogenannten Antibiotika Screenings[1]. Um neue bioaktive Verbindungen zu finden, können die Rohextrakte aus der Organismen-Kultivierung verschiedenen chemischen oder biologischen Testverfahren unterzogen werden. Gerade die letzteren ermöglichen die Fokussierung auf bioaktive Substanzen und weisen Vorteile auf, wie eine größere Sensitivität, oder die Möglichkeit eines hohen Probendurchsatzes, wie des industriellen „High Throughput Screenings“ (Grabley & Thiericke, 1999). Aber die bei diesen Tests begrenzte Anzahl an „screening targets“ wird zum Problem, wenn sowohl Wirkungszentrum als auch Wirkungsweise neuer Substanzen unbekannt sind, und führt womöglich zum frühzeitigen Ausschluss der Verbindungen aus

[1] Der Begriff Screening beschreibt eine biologische, chemische oder physikalische Interaktion von Metaboliten mit einem Testsystem, die anschließend quantitativ und qualitativ ausgewertet wird. (Omura, 1992)

dem Screening Prozess. Denn in den meisten Fällen kennt man die *in situ* Funktion der Naturstoffe nicht (Vining, 1992). Einige dienen als Signalstoffe zur inter- oder intrazellulären Kommunikation (Kleerebezem, et al, 1997; Shaw, et al, 1997), als Schreck- oder Lockstoff (Demain, 1992; Maplestone, et al, 1992), oder aber als Antifouling-Schutz (Steinberg, et al, 1997). Ist es bei der Produktion von antiviralen oder antibakteriellen Substanzen durch Mikroorganismen leicht ein biologisches Screeningverfahren anzuwenden, scheinen die immuno-supressive, neurotoxische oder antitumorale Wirkungsweisen anderer Substanzen mit einer *in situ* Funktion wenig gemein zu haben. Entsprechend gerät das biologische Screeningverfahren zu einem „try and error" Prozess.

Schon in den 1980er Jahren begannen Zähner und Mitarbeiter durch systematische Entwicklung chemischer Screeningmethoden sich diesem Problem zu nähern (Zähner, et al., 1982). Dünnschicht-Chromatographie und spezifische Färbereaktionen unter definierten Bedingungen erlauben eine Visualisierung fast des gesamten Sekundärstoffspektrums (Grabley, et al., 1999). Bei dieser Methode werden zuerst alle Metabolite isoliert, um unbekannte Substanzen gezielt einem biologischen Test zu unterziehen. Nachteilig ist die wiederholte Isolierung bereits bekannter Verbindungen.

Die höhere Sensitivität moderner MS und NMR Geräte und die Verfügbarkeit diverser Naturstoff-Datenbanken führte zu einer rasanten Entwicklung von HPLC oder GC-MS Screening-Verfahren. Hierbei kann direkt im gewonnenen Rohextrakt zwischen bekannten Verbindungen und neuen Produkten unterschieden werden. Auf diese Weise verhindert man die oft zeitraubende Isolierung bereits bekannter Substanzen, indem man gezielt diejenigen Bestandteile isoliert, die neue oder ungewöhnliche spektroskopische Eigenschaften aufweisen.

Die permanente Weiterentwicklung genetischer Untersuchungsmethoden, ermöglichte es während der letzen 10 Jahre, einen Zusammenhang zwischen den produzierten Sekundärstoffen und den dazugehörigen Genen herzustellen. Mittlerweile sind die Enzyme und Gene wichtiger Stoffklassen wie Polyketid-Synthasen, nicht-ribosomale Peptid-Synthetasen, dNDP-Glucodehydratasen und Halogenasen hinreichend charakterisiert und sequenziert, so dass PCR-basierte Screeningmethoden Anwendung finden (Decker, et al., 1996; Seow, et al., 1997; Rajendran, 1999; Hyun, et al., 2000; van Pee, 2001).

In den letzten Jahren galt die vermehrte Aufmerksamkeit der Naturstoffforschung marinen Organismen, die aber leider deutliche geringere Produktionsraten unter Standardinkubationsbedingungen aufwiesen als ihre terrestrischen Verwandten (Fusetani, 2000; Wagner-Döbler, et al., 2002). Ein großer Vorteil des genetischen Screenings liegt in der

geringen Menge benötigter DNA. Darüber hinaus ist eine schnelle Erkennung unproduktiver Organismen möglich und limitiert so die Anzahl der Spezies für die weiteren chemischen Tests. Andererseits liefert der Hinweis auf ein Gen, das in die Sekundärstoffproduktion involviert, ist noch kein Produkt. Es kann sich um ein sogenanntes stilles Gen handeln, das nicht expremiert wird, oder es bedarf besonderer Kultivierungsbedingungen, um die Genexpression auszulösen (Demain, 1998). Frühere Studien konnten diese Probleme erfolgreich lösen, indem die Produktion der vorhergesagten Sekundärstoffe durch biotechnologische Methoden stimuliert wurde. (Demain, 1998; Marwick, et al., 1999). Darüber hinaus können mit molekularbiologischen Verfahren stille Gene oder Gene aus bislang unkultivierbaren Mikroorganismen (z.B Symbionten) in anderen Wirtsstämmen expremiert werden (Haygood, et al., 1999; Davidson, et al., 2001; Piel, 2002).

Zielsetzung der Arbeit

Diese Arbeit wurde im Rahmen des niedersächsischen Forschungsschwerpunktes „Marine Biotechnologie“ (gefördert durch die VW-Stiftung) erstellt, dessen Schwerpunkt die Erforschung neuer mariner Naturstoffe aus neuen Bakterien bildete. Hierbei wurde die südliche Nordsee als Untersuchungs- und Probennahmegebiet gewählt, da dieses Habitat im Hinblick auf die Exploration von bakteriellen Sekundärstoffen weitgehend unerforscht ist. Auf Grundlage dieser Idee wurde ein interdisziplinärer niedersachsenweiter Forschungsverbund, aus 19 Arbeitsgruppen ins Leben gerufen. Durch Kooperation von Chemikern, Biologen, Biotechnologen und Pharmazeuten sollte die Effizienz der Forschung an neuen Sekundärmetaboliten erhöht werden.

Das Wattenmeer in der südlichen Nordsee ist mit einer Gesamtgröße von ca. 9300 km^2 eines der größten Wattgebiete der Welt (Hild, 1999) und reicht mit über 500 km Gesamtlänge von Esbjerg (Dänemark) bis Den Helder (Niederlande). Zeitlich und räumlich begrenzte Untersuchungen im Wattenmeer ergaben eine große temporäre und räumliche Variabilität, die als wesentliches Kennzeichen dieses Naturraumes anzusehen ist (Dittmann, 1999). Die Hydrodynamik ist hierbei das wohl auffälligste Merkmal.

Die dynamischen Prozesse, wie Gezeiten, Strömungen und Windveränderungen, führen durch Sedimentverlagerung zu ständigen Veränderungen der morphologischen Muster (Wolff, 1983). Ergebnis dieser Hydrodynamik sind starke Resuspensions- und Sedimentationsereignisse (van Leussen, 1996; Jago, et al., 2002), die zur Dynamik gelöster organischer (Poremba, et al., 1999) und anorganischer Stoffe führt (Asmus, et al., 1998), was

wiederum Auswirkungen auf Zusammensetzung und Abundanz der Bakteriengemeinschaft im Wattenmeer hat (Grossart, et al., 2004).

Im Rahmen des Teilprojektes „Sekundärstoffe in neuen Mikroorganismen aus der Nordsee“ wurde gezielt nach neuen Bakterien aus dem deutschen Wattenmeer gesucht (Stevens, 2004). Ein Teil dieser Isolate wurde in Zusammenarbeit mit Prof. A. Zeeck (Institut für organische Chemie, Univerität Göttingen) auf ihr Sekundärstoffpotential untersucht. Leider ist das chemische Screenning aufgrund der geringen Produktausbeuten, den langsamen Wachstumsraten mariner Bakterien und der Untersuchung von unproduktiven Stämmen sehr zeitintensiv. Um diesen Prozess zu beschleunigen, war es sinnvoll eine Vorauswahl der Isolate zu treffen, zumal die Kultivierungserfolge der biologischen Arbeitsgruppen eine chemische Untersuchung aller Bakterien im vorgegebenen Zeitrahmen unmöglich erscheinen ließen.

Zu den Hauptzielen dieser Arbeit zählten somit die Entwicklung und Tests von Primer-Systemen zum Nachweis von Genen des Polyketid- und nicht-ribosomalen Polypeptidstoffwechsels, von Tryptophan-Halogenase Genen, sowie von dNTP Glucose Dehydratase Genen, um eine Vorauswahl von Stämmen für das chemische Screening bereitzustellen und die zu erwartenden Stoffklassen vorab zu charakterisieren. Aufgrund des Sekundärstoffpotentials von terrestrischen *Actinobacteria* und der daraus resultierenden Fülle an bekannten Gen-Sequenzen wurden in einem ersten Schritt die gram-positiven Isolate aus dem Wattenmeer untersucht. Die Ergebnisse dieser Arbeiten sind in Kapitel II beschrieben.

Nach diesem erfolgreichen Test der Primer-Systeme konnten das Screening auf neue bisher wenig erforschte Bakteriengruppen ausgeweitet werden. Zusammen mit Prof. Lone Gram (Danish Institute for Fisheries Research, Lyngby, Dänemark) wurde eine intensive Untersuchen von Stämmen der *Roseobacter*-Gruppe aus unterschiedlichen Habitaten durchgeführt (Kapitel III). Darüber hinaus wurden eigene Tests von Sekundärstoffbildnern und deren Wirksamkeit (z.B. Wachstumshemmung, reduzierte Beweglichkeit) auf andere Isolate und Algen durchgeführt, um das Bildungs-Potential von Sekundärstoffen innerhalb von Bakteriengruppen aus dem Wattenmeer zu untersuchen. Die dabei gewonnenen Erkenntnisse, kennzeichnen die *Roseobacter*-Gruppe als potente Produzenten von Sekundärstoffen und Signalmolekülen.

Trotz einer relativ großen Auswahl an Teststämmen konnten Gene für Halogenasen nur in einem Isolat nachgewiesen werden. Die Ergebnisse dieser Untersuchung werden in einem gesonderten Kapitel (IV) vorgestellt.

Die meisten Studien zur Untersuchung von Sekundärstoffen bei Bakterien fokussieren auf große Metabolite mit mittlerer oder hoher Polarität, da diesen Verbindungen ein hohes Pharmakologisches Potential beigemessen wird. Sehr viel weniger Aufmerksamkeit wird den flüchtigen Substanzen von Bakterien geschenkt, obwohl diese Stoffe (z.B als Signalmoleküle) eine interessante ökologische Funktion besitzen können. Durch die Zusammenarbeit im Forschungsschwerpunkt „marine Biotechnologie“ konnte gemeinsam mit Jeroen Dickschaat und Prof. Stephan Schulz (Institut für Organische Chemie, Technische Universität Braunschweig, Braunschweig) mehrere Wattenmeer-Isolate auf die Produktion von flüchtigen Molekülen hin untersucht werden. Hierbei erwies sich der *Streptomyces* Stamm GWS-BW-H5 als Produzent bisher noch unbekannter Lacton-Verbindungen, deren mögliche Bioaktivität Ziel verschiedener Tests war. Die Ergebnisse wurden im „Journal of Natural Products“ veröffentlicht und im Kapitel V dieser Arbeit beschrieben.

Ein weiteres Ziel war die molekulargenetische und physiologische Identifizierung der Stämme bzw. Arten, um deren Vorkommen am Standort und in Modellökosystemen zu untersuchen und besser zu verstehen. Einer bezüglich der Sekundärstoffproduktion interessantesten Stämme (T5) konnte im Rahmen dieser Arbeit umfassend charakterisiert werden. Dieser Stamm ist eng mit *Roseobacter gallaeciensis* verwandt. Ein Vergleich mit den physiologischen Eigenschaften der namensgebenden Stämme *R. litoralis* und *R. denitrificans*, ergab jedoch entscheidende Unterschiede. Ergebnis unserer Untersuchung war der Vorschlag, *R. gallaeciensis* und Stamm T5 in die neu zu schaffenden Gattung „*Phaeobacter*“ einzuordnen. Das entsprechende Manuskript wurde beim „International Journal of Systematic and Evolutionary Microbiology“ eingereicht (Kapitel VI).

II

PCR-based screening of marine gram-positive bacteria for genes involved in secondary metabolite production

Submitted to Archives of Microbiology

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PCR-based screening of marine gram-positive bacteria for genes involved in secondary metabolite production

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Running title: *Secondary metabolite screening of marine gram-positive bacteria*

Abstract

To study the potential of marine gram-positive bacteria for secondary metabolite production we investigated 56 different strains, mainly isolated from the German Wadden Sea, a tidal flat system in the southern part of the North Sea. Analysis of the 16S rRNA genes of the isolates revealed that 42 strains affiliated with *Actinobacteria* and 14 with *Firmicutes* and exhibited a high phylogenetic diversity. The genetic potential of the isolates to produce secondary metabolites was explored using PCR-based screening approaches for non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) genes, followed by sequence analysis of the obtained PCR products. To detect antagonistic activity, agar diffusion tests with several target strains were performed. Overall the screening procedures gave evidence for secondary metabolite production for 37.5 % of the isolates. Genes for putative NRPS were detected in 11 and antagonistic activity was observed for 12 of the strains. No genes of PKS, however, were amplified. For two strains NRPS genes as well as antagonistic activity were detected. Comparison of the screening results and the phylogeny of the isolates indicated accumulations of positively tested strains within the genera *Kocuria*, *Nocardioides*, *Mycobacterium* and *Bacillus*. These genera included 52 % of the strains with potential for secondary metabolite production.

Keywords ***Actinobacteria, Firmicutes*, secondary metabolites, NRPS, PKS, Wadden Sea**

Introduction

The rediscovery rate of bioactive compounds from microorganisms currently in culture has been estimated to be 95 % (Fenical et al. 1999), what makes it necessary to isolate and investigate new strains from habitats which have not been intensively studied. Hence, secondary metabolites from marine microorganisms is a rapidly growing field (Faulkner 2000; Jensen and Fenical 1994; Jensen and Fenical 1996; Jensen and Fenical 2000), but still the great majority of new structures is found in terrestrial bacteria (Wagner-Döbler et al. 2002). This might be due to the fact that screening procedures for marine microorganisms are complicated since the compounds are often only produced under certain conditions and many strains grow slowly and yield only small amounts of substances (Chen et al. 2000; Wagner-Döbler et al. 2002).

So far most secondary metabolites from marine bacteria were isolated from gram-positive organisms, but mainly from *Streptomyces* species (Wagner-Döbler et al. 2002). Marine members of most other gram-positive genera have not been intensively screened. To investigate the potential for secondary metabolite production of marine gram-positive bacteria of a multitude of different taxa we based on recent results found by Stevens (2004), showing that the phylogenetic diversity of gram-positive bacteria isolated from water and sediment samples obtained from an intertidal mud flat of the German Wadden Sea is much higher compared to other marine environments (Jensen and Fenical 1995; Mincer et al. 2002; Suzuki et al. 1997). The Wadden Sea is located in the southern North Sea, stretching from the Netherlands (Den Helder) to Denmark (Esbjerg). Tidal dynamics and inputs of organic and inorganic nutrients from land, rivers and the North Sea influence this habitat in which microbial processes are of major significance (Poremba 1999). In the present study, 56 strains mainly isolated from the Wadden Sea and affiliated with *Actinobacteria* (42 strains) and *Firmicutes* (14 strains) were investigated.

A large number of pharmaceutical important natural products are of a polyketide origin, (Hopwood 1997; Staunton and Weissman 2001) or belong to the class of non-ribosomally produced peptides (Marahiel et al. 1997). Expanding availability of genes encoding for non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) made it possible to develop degenerated primers based on conserved sequence motifs and compare sequence information of amplified genes with those of known function. Thus, to prevent problems resulting from cultivation conditions, we explored the genetic potential of marine gram-positive bacteria to produce secondary metabolites using PCR based screening approaches for NRPS and PKS genes. Agar diffusion tests with bacteria of five phylogenetic classes as target strains were also conducted. Combination of phylogenetic analysis and screening results was finally used to indicate bacterial groups with increased potential for secondary metabolite production.

Materials and methods

Source and cultivation of bacteria

Overall 56 gram-positive strains were investigated in this study. Fifty-one of the strains were obtained from the German Wadden Sea. Isolation procedures were described by Grossart et al. (2004) (strains named "HP.."), Brinkhoff et al. (2004) (strains named "T.."), and Stevens (2004) (strains named "GWS.."). Strains H105, GWS-BW-H5, GWS-BW-H16, GWS-BW-H86, GWS-BW-H87, GWS-BW-H199, GWS-BW-H258, GWS-AG-H62, GWS-AG-H79, T10 and HP45/56 have not been published yet, however, isolation of these bacteria was performed according to the procedures described in the above listed publications. Four strains, i. e. GP-1, GP-3, GP-4 and GP-7 were isolated from different sections of the Weser estuary (Selje and Simon 2003), which is part of the German Wadden Sea. Strain GP-1 from the marine section, strains GP-3 and GP-4 from the brackish, and strain GP-7 from the limnic section. Strain TAF-2.4a was provided by Dr. Ocky Radjasa, (Leibniz-Institute of Marine Sciences [IFM-GEOMAR], Kiel, Germany) and was isolated from a coral reef in the sea of Jepara (Indonesia). All strains were cultivated and grew well on marine agar 2216 (MA) and in marine broth 2216 (MB) (Difco, Becton Dickinson Microbiology systems, USA).

Screening of isolates for inhibitory effects

To screen isolates for inhibitory effects agar diffusion assays were performed as described by Brinkhoff et al. (2004), except that the OD_{600} of cultures of the strains tested was ~ 1. Ten different isolates also obtained from the German Wadden Sea and belonging to five different phylogenetic classes were used target strains (Table 1). Plates with agar diffusion assays were incubated for 5 days at 20°C and inspected daily for zones of inhibition. Production of inhibitory compounds was determined positive when the diameter of the zone of inhibition was at least 4 mm greater than the diameter of the colony formed around the antibiotic assay disc by the producing bacteria. The experiments were performed in three parallels. An isolate was considered as producer of inhibitory compounds if inhibition was observed for at least two parallels.

PCR amplification and sequencing of 16S rRNA gene fragments

PCR amplification of 16S rRNA genes of the isolates, purification of PCR products and subsequent sequencing analysis were performed according to Brinkhoff and Muyzer (1997). For all isolates at least 550 base pairs (bp) were determined, except for H105, for which only 344 bp were sequenced. The sequences of screened strains were compared with similar sequences of reference organisms by BLAST search [http://www.ncbi.nlm.nih.gov/blast (Altschul et al. 1997)].

Phylogenetic analysis

Phylogenetic analysis was performed with the ARB software package [http://www.mikro.biologie.tu-muenchen.de (Strunk 1998)]. Phylogenetic trees were constructed with almost full length 16S rRNA gene sequences (>1300 bp) of type strains using maximum-likelihood analysis. Alignment positions at which less than 50 % of sequences of the entire set of data had the same residues were excluded from the calculations to prevent uncertain alignments within highly variable positions of the 16S rRNA genes, which cause mistakes in tree topology. Shorter sequences were added afterwards using maximum parsimony and the same filter.

PCR based screening for NRPS and PKS genes

To obtain genomic DNA of the isolates for PCR analysis cell material was taken from agar plates, suspended in sterile water (Sigma-Aldrich, Munich, Germany) and five cycles of freezing and thawing were applied. To avoid false negative results the DNA of each strain was tested in a PCR with the general primers GM5F and 907R for 16S rRNA genes (Brinkhoff and Muyzer 1997). Amplification of peptide synthetase gene fragments was carried out initially with the primers and PCR described by Rajendran (1999), however, no PCR products could be obtained with our strains. Therefore we developed new degenerated primers, i.e. A2f (5´-AAG GCN GGC GSB GCS TAY STG CC-3´) and A3r (5´-TTG GGB IKB CCG GTS GIN CCS GAG GTG-3´) designed from conserved regions of adenylation domains of various bacterial peptide synthetase sequences (GenBank accession numbers: AAK81824, AAK81827, AAK81826, AAC82549, CAA40561, CAC48362, CAA11796, CAC48369, CAC48369, AAF42473, BAB69322, CAB38518, AAG02364, AAG02355,

AAG02356, CAA67248, CAB93684, CAB93684, CAB93683, AAC68816, AAC44129, CAA65394, AAG05812, AAG05789, AAG05789, AAF40220, AAD51026, CAC11137, AAB96629). The sequence of the reverse primer is based on the signature sequence of the superfamily of adenylate forming enzymes TSGXTGXPK (motif A3), however, the sequence of the forward primer, based on the motif KAGGAY(LV)P (motif A2), is highly conserved for peptide synthetases, which are involved in non-ribosomal peptide synthesis (Doekel and Marahiel 2001). PCR was performed with an Eppendorf Mastercycler (Eppendorf Inc., Hamburg, Germany) as follows: 2 µl template DNA, 40 pmol of each of the appropriate primers, 12.5 nmol of each deoxyribonucleoside triphosphate, 5 µl of 10 x RedTaq™ PCR buffer (Sigma-Aldrich), 1.2 mg ml^{-1} (final concentration) bovine serum albumin (Sigma-Aldrich) and 0.75 units RedTaq™ DNA polymerase (Sigma-Aldrich) were adjusted to a final volume of 50 µl with sterile water (Sigma-Aldrich). A PCR run comprised 40 cycles with denaturing, annealing and extension temperatures for one minute at 95°C, one minute at 70°C and two minutes at 72°C. DNA from *Pseudomonas* sp. (DSM 50117) was used as positive control.

The ketosynthase specific primer pair KSDPQQF and KSHGTGR (Piel 2002) was used for the amplification of PKS I gene fragments including an initial denaturing step at 94°C for 2 min, followed by 45 cycles at 94°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 2 minutes. A 50 µl PCR-reaction mix contained 2 µl template DNA, 30 pmol of each of the appropriate primers, 12.5 nmol of each deoxyribonucleoside triphosphate, 5 µl of 10 x RedTaq™ PCR buffer (Sigma-Aldrich), 0.2 mg ml^{-1} (final concentration) bovine serum albumin (Sigma-Aldrich) and 0.75 units RedTaq™ DNA polymerase (Sigma-Aldrich). DNA from *Bacillus subtilis* strain 168 (DSM 402) was used as positive control.

Cloning and sequencing of PCR-products

PCR-products approximately of the expected length were gel-purified using the Perfectprep™ Gel cleanup Kit (Eppendorf Inc., Hamburg, Germany) and ligated into the pGEM-T vector (Promega, Mannheim, Germany) following the manufacturers protocol. Recombinant clones containing an insert were sequenced using the DYEnamic Direct cycle sequencing kit (Amersham Life Science Inc., Little Chalfont, UK) and a Model 4200 Automated DNA Sequencer (LI-COR Inc., Lincoln, USA). Both DNA strands were sequenced twice using M13F and M13R labeled with IRDye™800 as sequencing primers (Messing 1983). The deduced amino

acid sequences were compared with similar sequences by BLAST search [http://www.ncbi.nlm.nih.gov/blast] (Altschul et al. 1997).

DNA sequence accession numbers

The 16S rRNA gene sequences determined in this study have been deposited in the GenBank database under accession numbers AY731364 - AY731375. The putative non-ribosomal peptide synthetase sequences obtained from our isolates have been deposited in the GenBank database under accession numbers AY734509 - AY734519.

Results

Phylogenetic analysis of 16S rRNA gene sequences of the 56 investigated isolates demonstrated a wide distribution within the gram-positive bacteria (Fig. 1A and B). The majority of 42 isolates affiliate with the phylum *Actinobacteria*, 14 strains belong to the *Firmicutes*. Among the *Actinobacteria* most isolates could be assigned to members of nine families. As an exception, strain T10 showed only 93 % sequence similarity with *Conexibacter woeseii*, the closest related validated species. Among the *Firmicutes* all strains belong to the order *Bacillales*, and most of these strains (85 %) belong to the *Bacilliaceae*, however, two isolates affiliate with the genera *Planococcus* and *Staphylococcus*, respectively. Eleven of the 56 sequences exhibit a closest relative of marine origin, 19 of other environments like soil or freshwater (data not shown). From the remaining sequences no information on the source of isolation could be obtained.

Antagonistic activity was found for 12 (21.4 %) of the isolates (see Table 2 and Fig.1), however, the majority inhibited only one target strain. Only strains GWS-AG-H250-19, GWS-AG-H79 and HP9w were able to prevent two or more target strains from growing. HP9w inhibited even five strains of three different classes and produced zones of cleared agar with the largest diameter. The radius of all inhibition zones varied and ranged between 2 and 11 mm, with a clear trend towards smaller inhibition zones. The mean percentage of inhibiting strains was equal for *Actinobacteria* and *Firmicutes*. Within the *Actinobacteria* all three tested strains affiliated with the genus *Nocardioides* showed antagonistic activity. Within the *Bacilli* inhibition was only observed within the *Bacillus subtilis* cluster (Fig. 1B) (three out of five strains tested).

A strategy for amplification and identification of peptide synthetase genes with primers based on conserved A2 and A3 motifs has been described earlier (Borchert et al. 1992; Rajendran 1999), but only PCR with the primer pair A2f and A3r reproducibly resulted in products of the expected size. Out of 56 isolates examined, 12 strains yielded a PCR product. Analysis of the derived amino acid sequences of these products revealed high similarities for 11 sequences to non-ribosomal peptide synthetase genes in the GenBank database. One gene fragment with the expected length showed 81 % identical amino acid positions with the acyl-CoA carboxyltransferase beta chain from *Xanthomonas axonopodis* (Table 3).

Type I polyketide synthases consist of repeated modules, which process biosynthesis of a polyketide chain in an assembly line fashion (Hopwood 1997). To perform one chain elongation cycle at least a keto-acyl synthase (KS), acyltransferase and acyl carrier protein (ACP) domains are necessary. Screening for modular type I PKS genes was performed using the degenerated oligonucleotide primers described by Piel (2002) based on universally conserved motifs of KS-domains as described before (Beyer et al. 1999). Even though PCR with primers for 16S rRNA genes yielded products with DNA of the tested strains and products could always be obtained with the positive control for the PCR with primers for type I PKS genes, no genes could be amplified in the tested strains.

Accumulations of positive screening results were found for strains affiliated with the genera *Kocuria*, *Nocardioides*, *Mycobacterium* and *Bacillus*, i. e. the *Bacillus subtilis* cluster (see Fig. 1A and B). These genera include 52 % of the strains with potential for secondary metabolite production. For all tested strains affiliated with *Kocuria*, *Nocardioides* and *Mycobacterium* positive results were obtained, as well as for three out of five strains affiliated with the *B. subtilis* cluster.

Discussion

The screened isolates obtained from the German Wadden Sea showed a greater phylogenetic diversity among the *Actinobacteria* on genus level compared with other studies, using either culture-dependent (Jensen and Fenical 1995; Mincer et al. 2002, Suzuki et al. 1997) or culture–independent approaches (Fuhrman et al. 1993; Gray and Herwig 1996; Rappé et al. 1999; Suzuki et al. 1997; Urakawa et al. 1999). This might be caused by the various isolation procedures applied (Stevens 2004), but may also reflect the specific signature of the Wadden Sea ecosystem, with marine as well as limnic and terrestrial impacts. Approximately 20 % of

the closest related sequences to our strains are of marine origin, but none affiliates with the marine *Actinobacteria* clade (Rappé et al. 1999) or other exclusively marine clusters (Mincer et al. 2002). The impossibility to distinguish between marine and terrestrial origin on phylogenetic data necessitates other criteria to define marine bacteria. One of the organisms screened within this study is the recently described species *Aeromicrobium marinum*, which was also isolated from the Wadden Sea (Bruns et al. 2003). *A. marinum* clusters with terrestrial *Aeromicrobium* spp., but exhibits a requirement for Na^+ and a salinity optimum at 53.5‰ what identifies the strain as clearly marine. All tested strains grew well on marine broth 2216 (Difco) as medium, and since they were isolated from a marine habitat on marine media, they could be defined as marine, like other authors proposed (Faulkner 1999; Wagner-Döbler et al. 2002). Since most of the new compounds reported from marine bacteria were obtained from species that can, in principle, be isolated from land and sea (Jensen and Fenical 2000), the Wadden Sea with impacts from land and sea might be a promising habitat for containing bacteria with a potential for secondary metabolite production.

A fraction of 21 % of the screened isolates exhibited antagonistic properties against one or more background strains. Okami (1986) as well as Nair and Simidu (1987) found lower percentages of bacteria with inhibiting properties (5 – 8 %), using very limited numbers of target species. In two more recent studies antagonistic activity was reported for more than 50 % of the screened isolates (Grossart et al. 2004; Long and Azam 2001), suggesting inhibition is a common phenomenon among pelagic marine bacteria. The experiments of the latter two studies were carried out with a much higher variety of target organisms. This could be a reason, why some of the results concerning inhibiting properties of some strains tested already by Grossart et al. (2004) differ from our findings, since the chance to find sensitive species obviously increases with increasing number of target organisms. Moreover the agar diffusion assay by Grossart et al. (2004) was carried with doubled salinity, a stress situation, which can enhance production of secondary metabolites (Marwick et al. 1999). Additionally one should note that inhibition must not necessarily be based on an antibiotic. Interspecies growth control by signal molecules, availability of nutrients (e.g. iron uptake with siderophores) or alteration of pH may have similar effects.

We observed that background strains were affected by the antagonistic bacteria very differently. Only strains HP9w and GWS-AG-H79 were able to inhibit 5 and 3 background strains, respectively, each out of 3 different bacterial classes. The majority of bacteria with antagonistic activity inhibited growth of only one or two target strains, but no clear preference towards one phylogenetic group could be observed (Table 2). Target strains T1 and T4,

affiliating with γ-*Proteobacteria* and *Actinobacteria*, were most frequently inhibited. A possible reason could be lower resistance against antibiotics of these particular species.

No PCR products could be obtained with the screened isolates and the primers used for the type I PKS domain, even though successful amplification with DNA from symbiotic bacteria was proven in a former study (Piel 2002), and the positive control used in our study, i. e. chromosomal DNA from *Bacillus subtilis* 168 (DSM 402), resulted in intense PCR products. Furthermore, using the same PCR conditions, we found putative PKS genes in several other bacteria of various lineages, i. e. alpha- and gamma-*Proteobacteria* as well as *Bacteriodetes* (Martens et al., unpublished). This indicates that PKS genes in the screened bacteria are truly absent or the target site for at least one of the primers differs extremely from the consensus sequence derived from the known genes and used for the primer design.

In contrast we found high similarities to known NRPS with 11 amino acid sequences derived from sequenced PCR products, obtained with the screened isolates and primers specific for conserved regions of adenylation domains of bacterial peptide synthetase sequences. However, this is a small yield of positively screened bacteria, compared to a previous study by Sosio et al. (2000) who investigated 19 *Actinomycetes* spp. belonging to 8 different genera, and found various NRPS modules in all species. Comparable investigations of *Cyanobacteria* using a PCR based approach, or hybridisation techniques discovered NRPS genes in more than 75 % of 36 genera including all five cyanobacterial sections (Neilan et al. 1999; Christiansen et al. 2001). In principle it is possible that at least some of the 44 strains for which no PCR products were detected, may contain NRPS genes with less conserved adenylation core motifs. Nevertheless a great variety of genes available in the GenBank database was considered for primer modification. The amplification strategy, with binding sites of both primers in the adenylation domain, includes even type II NRPS gene clusters, which own an additional N-methylation domain between the essential A and T domains or NRPS systems with an unusual domain organization (Konz and Marahiel 1999). Therefore we are confident that the lack of NRPS amplicons truly reflects the absence of corresponding genes.

Lengths of the obtained putative peptide synthetase gene fragments differ much (Table 3) and show relatively low sequence similarities between the screened strains of different genera. This is in line with previous investigations (Neilan et al. 1999; Sosio et al. 2000; Christiansen et al. 2001) indicating that no phylogenetic relationship can be concluded from peptide synthetase sequence similarity, since the randomly amplified sequences of different modules have different functions and belong to different NRPS genes (again, having different

function). Comparison of putative NRPS sequences obtained from strains closely related on 16S rRNA level (i. e., GWS-BW-H260 and GWS-BW-H120M, as well as GWS-BW-H16, GWS-BW-H199 and GP-7), however, revealed high similarities of the NRPS genes as well. But due to the fact, that the function of these modules is not clarified yet, we can only speculate whether the sequence similarity reflects a phylogenetic relationship of bacteria with enzymes of equal function.

Comparison of the screening results for antagonistic activity and screening for secondary metabolite genes showed only concordance for strains GWS-BW-H220M and GWS-BW-H199. For strain GWS-BW-H220M also the highest similarity to a known NRPS amino acid sequence was obtained. This indicates that GWS-BW-H220M is indeed able to produce a non-ribosomally synthesized antibiotic.

Production of bioactive compounds must often be stimulated by alteration of media or growth conditions (Chen et al. 2000; Demain 1998). Recent results by Liang (2003) complemented our finding of a NRPS gene in strain GWS-BW-H260. Production of a bioactive peptide by this strain, assumed to be synthesised in a non-ribosomal mode, was demonstrated. While this strain produced only very low concentrations of the bioactive compound with marine broth 2216 (Difco) as medium, use of a defined medium with D-mannitol and soybean meal as carbon sources enhanced its production.

NRPS are generally considered as typical enzymes of secondary metabolism responsible for the biosynthesis of oligopeptides on a protein template (Kleinkauf and von Döhren 1996). Among the vast number of known non-ribosomal peptides are important pharmaceuticals including antibiotics, immunosuppressants, as well as antitumor or antifungal agents. However, synthesis of siderophores, essential for iron uptake in many microbial systems, is also based on NRPS (Crosa and Walsh 2002; Gehring et al. 1998). Therefore it is possible that some NRPS genes are involved in primary metabolism.

The combination of screening results and phylogenetic analysis directed the focus on the four genera *Kocuria*, *Mycobacterium*, *Nocardioides* and *Bacillus* (*Bacillus subtilis* cluster), which include 52 % of our positively tested strains. *Nocardioides* and *Bacillus* species of terrestrial origin are well known producers of bioactive compounds, but even though *Bacillus* spp. were frequently isolated from marine habitats (e. g., Sieburth 1976; Urakawa et al. 1999), the number of products reported from marine strains so far is comparatively low (Jensen and Fenical 1994; Wagner-Döbler et al. 2002). To our knowledge no secondary metabolite production of marine *Nocardioides* spp. has been described. We found antagonistic activity for all three tested *Nocardioides* spp., but despite of the phylogenetic similarity, differences in

the inhibition patterns against the target strains (see Table 2) suggest the production of different antimicrobial compounds or different antagonistic mechanisms. Terrestrial or obligate pathogen members of the genus *Mycobacterium* are also known as producers of secondary metabolites and genes for PKS and NRPS have been detected (Daniel et al. 2004; Fiss et al. 1994; Saxena et al. 2003; Stinear et al. 2004). Little is reported on the genus *Kocuria*. Berg et al. (2002) observed antagonistic activity of *Kocuria kristiniae* isolated from a rhizophere and *Kocuria varians* was identified as producer of the lantibiotic variacin (Pridmore et al. 1996). Although *Kocuria* species have been isolated from marine sediments before (Kim et al. 2004), our results are the first report of NRPS genes and antagonistic effects of marine isolates from this genus.

Our results demonstrate that potential for secondary metabolite production seems also to be present in marine members of genera, of which the terrestrial species are well known for secondary metabolite production. However, we found hints for secondary metabolite production in 8 further genera, including *Plantibacter* and *Agrococcus* of which no secondary metabolite production has been reported to our knowledge. The detection of new NRPS genes and antagonistic activities may encourage further studies to study gene-expression of the tested strains, to isolate and identify new compounds, or to clone the complete gene clusters for subsequent genetic approaches (Mootz and Marahiel 1999).

Acknowledgements

We thank Jörn Piel for helpful ideas and comments. This work was supported by the Volkswagen Foundation within the Lower Saxonian priority program "Marine Biotechnology".

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Table 1 Phylogenetic classes and closest related organisms (based on 16S rRNA gene similarity) of German Wadden Sea isolates used as target strains in agar diffusion assays

No.	Phylogenetic class	Strain	Acc. no.	Closest relative* (acc. no.)	16S rRNA gene similarity (%)
1)	α- *Proteobacteria*	HP30	AY239009	*Sulfitobacter mediterraneus* CH-B427 (Y17387)	96
2)		T3	AY177713	*Erythrobacter citreus* RE35F/1 (AF118020)	98
3)	γ- *Proteobacteria*	HP1	AY241547	*Rheinheimera baltica* OSBAC5 (AJ441082)	98
4)		T1	AY177717	*Shewanella waksmanii* KMM 3823 (AY170366)	95
5)	*Flavobacteria*	HP2	AY241555	*Arenibacter troitsensis* KMM 3674 (AB080771)	97
6)		HP25	AY241561	*Zobellia laminariae* KMM3676 (AB121875)	96
7)		TN	AY177724	*Arenibacter troitsensis* KMM 3674 (AB080771)	92
8)	*Actinobacteria*	HP20	AY177728	*Plantibacter flavus* DSM 14012 (AJ310417)	94
9)		T4	AY177725	*Pseudonocardia alni* IMSNU 20049 (AJ252823)	99
10)	*Bacilli*	HP10	AY172664	*Bacillus benzoevorans* DSM5391 (D78311)	96

* Affiliation determined by BLAST analysis (http://www.ncbi.nlm.nih.gov/blast); only validly published organisms were taken into account

Table 2 Summary of screening results

Phylogenetic **affiliation**	Strain (bp. sequenced)	Accession **no.**	NRPS **genes**	Denotation of **inhibited strains**
Actinobacteria				
Brevibacteriaceae	TAF-2.4a (813)	AY731368	+	
Gordoniaceae	GP-7 (694)	AY145535	+	
Microbacteriaceae	GP-8 (1074)	AY145536	+	
	GWS-BW-H145 (801)	AY332104	+	
	GWS-SE-H149 (616)	AY332164		4
Micrococcaceae	GWS-BW-H260 (838)	AY332122	+	
	GWS-BWrt-H120M (659)	AY370620	+	
	GWS-BW-H45M (1354)	AY370618		9
	GWS-BW-H258 (549)	AY731367		9
Mycobacteriaceae	GWS-BW-H16 (702)	AY731369	+	
	GWS-BW-H199 (704)	AY731370	+	4
Nocardiaceae	GWS-BW-H125 (704)	AY332094	+	
Nocardioidaceae	GP-1 (1102)	AY145529		1
	GWS-AG-H250-19 (718)	AY332140		3; 4
	GWS-AG-H206 (699)	AY332134		4
	Aeromicrobium marinum T2 (1357)	AY166703		4
Uncertain actinomycetes	T10 (1264)	AY731374		5
Firmicutes				
Bacillaceae	GWS-SE-H136 (717)	AY332154	+	
	GWS-BW-H220M (759)	AY370628	+	9
	GWS-AG-H79 (683)	AY731372		2; 5; 9
	HP9w (818)	AY172663		6; 7 ; 8 ; 9 ; 10

Table 3 Comparison of deduced NRPS amino acid sequences with similar GenBank sequences determined by BLAST analysis

Strain	Amino acids	Sequence similarity (protein, origin, acc. no.)	Expectation value [a]	Identity /similarity [b]
Actinobacteria				
GP-7	69	PstB, *Mycobacterium avium* (AAD44234)	E = 3e-13	54% / 70%
GP-8	75	NcpB, *Nostoc* sp. ATCC 53789 (AAO23334)	E = 2e-08	42% / 61%
GWS-BWrt-H120M	55	non-ribosomal peptide synthetase, *Streptomyces avermitilis* (BAB69370)	E = 5e-06	54% / 60%
GWS-BW-H125	78	SimH, *Streptomyces antibioticus* (AAG34184)	E = 7e-06	38% / 46%
GWS-BW-H145	69	putative non-ribosomal peptide synthetase, *Corynebacterium efficiens* YS-314 (NP_739167)	E = 5e-04	38% / 47%
GWS-BW-H16	67	peptide synthetase, *Mycobacterium smegmatis* (CAB55600)	E = 4e-05	40% / 55%
GWS-BW-H199	68	PstB, *Mycobacterium avium* (AAD44234)	E = 9e-13	52% / 70%
GWS-BW-H260	55	non-ribosomal peptide synthetase, *Streptomyces avermitilis* (BAB69370)	E = 1e-05	54% / 61%
TAF-2.4a	82	non-ribosomal peptide synthetase, *Pseudomonas syringae* pv. tomato str. DC3000 (AAO58141)	E = 2e-04	41% / 52%
Firmicutes				
GWS-SE-H136	70	pyoverdine sidechain peptide synthetase III, *Pseudomonas syringae* pv. tomato str. DC3000 (AAO55666)	E = 3e-08	46% / 59%
GWS-BW-220M	74	pyoverdine synthetase A, *Pseudomonas fluorescens* (AAF40219)	E = 7e-36	97% / 97%

[a] The Expectation value (E) is a parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size

[b] percentage of sequence position with identical or similar amino acids

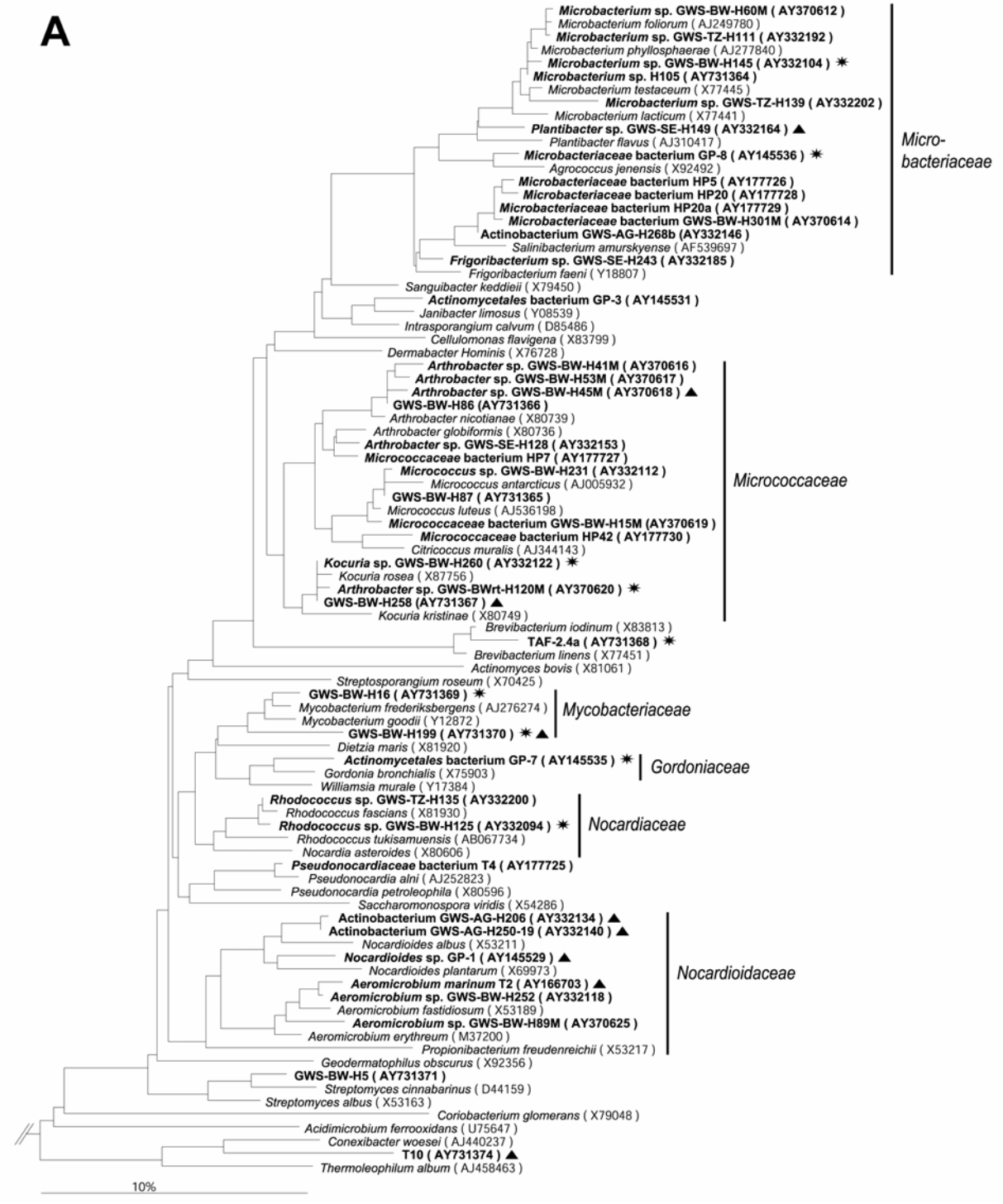
A
Microbacterium sp. GWS-BW-H60M (AY370612)
Microbacterium foliorum (AJ249780)
Microbacterium sp. GWS-TZ-H111 (AY332192)
Microbacterium phyllosphaerae (AJ277840)
Microbacterium sp. GWS-BW-H145 (AY332104)
Microbacterium sp. H105 (AY731364)
Microbacterium testaceum (X77445)
Microbacterium sp. GWS-TZ-H139 (AY332202)
Microbacterium lacticum (X77441)
Plantibacter sp. GWS-SE-H149 (AY332164)
Plantibacter flavus (AJ310417)
Microbacteriaceae bacterium GP-8 (AY145536)
Agrococcus jenensis (X92492)
Microbacteriaceae bacterium HP5 (AY177726)
Microbacteriaceae bacterium HP20 (AY177728)
Microbacteriaceae bacterium HP20a (AY177729)
Microbacteriaceae bacterium GWS-BW-H301M (AY370614)
Actinobacterium GWS-AG-H268b (AY332146)
Salinibacterium amurskyense (AF539697)
Frigoribacterium sp. GWS-SE-H243 (AY332185)
Frigoribacterium faeni (Y18807)
Micro-
bacteriaceae
Sanguibacter keddieii (X79450)
Actinomycetales bacterium GP-3 (AY145531)
Janibacter limosus (Y08539)
Intrasporangium calvum (D85486)
Cellulomonas flavigena (X83799)
Dermabacter Hominis (X76728)
Arthrobacter sp. GWS-BW-H41M (AY370616)
Arthrobacter sp. GWS-BW-H53M (AY370617)
Arthrobacter sp. GWS-BW-H45M (AY370618)
GWS-BW-H86 (AY731366)
Arthrobacter nicotianae (X80739)
Arthrobacter globiformis (X80736)
Arthrobacter sp. GWS-SE-H128 (AY332153)
Micrococcaceae bacterium HP7 (AY177727)
Micrococcus sp. GWS-BW-H231 (AY332112)
Micrococcus antarcticus (AJ005932)
GWS-BW-H87 (AY731365)
Micrococcus luteus (AJ536198)
Micrococcaceae bacterium GWS-BW-H15M (AY370619)
Micrococcaceae bacterium HP42 (AY177730)
Micrococcaceae
Citricoccus muralis (AJ344143)
Kocuria sp. GWS-BW-H260 (AY332122)
Kocuria rosea (X87756)
Arthrobacter sp. GWS-BWrt-H120M (AY370620)
GWS-BW-H258 (AY731367)
Kocuria kristinae (X80749)
Brevibacterium iodinum (X83813)
TAF-2.4a (AY731368)
Brevibacterium linens (X77451)
Actinomyces bovis (X81061)
Streptosporangium roseum (X70425)
GWS-BW-H16 (AY731369)
Mycobacterium frederiksbergens (AJ276274)
Mycobacterium goodii (Y12872)
GWS-BW-H199 (AY731370)
Mycobacteriaceae
Dietzia maris (X81920)
Actinomycetales bacterium GP-7 (AY145535)
Gordonia bronchialis (X75903)
Gordoniaceae
Williamsia murale (Y17384)
Rhodococcus sp. GWS-TZ-H135 (AY332200)
Rhodococcus fascians (X81930)
Rhodococcus sp. GWS-BW-H125 (AY332094)
Rhodococcus tukisamuensis (AB067734)
Nocardia asteroides (X80606)
Nocardiaceae
Pseudonocardiaceae bacterium T4 (AY177725)
Pseudonocardia alni (AJ252823)
Pseudonocardia petroleophila (X80596)
Saccharomonospora viridis (X54286)
Actinobacterium GWS-AG-H206 (AY332134)
Actinobacterium GWS-AG-H250-19 (AY332140)
Nocardioides albus (X53211)
Nocardioides sp. GP-1 (AY145529)
Nocardioides plantarum (X69973)
Aeromicrobium marinum T2 (AY166703)
Aeromicrobium sp. GWS-BW-H252 (AY332118)
Aeromicrobium fastidiosum (X53189)
Aeromicrobium sp. GWS-BW-H89M (AY370625)
Aeromicrobium erythreum (M37200)
Propionibacterium freudenreichii (X53217)
Nocardioidaceae
Geodermatophilus obscurus (X92356)
GWS-BW-H5 (AY731371)
Streptomyces cinnabarinus (D44159)
Streptomyces albus (X53163)
Coriobacterium glomerans (X79048)
Acidimicrobium ferrooxidans (U75647)
Conexibacter woesei (AJ440237)
T10 (AY731374)
Thermoleophilum album (AJ458463)
10%

Figure 1A

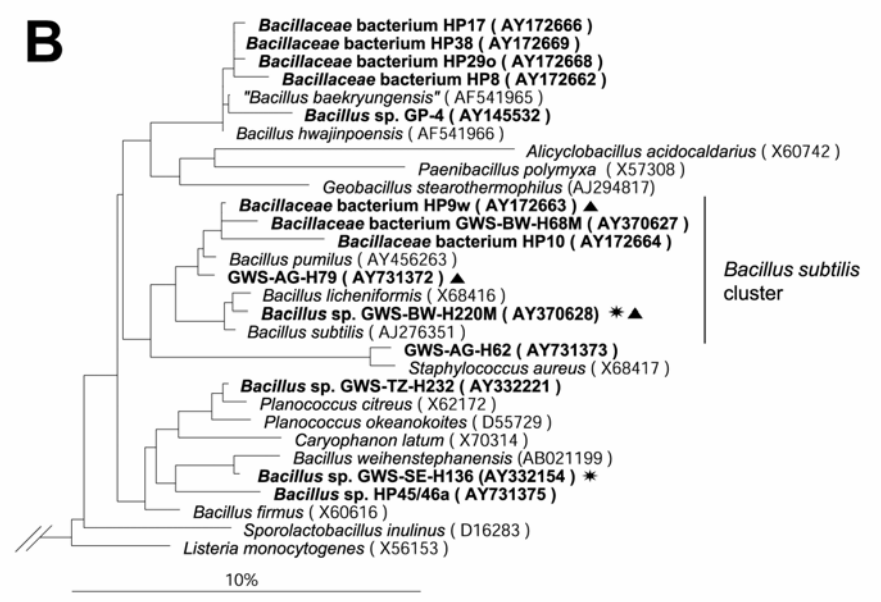

Figure 1B

Fig. 1 Maximum likelihood trees of *Actinobacteria* (A) and *Firmicutes* (B) showing the affiliation of the gram-positive isolates investigated in this study (bold). Sequences <1300 bp were added with maximum parsimony. Scale bars indicate 10 % sequence divergence. ✳ = NRPS genes detected; ▲ = inhibition of test strains in agar diffusion assays.

III

Bacteria of the *Roseobacter* clade show high potential for secondary metabolite production

Submitted to Applied and Environmental Microbiology

Bacteria of the *Roseobacter* clade show high potential for secondary metabolite production

Running title: Secondary metabolite production of *Roseobacter* spp.

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Keywords: *Roseobacter*, secondary metabolites, polyketide synthase, non-ribosomal polypeptide synthetase, AHL

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ABSTRACT

Members of the *Roseobacter* clade are abundant and widespread in marine habitats and have very diverse metabolisms. Production of acylated homoserine lactones (AHL) and secondary metabolites, e. g. antibiotics has been described sporadically. This prompted us to screen 36 strains of this group for production of signalling molecules, antagonistic activity and the presence of genes encoding for non ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS), both enzymes involved in the synthesis of various pharmaceutically important natural products. The screening approaches for NRPS and PKS genes were based on PCR with degenerated primers specific for conserved sequence motifs. Obtained PCR products were cloned, sequenced, and compared with genes of known function. Genes showing similarity to known NRPS and PKS genes were found in 7 and 5 strains, respectively. Three strains exhibited antagonistic activity. Antagonistic activity is in some bacteria regulated by cell-to-cell signalling and we detected AHLs in 11 isolates. Phylogenetic analysis of the 16S rRNA gene sequences of the tested organisms showed that several of the AHL positive strains clustered together, but no link was found between antagonistic activity and AHL production. Four strains were positive for three or four categories tested, and three of these organisms were found to be closely related. The simultaneous presence of NRPS and PKS genes in some of the strains and similarities of the PKS amino acid sequences to genes with known function indicated that the *Roseobacter* spp. contain genes coding for hybrid multienzyme complexes, containing both, PKS and NRPS modules. Our screening results indicate that the *Roseobacter* clade might be a rich, but largely untapped source for secondary metabolites.

INTRODUCTION

The eponymous genus of the *Roseobacter* clade (25) was originally established for two aerobic bacteriochlorophyll *a* containing species, *Roseobacter litoralis* and *Roseobacter denitrificans*, isolated from surfaces of green seaweeds (67). During the last decade, several new species were described as members of the *Roseobacter* clade, which presently comprises 21 genera. Isolates belonging to this group form a coherent phylogenetic cluster within α-*Proteobacteria* and have been obtained from a wide range of globally distributed marine habitats, open ocean as well as coastal environments, and all require a saline environment (25). They have a broad range of physiological properties and use a multitude of different carbon sources. Additional energy is gained by some organisms by oxidizing reduced sulfur compounds like sulfite or thiosulfate (71, 77), and, as stated above, some organisms possess bacteriochlorophyll *a* and are capable of aerobic photosynthesis, however, they are unable to grow autotrophically (67, 68).

Members of the *Roseobacter* clade are abundant in marine environments (26, 58, 64, 87) and sequences falling into this group form the second most abundant SSU rRNA gene cluster recovered from marine plankton clone libraries (25). Organisms of this group are also associated with cephalopods (4) or algae (2, 30, 38, 67).

Studying bacterial primary colonization of surfaces in coastal environments Dang and Lovell (15) found that the majority of their investigated clones belong to the *Roseobacter* clade, indicating that these organisms are excellent biofilm formers and/or have the ability to outcompete other organisms. This success might include production of secondary metabolites, like antibiotics. Indeed some species of this cluster are capable of producing antibiotic compounds (61, 80).

In the present study we screened 36 strains of the *Roseobacter* clade for antagonistic activity and presence of genes encoding for enzymes involved in the production of important natural products, i. e., non ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS). Polyketides and non ribosomal peptides represent large families of natural products that are widely used as pharmaceuticals, agrochemicals and antibacterial agents (35, 37, 73). Polyketides are synthesized from acyl CoA precursors by PKS, which have been divided into PKS of type I, II or III in terms of their organisation. Type I PKSs are multifunctional enzymes that are organised into modules, each of which harbours a set of distinct, non iteratively acting activities responsible for the catalysis of one cycle of polyketide chain elongation. Type II PKSs are made up of several separate monofunctional enzymes and

catalize the formation of cyclic aromatic compounds. Type III PKS, also known as chalcone synthase-like PKS, are homodimeric enzymes that essentially are iteratively acting condensing enzymes (22). A growing number of studies have demonstrated biosynthesis of complex natural products by mixed or hybrid multienzyme complexes containing both PKS and NRPS modules. First identified was the rapamycin biosynthetic gene cluster of *Streptomyces hygroscopicus* (62), and mixed multienzyme complexes have so far been found in *Actinobacteria*, *Cyanobacteria*, γ-*Proteobacteria* and *Myxobacteria* (e.g. 24, 48, 56,70, 79).

Production of antibiotics and other antagonistic compounds is in some gram-negative bacteria regulated in a cell density dependant manner (3, 45, 55, 83). AHLs are important messenger molecules in quorum sensing regulation systems, widely distributed among gram-negative bacteria (18), and have already been found in some organisms of the *Roseobacter* clade (9, 28, 38).

Combination of phylogenetic analyses and screening results was subsequently used in this study to identify clusters with increased potential for secondary metabolite production, which could serve as new potent sources for natural products.

MATERIALS AND METHODS

Isolation and cultivation of bacteria. Environmental samples were taken from the German Wadden Sea (southern North Sea) and the marine part of the Weser Estuary from which 25 strains affiliated with the *Roseobacter* clade were isolated. Additionally four *Roseobacter* strains were isolated from water samples taken from the Red Sea near Marsa Alam (Egypt). Bacteria were enriched in liquid cultures using various media and subsequently isolated through direct plating of culture aliquots. For a detailed description see Grossart et al. (31) (strains named "HP.." and strains named “ROS...”), Brinkhoff et al. (7) (strains named "T.."), and Stevens (74) (strains named "GWS.." or “H..”). Strains D1, D4, TY, HP14w, HP32, HP44w, HP47, HP47a, HP50, ROS2, ROS4, ROS7, ROS8, and H43-35 have not been described before, however, isolation of these bacteria was performed according to the procedures described in the above listed publications. Strains D1 and D4 were isolated from rolling tanks with surface water from the German Wadden Sea and amended with dried and autoclaved *Fucus vesiculosus* as substrate, according to Stevens (74). Two strains, i. e. AP-26 and AP-27, were isolated from the Weser estuary (63), which is part of the German Wadden

Sea. In addition, 7 validated species of the *Roseobacter* clade were tested (*Roseobacter denitrificans*, DSM 7001; *Roseobacter gallaeciensis* DSM 12440; *Roseovarius tolerans* DSM 11457; *Ruegeria algicola* DSM 10251; *Ruegeria gelatinovorans* DSM 5887; *Leisingera methylohalidivorans* DSM 14336; *Sulfitobacter pontiacus* DSM 10014). The strains were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Pure cultures of all strains were grown on Marine Broth (MB) 2216 media (DIFCO^{TM}, Becton Dickinson Microbiology systems, USA).

Screening of isolates for inhibitory effects. We used an agar diffusion assay modified after Brinkhoff et al. (7) to screen for inhibitory effects. The 8 target strains were isolated from the German Wadden Sea as most of the test strains, belong to 5 different phylogenetic classes (Table 1), and did not produce inhibitory compounds in preliminary tests [(31), T. Brinkhoff, unpublished results]. To produce a bacterial lawn 100 µl culture broth of a target strain (OD_{600} ~ 0.5), were spread on an agar plate with MB 2216 as medium. Sterile antibiotic assay discs (∅ 6mm, Schleicher & Schuell) were placed on the plates, and 10 µl culture solution of a potential producer (OD_{600} ~ 1) was applied. The plates were incubated for 6 days at 20°C and daily inspected for inhibition zones. Inhibition of a target strain was determined positive when the diameter of the zone of inhibition was at least 4 mm greater than the diameter of the colony formed around the antibiotic assay disc by the producing bacteria. The experiments were performed in three replicates. An isolate was considered as inhibiting if at least two replicates were positive.

PCR amplification and sequencing of 16S rRNA gene fragments. PCR amplification of 16S rRNA genes, purification of PCR products and subsequent sequencing analysis were performed according to Brinkhoff and Muyzer (8). For all isolates at least 800 bp were determined, except for HP14w, for which only 710 bp were sequenced. Sequences of screened strains were compared with similar sequences of reference organisms by BLAST search [http://www.ncbi.nlm.nih.gov/BLAST/] (1).

Phylogenetic analysis based on 16S rRNA gene sequences. Phylogenetic analysis was performed with the ARB software package [http://www.arb-home.de] (44). A phylogenetic tree was constructed using maximum likelihood analysis of almost full length 16S rRNA gene sequences (>1300 bp). Alignment positions at which less than 50 % of sequences of the entire set of data had the same residues were excluded from the calculations to prevent uncertain alignments within highly variable positions of the 16S rRNA genes, which cause mistakes in tree topology. Shorter sequences were added afterwards using maximum parsimony and the same filter.

Amplification, cloning and sequencing of NRPS and PKS gene domains. To obtain genomic DNA of the isolates for PCR analysis, cell material was taken from agar plates, suspended in sterile water (Sigma-Aldrich, Munich, Germany) and five cycles of freezing and thawing were applied. To avoid false negative results, the DNA of each strain was tested in a PCR with the general primers GM5F and 907R for 16S rRNA genes (8). Amplification of peptide synthetase gene fragments was carried out initially with the primers and PCR described by Rajendran (57), however, no PCR products could be obtained with our strains. Therefore we developed new degenerated primers, i.e. A2f (5´-AAG GCN GGC GSB GCS TAY STG CC-3´) and A3r (5´-TTG GGB IKB CCG GTS GIN CCS GAG GTG-3´), designed from conserved regions of adenylation domains of various bacterial peptide synthetase sequences (GenBank accession numbers: AAK81824, AAK81827, AAK81826, AAC82549, CAA40561, CAC48362, CAA11796, CAC48369, CAC48369, AAF42473, BAB69322, CAB38518, AAG02364, AAG02355, AAG02356, CAA67248, CAB93684, CAB93684, CAB93683, AAC68816, AAC44129, CAA65394, AAG05812, AAG05789, AAG05789, AAF40220, AAD51026, CAC11137, AAB96629). The sequence of the reverse primer is based on the signature sequence of the superfamily of adenylate forming enzymes TSGXTGXPK (motif A3), however, the sequence of the forward primer, based on the motif KAGGAY(LV)P (motif A2), is highly conserved for peptide synthetases, which are involved in non-ribosomal peptide synthesis (17). PCR was performed with an Eppendorf Mastercycler (Eppendorf Inc., Hamburg, Germany) as follows: 2 µl template DNA, 40 pmol of each of the appropriate primers, 12.5 nmol of each deoxyribonucleoside triphosphate, 5 µl of 10 x RedTaq^{TM} PCR buffer (Sigma-Aldrich), 1.2 mg ml^{-1} (final concentration) bovine serum albumin (Sigma-Aldrich) and 0.75 units RedTaq^{TM} DNA polymerase (Sigma-Aldrich) were adjusted to a final volume of 50 µl with sterile water (Sigma-Aldrich). A PCR run comprised 40 cycles with denaturing, annealing and extension temperatures for one minute at 95°C, one minute at 70°C and two minutes at 72°C. DNA from *Pseudomonas* sp. (DSM 50117) was used as positive control.

The ketosynthase specific primer pair KSDPQQF and KSHGTGR (54) was used for amplification of PKS I gene fragments, including an initial denaturing step at 94°C for 2 min, followed by 45 cycles at 94°C for 1 minute, annealing at 55°C for 1 minute, and elongation at 72°C for 2 minutes. A 50 µl PCR-reaction mix contained 2 µl template DNA, 30 pmol of each of the appropriate primers, 12.5 nmol of each deoxyribonucleoside triphosphate, 5 µl of 10 x RedTaq^{TM} PCR buffer (Sigma-Aldrich), 0.2 mg ml^{-1} (final concentration) bovine serum

albumin (Sigma-Aldrich) and 0.75 units RedTaq™ DNA polymerase (Sigma-Aldrich). DNA from *Bacillus subtilis* strain 168 (DSM 402) was used as positive control.

PCR-products approximately of the expected length were gel-purified using the Perfectprep™ Gel cleanup Kit (Eppendorf Inc., Hamburg, Germany) and ligated into the pGEM-T vector (Promega, Mannheim, Germany) following the manufacturers protocol. Recombinant clones containing an insert were sequenced using the DYEnamic Direct cycle sequencing kit (Amersham Life Science Inc., Little Chalfont, UK) and a Model 4200 Automated DNA Sequencer (LI-COR Inc., Lincoln, USA). Both DNA strands were sequenced twice using M13F and M13R labeled with IRDye™800 as sequencing primers (46). The deduced amino acid sequences were compared with similar sequences by BLAST search [(http://www.ncbi.nlm.nih.gov/blast)] (1).

Phylogenetic analysis based on PKS amino acid sequences. PKS amino acid sequences obtained in this study, were aligned using the program BioEdit v5.09 (www.mbio.ncsu.edu/BioEdit/bioedit.html) and the multiple-sequence alignment tool Clustal X v1.83 (78). To construct a phylogenetic tree based on these alignments we used the PHILIP v 3.6a3 package (21). Protein distances were inferred by using a maximum likelihood method implemented in the program PRODIST, with the JTT matrix as the amino acid replacement model. A tree was inferred from the distances by using the neighbour joining method of the NEIGHBOR program and tested statistically by bootstrap analysis. Bootstrap values were calculated for the protein tree from 1000 replicates.

Detection of acylated homoserine lactones. The presence of acylated homoserine lactones (AHL), was tested with sterile filtered supernatants from cultures incubated for 1.5 to 2 weeks at 15°C. Samples were analysed in three AHL monitor systems with *Agrobacterium tumefaciens* (10) and *Chromobacterium violaceum* CV026 (45) using the well-diffusion assay described by Ravn et al., (59) and in the *Escherichia coli* pSB403 LuxR assay (19, 76) as described (27). *C. violaceum* was cultured in LB broth (5) and *A. tumefaciens* in ABTG medium (14). Because AHLs are unstable at high pH, all cultures were grown in MB which pH was adjusted to 6.2.

DNA sequence accession numbers. The 16S rRNA gene sequences determined in this study have been deposited in the GenBank database under AY841770 - AY841784. The putative peptide synthetase and polyketide synthase sequences obtained from our isolates have been deposited in the GenBank database under AY841763 - AY841769 and AY841785 - AY841791, respectively.

RESULTS

Inhibitory effects. Growth inhibition of target strains was observed for 3 of the isolates. While *Roseobacter gallaeciensis* DSM 12440 and strain T5 were able to inhibit the growth of all target strains with an inhibition-zone of a mean diameter of 20 mm, strain D1 inhibited only two strains (HP39 and HP10) and showed a weaker activity (10 mm diameter of the inhibition zones). The majority of the tested bacteria did not show any effect, however, 7 strains (*R. gelatinovorans*, HP29w, HP30, HP47, HP50, ROS4 and GWS-BW-H22M) produced zones of reduced growth, in which the cell density of the target strains (mainly T3) was reduced.

Screening for AHL production. Of 36 strains tested for production of AHLs, 11 isolates gave positive results in at least one of the monitoring systems. Comparison of these screening results and those from the phylogenetic analysis revealed that 8 of the AHL positive strains grouped in two clusters (Fig. 1). Five positive strains were affiliated with *Staleya guttiformis*, and 3 positive strains grouped in the *R. gallaeciensis* cluster.

Cloning and sequencing of NRPS and PKS I gene sequences. PCR with the new primer pair A2f and A3r reproducibly resulted in products of the expected size. Out of 36 isolates examined, DNA of 7 strains yielded a PCR product. Sequence analysis of these products revealed high similarity to non ribosomal peptide synthetases in the GenBank database (Table 2).

Type I polyketide synthases consist of repeated modules, which process biosynthesis of a polyketide chain in an assembly line fashion (35). To perform one chain elongation cycle at least keto-acyl synthase (KS), acyltransferase and acyl carrier protein (ACP) domains are necessary. Among PKS I domains, the KS domain is the most conserved (43, 47). Therefore detection of modular type I PKS genes was performed using degenerated oligonucleotide primers based on universally conserved motifs of KS-domains as described before (6). Sequences of KS-domains were obtained from *Roseobacter gallaeciensis*, *Ruegeria gelatinovorans*, and the strains T5, HP12 and TY (Table 3). Random screening for PKS I genes of some other isolates obtained from the Wadden Sea gave positive results for two other strains, i. e. GWS-SE-H246 affiliated with the genus *Halomonas* (γ-*Proteobacteria*) and HP25 affiliated with the genus *Zobellia* (*Flavobacteria*) (75) (Table 3). The most similar GenBank sequences for GWS-SE-H246 belong to β-*Proteobacteria* like WcbR from *Bordetella bronchiseptica* (Acc. no.: NP_889454; 70 % identity) and *Burkholderia mallei* (Acc. no.: AAK26474; 70 % identity). The most similar gene product with known function is

MelF from, *Melittangium lichenicola*, (Acc. no. CAD89777), involved in Melithiazol biosynthesis (82). Except for strain GWS-SE-H246, which closest related KS sequences with known function belong to a cluster with conventional PKS I sequences, all other detected KS fragments were similar to mixed or hybrid PKS/NRPS complexes (11, 20, 33) (Fig. 2).

DISCUSSION

Acylated homoserine lactones were detected in 11 out of 36 strains tested. AHL production has been reported earlier for other members of the α-*Proteobacteria* such as *Rhizobium* and *Agrobacterium* (60, 86) as well as from the *Roseobacter* clade (9, 28). Our study confirms and broadens the knowledge of AHL production and possible quorum sensing in this phylogenetic group.

So far there are no reports about the function of the AHLs produced by bacteria of the *Roseobacter* clade. Gram et al. (28) speculated that AHLs in *Roseobacter* species govern phenotypic traits (biofilm formation, exoenzyme production, and antibiotic production), which are required mainly when the population reaches high densities, e. g., in marine snow communities, but this remains to be tested. Even though 8 of the 11 AHL positive *Roseobacter* strains appear in two phylogenetic clusters (Fig. 1) AHL production is obviously widespread within this group. Since there are more than 50 different structures of biologically active AHLs known (23) it is even possible that strains which showed no signal might have given a response with other AHL receptors or under different culture conditions. Broad functionality of these systems, however, was proven in previous studies (59, 66), supporting the reliability of our results.

Only very few isolates, i. e. 3 out of 36 isolates, showed clear antagonistic properties against 2 or even all background strains. This is in line with two previous studies, which reported antagonistic interactions of 5 – 8 % of the isolates examined (51, 53). Much higher percentages of antagonistic strains were reported in two more recently published papers (31, 41), suggesting inhibition is a common phenomenon among pelagic bacteria. Tests of the latter studies, however, were carried out with much higher numbers of target organisms. Higher numbers of target strains in our study presumably had also resulted in detection of higher numbers of antagonistic *Roseobacter* strains. Thus the low detection rate may only reflect a statistically effect.

Instead of a clear inhibition zone, more often the isolates produced a zone of reduced growth of the target strains. This phenomenon was observed regularly in all replicates. The isolates reduced the cell density of the background strain independently from the incubation time. It is known that many agents can effectively kill isolated bacterial cells, but are ineffective against dense populations of a species. This was shown for biofilms and colonies, which showed enhanced resistance against a wide range of antibacterial compounds (65). Consequently, a strain may produce inhibitory compounds, which are non or only minor effective in the filter disc assay.

Comparison of the derived amino acid sequences from 17 % of all isolates revealed a high homology to sequence fragments of known peptide synthetases by a BLAST analysis (Table 2). Sosio et al. (72) detected among 19 Actinomycetes belonging to 8 different genera various NRPS modules in all species. Investigations of *Cyanobacteria* discovered, either with a PCR based approach, or with hybridisation techniques, NRPS genes in more than 75 % of almost all genera (13, 52). Although very little is known about the distribution of NRPS gene clusters among α-*Proteobacteria*, these results indicate that a smaller number of these bacteria contains NRPS modules as compared to other groups, or that the PCR system did not detect all present genes.

PKS genes have been reported for many microorganisms including various *Actinobacteria* (35), *Cyanobacteria* (49, 79), and *Proteobacteria* (69). KS-domain sequence data in the alpha subgroup of the *Proteobacteria* were so far mainly obtained from terrestrial species of the order *Rhizobiales* (36, 39, 47, 84). The deduced amino acid sequences for type I KS-synthase domains showed not only a striking identity with PKS sequences in the GenBank database, detection of the highly conserved motif DTACSSSMVA, present in almost all KS domains (47), is an additional proof for the existence of real PKS genes in the positive tested *Roseobacter* isolates.

As shown in Fig. 2, type I KS domain protein sequences cluster phylogenetically into two functional groups. KS domains which use acyl CoA as their starter or extender unit are distinct from domains of mixed or hybrid PKS/NRPS gene clusters (47). The mixed PKS/ NRPS sequences from the synthesis cluster of immunorepressants FK 506 (AAC68815) and FK 520 (AAF86393; AAF86396) cluster within the PKS I group, however, seem to be phylogeneticelly distinct from other mixed sequences. The condensation domain of the NRPS modules from these biosynthetic systems catalyzes the condensation of the amino acid moiety to the end of the polyketide chain (50, 85). Therefore, only KS-domains catalyzing the condensation of a polyketide moiety onto an amino acid moiety, appear to be structurally

different from non hybrid KS-domains and can be distinguished. Phylogenetic analysis of our sequences exhibited that all *Roseobacter* KS fragments form a separate cluster among the sequences from mixed or hybrid PKS/NRPS systems. The sequence obtained from strain HP25, belonging to *Flavobacteria*, also affiliates with these sequences. This strongly indicates that these species have genes for hybrid PKS/NRPS complexes. According to the phylogenetic analysis of the PKS sequences, we also found NRPS modules in 4 of these *Roseobacter* isolates, supporting this assumption.

Phylogenetic analysis of the PKS I fragments from our isolates and various published KS domain sequences showed that the phylogeny of KS domains is related to the 16S rRNA based phylogeny of the organisms containing these KS-domains. This is in line with results from a previous study (47).

The putative keto-acyl synthase domain sequence of strain GWS-SE-H246 was most similar to those of β-*Proteobacteria*, but the 16S rRNA gene sequence clearly affiliates with the genus *Halomonas* within the γ-*Proteobacteria*. Today many KS-synthase domain sequences are available for *Cyanobacteria*, *Actinobacteria*, *Firmicutes* and *Proteobacteria*, demonstrating that the phylogeny of KS domains is related to the organism of origin (47). Thus the similarity of the putative KS-synthase domain found in strain GWS-SE-H246 to those found in β-*Proteobacteria* may simply reflect the close relationship of γ- and β-*Proteobacteria* on the 16S rRNA level.

Comparison of the screening results for inhibitory compounds and screening for secondary metabolite genes showed only little concordance with positive results in both assays. Production of bioactive compounds by bacteria must often be stimulated by alteration of media or growth condition (12, 16), presence of other bacteria (61), or growth on a solid surface (42). Previous findings indicated that the limit of the number of secondary metabolites produced by a species is systematically underestimated (81). Today researchers favor the “one strain many compound” (OSMAC) theory, suggesting a species is able to produce more secondary metabolites when changing the culture conditions (34). This was exemplified for *Streptomyces griseoviridis* Tü 3634 (29). Therefore it is possible that the application of standard media has no effect on the production of inhibitory compounds. For *Roseobacter gallaeciensis* and strain T5 it was shown that the inhibiting compound produced under the conditions used also in the present study is the tropone derivative tropodithietic acid, an antibiotic with a broad antibacterial activity (7). Therefore the observed inhibitory effects are probably completely uncoupled from the detected genes involved in secondary metabolism.

Ruiz-Ponte et al. (61) and Hjelm et al. (32) showed that *Roseobacter* strains are antagonistic against several γ-*Proteobacteria*. The former study found that the antagonistic compound produced by *Roseobacter gallaeciensis* BS107 was sensitive to trypsin and stable at 100°C and it was assumed that the substance is a peptide. Since tropodithietic acid is no peptide (40) and therefore not sensitive to trypsin, *R. gallaeciensis* must be able to produce another, yet undescribed antibiotic. The putative NRPS detected in the present study might be involved in the synthesis of this peptide.

Among the phylogenetic subclusters of screened *Roseobacter* species, two clusters contain most of the bacteria, which were determined positive in at least one of our assays: the HP44w cluster and the *Roseobacter gallaeciensis* cluster. In the HP44w cluster 5 out of 7 strains were able to produce AHLs, but no direct hint for secondary metabolite production was given for one of these strains. For strains affiliated with other clusters, however, the potential to produce AHLs was mostly coupled to a potential for secondary metabolite production.

Strains affiliated with the *Roseobacter gallaeciensis* cluster harbour the broadest spectrum of positive screening results, as demonstrated by this and previous studies (7, 61). These results are in line with the OSMAC theory. Thus, especially this cluster might be a rich, but largely untapped source for novel natural compounds, like secondary metabolites and signaling molecules.

Despite of the fact that the biological function of the detected genes and AHLs remains unclear, our results indicate the presence of many, probably new compounds and mechanisms in species of the *Roseobacter* clade. Future studies have to elucidate growth conditions for the expression of the detected PKS and NRPS genes and to isolate and identify the new compounds. Furthermore the high potential for production of secondary metabolites and signaling molecules could be ecologically important and partially explain the success of these organisms.

ACKNOWLEDGEMENTS

We thank Jörn Piel for helpful comments. We are grateful to B. Rink for providing us with strains D1 and D4. This work was supported by grants from the Volkswagen Foundation within the Lower Saxonian priority program Marine Biotechnology and the Universitäts-Gesellschaft Oldenburg.

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TABLE 1. Phylogenetic affiliation and closest related described species of German Wadden Sea isolates used as target strains in the agar diffusion assay.

No.	Strain	Acc. no.	Phylogenetic affiliation (class)	Closest described relative* (acc. no.)	16S rRNA gene similarity (%)
1)	HP39	AY239012	α- *Proteobacteria*	*Stappia aggregata* (D88520)	91
2)	T3	AY177713		*Erythrobacter citreus* (AF118020)	98
3)	HP3	AY241548	γ- *Proteobacteria*	*Alcanivorax borkumensis* (Y12579)	98
4)	HP6	AY241550		*Marinobacter aquaeolei* (AF173969)	97
5)	HP23	AY241560	*Sphingobacteria*	*Belliella baltica (*AJ564643)	89
6)	HP28	AY241563		*Belliella baltica* (AJ564643)	90
7)	HP7	AY177727	*Actinobacteria*	*Arthrobacter globiformis* (AB098573)	99
8)	HP10	AY172664	*Bacilli*	*Bacillus pumilus* (AY456263)	99

* Affiliation determined by BLAST analysis (http://www.ncbi.nlm.nih.gov/blast). Only validly published organisms were taken into account.

TABLE 2. Similarity analysis of deduced NRPS amino acid sequences from investigated strains with GenBank sequences determined by BLAST analysis.

Strain	Amino acids	Sequence similarity (protein, gene, origin, accession no.)	Expect value [a]	Identity/ similarity [b] (%)
GWS-BW-H55M	70	tyrocidine synthetase 3, tycC, *Brevibacillus brevis*, AAC45930	E = 4e-07	38 / 61
HP12	75	non-ribosomal peptide synthetase, mxaA, *Stigmatella aurantiaca*, AAK57184	E = 4e-10	45 / 61
HP32	70	nonribosomal peptide synthetase, lgrC, *Brevibacillus brevis*, CAD92851	E = 2e-07	42 / 63
Leisingera methylohalidivorans	69	nonribosomal peptide synthetase, ctaG, *Cystobacter fuscus*, AAW03330	E = 5e-09	42 / 67
Ruegeria gelatinovorans	71	peptide sythetase, mcyA, *Anabaena* sp. 90, AAO62586	E = 8e-10	40 / 73
Roseobacter gallaeciensis	90	nonribosomal peptide synthetase, vioA, *Streptomyces vinaceus*, AAP92491	E = 1e-05	36 / 45
T5	71	nonribosomal peptide synthetase, lgrC, *Brevibacillus brevis*, CAD92851	E = 2e-07	40 / 56

[a] The Expect value (E) is a parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size.
[b] percentage of sequence position with identical or similar amino acids.

TABLE 3. Similarity analysis of deduced keto-acyl synthase sequences from investigated strains with GenBank sequences determined by BLAST analysis.

Strain	Amino acids	Sequence similarity (protein, origin, acc. no.)	Expect value [a]	Identity/ similarity [b] (%)
α-Proteobacteria				
TY	223	NosB, *Nostoc* sp. GSV224, AAF15892	1e-62	53 / 69
Ruegeria gelatinovorans	223	NosB, *Nostoc* sp. GSV224, AAF15892	9e-61	54 / 67
Roseobacter gallaeciensis	223	BarE, *Lyngbya majuscula*, AAN32979	4e-64	53 / 71
T5	223	CtaD, *Cystobacter fuscus*, AAW03327	7e-66	52 / 70
HP12	223	NosB, *Nostoc* sp. GSV224, AAF15892	4e-62	57 / 70
Flavobacteria				
HP25	222	JamM, *Lyngbya majuscula*, AAS98784	1e-59	50 / 70
γ-Proteobacteria				
GWS-BW-H246	217	MelF, *Melittangium lichenicola*, CAD89777	2e-59	51 / 70

[a] The Expect value is a parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size.
[b] Percentage of sequence position with identical or similar amino acids.

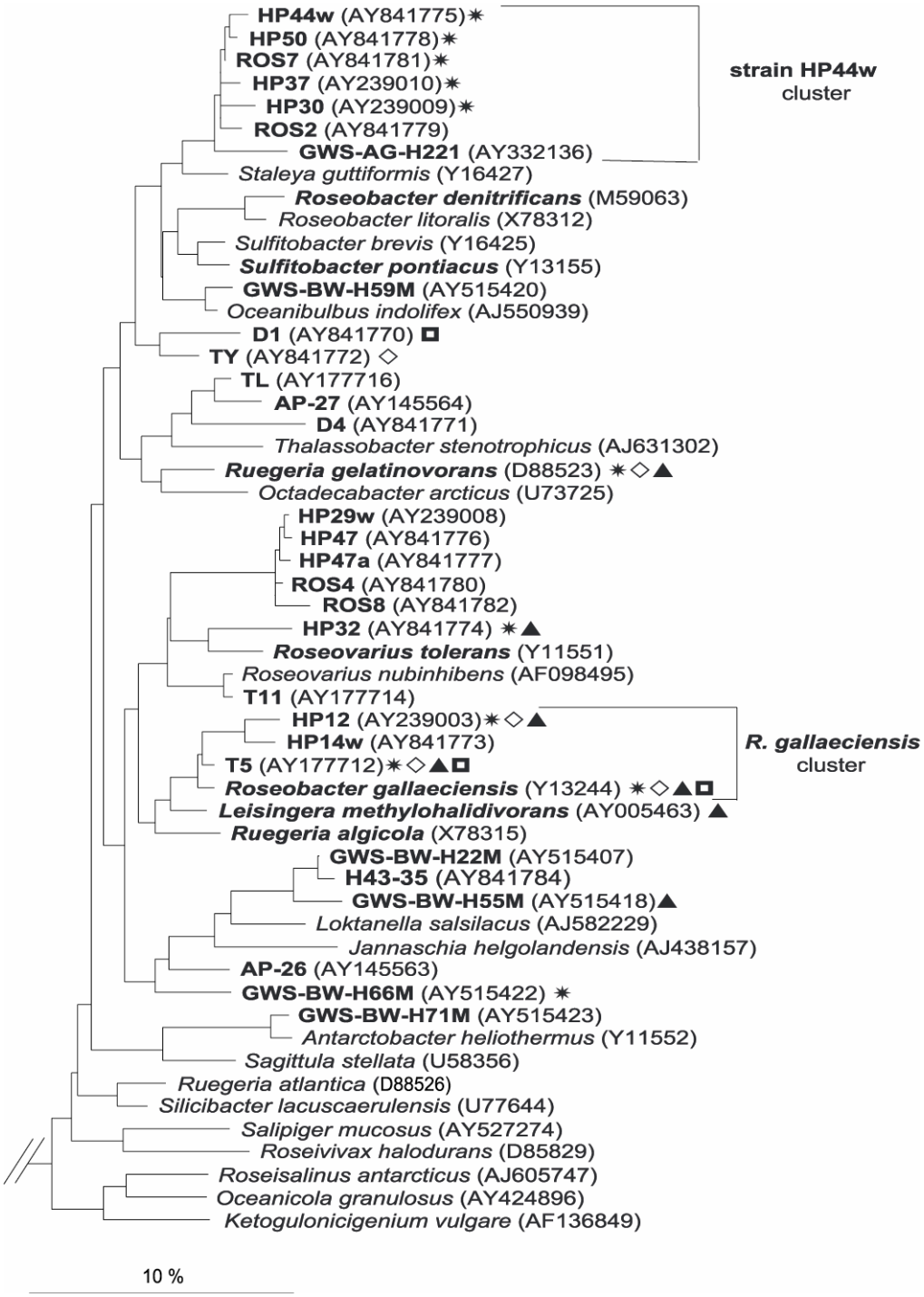

FIG. 1. Maximum likelihood tree based on 16S rRNA gene sequences showing the phylogenetic affiliation of isolates investigated in this study (boldface) within the *Roseobacter* clade. Sequences <1300 bp were added with maximum parsimony. Scale bar indicates 10 % sequence divergence. ✳ = AHL production; ▲= NRPS gene detected; ◇= PKS gene detected; ◘ = inhibition of test strains in agar diffusion assays.

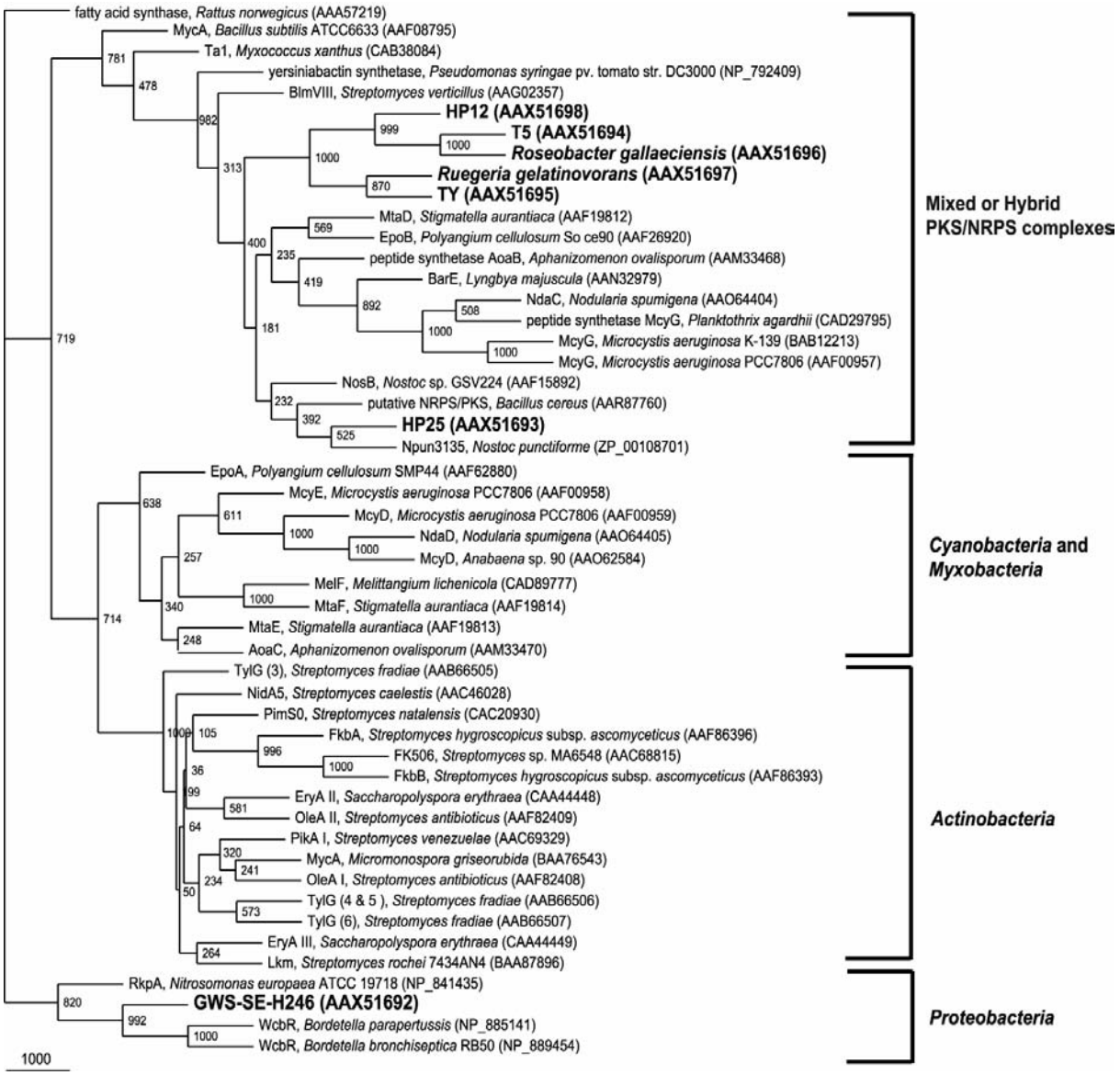

FIG. 2. Neighbour-joining tree showing the relationships of protein sequences of type I KS-domains. Sequences obtained during this study are given in boldface. GenBank accession numbers are given in parenthesis. The tree was printed out with CONSENSE using the Majority Rule consensus method (21), bootstrap values calculated from 1000 trees are indicated at the nodes. Note that the lengths on the tree on the output tree file are not branch lengths but the number of times that each group appeared in the input trees. The scale bar represents 100 % appearance frequency (= 1000 trees). The sequence of the fatty acid synthase from *Rattus norwegicus* was used as outgroup and to root the tree.

IV

PCR-basiertes Screening nach Genen für $FADH_2$-Halogenasen

Einleitung

Bereits im Jahr 1904 wurde Diploicin als erster halogenierter Sekundärstoff aus einer Flechte isoliert (Zopf, 1904). Trotz der anfänglich geringen Anzahl gefundener Produkte, bis 1961 waren nur 29 natürliche halogenierte Verbindungen bekannt, führte die systematische Ausweitung des Antibiotika Screenings zu der Entdeckung von heute mehr als 3000 bekannten Halogen-Metaboliten (van Pee, 2001). Auch die Ausweitung des Screenings auf marine Organismen hat zur Fülle der publizierten Verbindungen beigetragen. Genau so vielfältig wie die Verbindungen selbst sind auch die produzierenden Organismen. Neben Mikroorganismen, sind auch Algen, Pflanzen, marine Invertebraten, Insekten und auch Säugetiere in der Lage halogenierte Verbindungen zu synthetisieren.

Der größte Teil (ca 50 %) alle natürlichen halogenierten Naturstoffe wurde in Pilzen oder in terrestrischen Bakterien entdeckt (Naumann, 1993; Naumann, 1994). Dazu zählen auch wichtige Antibiotika wie z.B. Aureomycin, Avilamycin, Chloramphenicol, Chlorotricin, Clindamycin und Griseofulvin (Mason et al., 1982).

Obwohl eine große Anzahl an halogenierten Naturstoffen bekannt ist, gibt es immer noch wenig Hinweise auf die biologische Funktion vieler Verbindungen. Dies liegt zum Teil daran, dass es keine eindeutige Regel für den Einfluss auf die biologische Aktivität des halogen Atoms, deren Anzahl oder Position gibt. So kann, verglichen mit dem entsprechenden unhalogenierten Produkt, Halogenierung die biologische Aktivität steigern oder vermindern, (Neidleman & Geiger, 1986; van Pee & Ligon, 2000)

Die halogen Atome werden durch enzymatische Reaktion in organische Verbindungen inkooperiert, wobei Halogen-Ionen als Halogen Quelle dienen (Gribble, 1994). In Organismen die halogenierte Sekundärmetabolite produzieren, wurden insgesamt vier Typen von Halogenasen entdeckt. Zu den am besten untersuchten Enzymen gehören die Haloperoxidasen und Perhydrolasen, zwei Enzymklassen denen nach jüngeren Erkenntnissen die Substratspezifität fehlt, die für die Synthese vieler natürlich vorkommender Halometabolite notwendig ist (van Pee, 2001). Der dritte Typ ist die S-Adenosinmethionine-methyltransferase, die in zellfreien Extrakten des Pilzes *Phellinus pomacius* gefunden wurde (Wuosmaa and Hager, 1990). Neben diesen seit Jahrzehnten bekannten Halogenasen, haben jüngere molekulargenetische Untersuchungen gezeigt, dass zumindest in Bakterien noch eine vierte Enzymklasse an der Bildung von Halometaboliten beteiligt ist (Dairi et al., 1996; Hammer et al., 1997). Es handelt sich um $FADH_2$-abhängige Halogenasen, die sowohl Substrat-, als auch

Regioselektivität aufweisen (van Pee & Holzer, 1999) und von denen bisher nur wenige bekannt sind.

Entsprechend gering ist auch die Anzahl der gut untersuchten $FADH_2$- Halogenasegene, die für die Biosynthese von Pyrrolnitrin (prnA und prnC) in *Pseudomonas flourescens* (Kirner et al., 1998) und für die Pyoluterin-Biosythese in *Pseudomonas flourescens* Pf-5 (Nowak-Thompson, et al., 1999) durch Tn5 Mutagenese der produzierenden Stämme entdeckt wurden. Obwohl diese Enzyme verschieden Substrate umsetzen und eine entsprechende Selektivität aufweisen, besitzen sie doch einen gemeinsamen Reaktionsmechanismus. Der für das katalytische Zentrum codierende DNA-Abschnitt kann somit als Ziel-Region für die Klonierung verschiedener Halogenasegene dienen. Obwohl über eine fehlende Homologie zwischen geklonten Halogenasegenen PrnA und PrnC berichtet wurde (Kirner et al., 1998) gibt es bis bereits eine Studie die mit Hilfe der PCR Genfragmente amplifizieren konnte (Piraee & Vinning, 2002). Es handelt sich jedoch um eine Publikation in der gezielt nach PrnC homologen Genen gesucht wurde, die hier vorliegende Arbeit ist unseres Wissens nach der erste Versuch mittels degenerierten Primern PrnA homologe $FADH_2$-abhängige Halogenasen zu identifizieren.

Material und Methoden

Herkunft und Isolierung der Bakterien

Insgesamt wurden 101 Stämme Untersucht, von denen 42 zum Stamm *Actinobacteria* gehörten, 14 zu *Firmicutes,* 42 zu *Proteobacteria* und 3 zu *Bacteriodetes*. Die Stämme wurden überwiegend aus dem deutschen Wattenmeer und aus dem Weserästuar isoliert. Für eine genaue Beschreibung von Herkunft, Phylogenie und Isolierung siehe Abschnitt „Material and Methods“ in Kapitel 1 und 2 dieser Arbeit. *Burkholderia pyrrocinia* (DSM 10685) wurde von der Deutschen Sammlung von Mikroorganismen und Zellkulturen bezogen und nach den Angaben der Stammsammlung kultiviert, alle anderen Stämme wurden auf Marine Broth 2216 (Difco, Becton Dickinson Microbiology systems, USA) kultiviert.

PCR basiertes Screening auf Gene für $FADH_2$-abhängige Halogenasen

Die für hochkonservierte Bereiche von $FADH_2$-abhängigen Halogenase-Genen entwickelten Primer Hal1F (5'- TCG G(CT)G T(GC)G GCG A(AG)G CGA CC(AG) TCC C -3'), Hal2F (5'- T(GC)G GCG GCG GCA C(CT)G C(GC)G G(AC)T GGA TG -3', Hal3R (5'- AGC AT(GC) GG(AG) ATC TTC CAG GTC CA(GCT)CC -3') und Hal4R (5'- GCC GGA GCA GTC

GA(CT) GAA (GC)AG GTC -3') wurden von Susanne Zehner (AG Biochemie, TU Dresden) zur Verfügung gestellt. Die zwei Forward-Primer und Zwei Reverse-Primer wurden in verschiedenen Kombination ausprobiert, um durch mehrfache PCR-Produkte die Nachweiswahrscheinlichkeit zu erhöhen. Abbildung 1 zeigt ein Schema mit den Primer-Bindestellen. Es ergeben sich vier Kombinationsmöglichkeiten die Produkte mit folgender Länge ergeben können: Primer Hal1F + Hal3R: 720bp, Primer Hal1F+ Hal4R: 540bp, Primer Hal2F +Hal3R: 900bp und Primer Hal2F + Hal4R: 630bp.

Um genomische DNA für das PCR-Screening zu gewinnen, wurde Zellmaterial von Agarplatten in sterilem Wasser (Sigma-Aldrich, München, Deutschland) suspendiert und mehrere Einfrier- und Auftauzyklen durchgeführt. Um falsch negative PCR Ergebnisse zu vermeiden, wurde vorab die Amplifizierbarkeit des Templates mit universellen Bakterienprimern für 16S rRNA Gene gemäß Brinkhoff und Muyzer (1997) getestet.

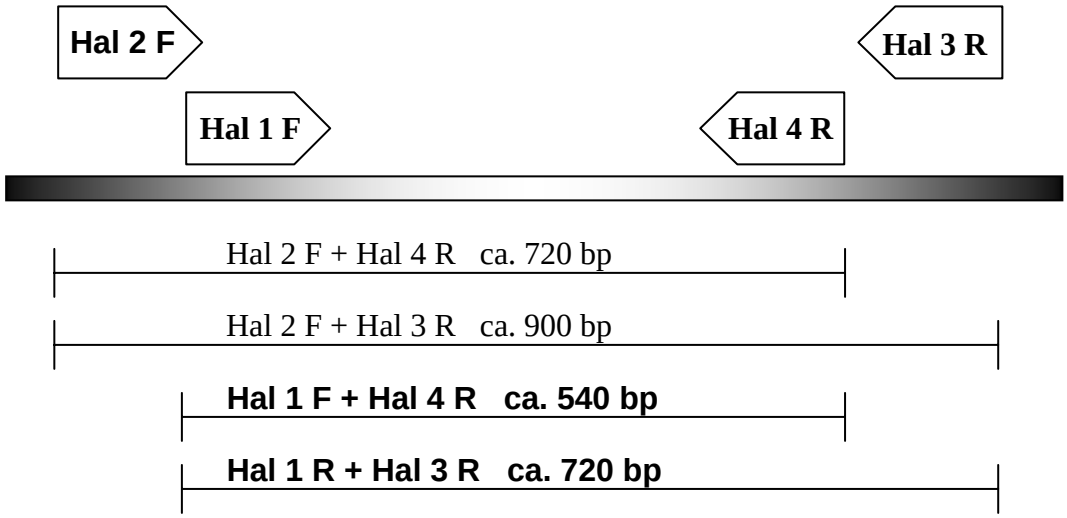

Abb. 1 Schematische Darstellung von Bindungsstellen der Primer im Halogenasegen und der Größe der entsprechenden PCR – Produkte.

Ein 50 µl PCR-Ansatz mit Halogenase-Primern enthielt ca. 2 µl DNA-Template, 50 pmol von jedem Primer, 12,5 nmol von jedem Deoxyribonucleosid-Triphosphat (dNTP), 5 µl des 10 x RedTaq™ PCR Puffers (Sigma-Aldrich), 0.2 mg ml^{-1} (Endkonzentration) Rinerserum-Albumin (Sigma-Aldrich) und 0.75 units RedTaq™ DNA Polymerase (Sigma-Aldrich). DNA aus *Burkholderia pyrrocinia* (DSM 10685) diente als Positiv-Kontrolle und wurde zur Optimierung des PCR Programms eingesetzt. Eine PCR Amplifizierung umfasste einen ersten Denaturierungsschritt von 2 min. bei 95° C, gefolgt von einer Stepdown-Phase (Don et al., 1991) mit Denaturierung bei 95 °C für 1 min, *annealing* bei 70-66° C für 1 min und Extension

bei 72° C für 3 min, bei der alle zwei Zyklen die *annealing*-Temperatur um 1° C erniedrigt wurde. Anschließend erfolgten 30 Zyklen mit einer Annealing-Temperatur von 65° C mit abschließendem Verlängerungsschritt bei 72° C für 7 min.

Klonierung und Sequenzierung von PCR-Produkten

PCR-Produkte mit der erwarteten Länge, wurden mit Hilfe von 1,5 % Agarose-Gelen und dem Perfectprep™ Gel cleanup Kit (Eppendorf, Hamburg, Deutschland) aufgereinigt und nach den Instruktionen des Herstellers in den pGEM-T Vector (Promega, Mannheim, Deutschland) ligiert. Kompetente Zellen von *E. coli* DH5-α wurden mit dem Roti-Transform-Kit (Roth GmbH, Karlsruhe, Deutschland) hergestellt und rekombinante Klone anschließend mit dem DYEnamic Direct cycle sequencing kit (Amersham Life Science Inc., Little Chalfont, UK) in einem Model 4200 DNA Sequencer (LI-COR, Lincoln, USA) sequenziert. Beide DNA-Stränge wurden doppelt mit den IRDye™800 markierten Primern M13F und M13R (Messing, 1983) sequenziert. Anschließend wurde die übersetzte Aminosäuresequenz mit dem Programm BLAST (http://www.ncbi.nlm.nih.gov/blast) mit ähnlichen Sequenzen in der GenBank Datenbank verglichen (Altschul et al., 1997).

Phylogentische Analyse von Stamm T3

PCR Amplifikation und Sequenzierung des 16S rRNA Gens von Stamm T3 wurde gemäß den Angaben bei Brinkhoff und Muyzer (1997) durchgeführt und die erhaltene Sequenz anschließend anhand einer BLAST Analyse mit Sequenzen aus der GenBank Datenbank verglichen (Altschul et al., 1997). Die 16S rRNA Gensequenz von Stamm T3 ist bei GenBank hinterlegt (Acc. No. AY177713).

Ergebnisse und Diskussion

Bei der Untersuchung von 101 Isolaten konnte lediglich aus Stamm T3 mit der Primerkombination Hal2F- Hal4R und Hal1F- Hal4R ein 540 bp beziehungsweise 640 bp langes Fragment amplifiziert werden. Die Sequenzierung des längern Produktes und der Vergleich der gewonnenen Aminosäuresequenz ergab 31 % Übereinstimmung mit einer Tryptophanhalogenase aus *Pseudomonas chlororaphis* (Acc. No. AAD46360), die in die Pyrrolnitrin-Biosynthese involviert ist (Hammer et al., 1999). Höhere Ähnlichkeit (49 %) besteht zu annotierten Genen aus *Caulobacter crescentus* CB15 (NP_421601; NP_421604), deren genaue Funktion aber noch nicht nachgewiesen werden konnte. Die Sequenz aus T3 ist

somit dem Tryptophan-Halogenase-Gen PrnA ähnlich, zumal auch keine signifikante Sequenzähnlichkeit (GenBank Expect-Value < 0,0005) zu PrnC oder homologen Genen wie Chl (aus *S. aureofaciens*) (Hammer et al., 1997) gefunden wurde.

PrnA ist genau wie PrnC substratspezifisch und synthetisiert den ersten Schritt in der Pyoluterin-Biosynthese von Tryptophan zu 7-Chlorotryptophan. Sowohl PrnA als auch PrnC enthalten eine hochkonservierte FAD Bindestelle mit dem Aminosäuremotiv GxGxxG. Ein direkter Nachweis, dass das in dieser Arbeit sequenzierte Fragment ebenfalls ein solches Motiv enthält war nicht möglich, da die Stelle aufgrund der Sequenzhomologie als Primermotiv für Hal2F gewählt wurde. Die erfolgreiche Amplifikation eines Genfragmentes von erwarteter Länge, mit diesem Primer ist allerdings ein deutlicher Hinweis auf das Vorhandensein eines solchen funktionellen Genmotivs. Die meisten bis jetzt entdeckten $FADH_2$-abhängigen Halogenasen sind in die Halogenierung von Indol-, Phenol- oder Pyrrolringen involviert, wie z.B. Pyrrolnitrin (Hammer et al., 1997), Chloroeremomycin (van Wageningen et al., 1998), Balhimycin (Pelzer et al., 1999), Pyoluterin (Nowak-Thompson et al., 1999) oder Rebeccamycin (Sanchez et al., 2002; Onaka et al., 2003), aber jüngere Veröffentlichung berichten über die Chlorinierung von aliphatischen Kohlenstoff Verbindungen (Otsuka et al., 2004). Obwohl damit die zentrale Rolle dieser Enzymklasse für biologische Chlorierungs-Reaktionen offensichtlich wird, macht es gleichzeitig eine Vorhersage über die wahrscheinliche enzymatische Reaktion unmöglich.

Ein phylogenetischer Vergleich der 16S rRNA Sequenz von T3 weist mit 98 % Ähnlichkeit *Erythrobacter citreus* (Acc. No. AF118020) als nächsten validierten Verwandten aus. Die Tatsache, dass Stamm T3 aus dem Wattenmeer isoliert wurde und mit marinen Medien wächst steht in einem sinnvollen Zusammenhang mit der phylogenetischen Einordnung, da die Gattung *Erythrobacter* bisher ausschließlich marine Vertreter umfasst. Andere Tryptophan-Halogenasen, die eine Sequenzähnlichkeit mit PrnA aufwiesen, wurden bei alpha-*Proteobacteria* bisher lediglich im Rahmen zweier Genom Vollsequenzierung von *Erythrobacter litoralis* HTCC2594 (Acc. No. NZ_AAGG01000008) und *Caulobacter crescentus* (Acc. No. AE005774) annotiert. Über die in situ Funktion von Halogenasen ist wenig bekannt, zumal nach unserem Wissen erst an drei Halogenasen die entsprechende Enzym-Aktivität *in vitro* nachgewiesen werden konnte (Keller et al., 2000; Wynands & van Pee, 2004). Halogenierte Verbindungen weisen sehr häufig eine antibiotische Aktivität auf (Burd & van Pee, 2003) und Halogenverbindungen aus marinen Habitaten besitzen meist eine Verteidigungsfunktion (Gribble et al, 1999). Die vermeintliche Rolle vieler bakterieller Halometabolite, durch Inhibierung von Kontrahenten dem Produzenten einen Vorteil zu

verschaffen, ist allerdings fraglich, da nur äußerst geringe Mengen produziert werden (van Pee, 1996). Da der Stamm T3 in Hemmtest keine antagonistische Wirkung zeigte (Ergebnisse nicht gezeigt), sondern vielmehr zu denjenigen Isolaten gehört die von Bakterien aus dem gleichen Habitat besonders häufig gehemmt wurden, bleibt nach wie vor unklar welche biologische Funktion das gefundene Gen besitzt.

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Volatiles Released by a *Streptomyces* sp. Isolated from the North Sea

Journal of Natural Products, in press

Volatiles Released by a *Streptomyces* sp. Isolated from the North Sea

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The North Sea *Streptomyces* strain GWS-BW-H5 was investigated by analyzing headspace extracts of agar-plate cultures (HE) or liquid cultures (LCE), obtained with a closed-loop stripping apparatus (CLSA), by GC-MS. The volatile profile of the HE is dominated by the known volatiles (–)-geosmin (**4**) and 2-methyisoborneol (**1**). Small amounts of sesquiterpens occur, which are present in a more diverse structural variety and higher quantities in the LCE. The different structures can be rationalized by few cationic intermediates along their biosynthetic pathway. The most prominent difference between the two culture methods were the presence of eight Me-branched γ- and δ-lactones, not previously reported from nature, in the LCE. Major components were 10-methyldodecan-5-olide (**34**), 10-methyldodec-2-en-4-olide (**36**), and 10-methyldodec-3-en-4-olide (**38**). The structures of all eight lactones were verified by synthesis. Furthermore, more volatiles in higher amounts were produced by the liquid culture compared to the agar plate cultures. Since **36** showed inhibitory growth effects against strain GWS-BW-H5, growth inhibition against twelve other strains isolated from the same habitat was tested. Antagonistic activity against four of the strains was observed with a slightly higher threshold level than found for penicillin G that was used in control experiments.

Introduction. – Streptomycetes are well known for their potential to produce a large variety of secondary natural products. Most of the studies focussing on the metabolic potential of these bacteria are dealing with metabolites of medium or high polarity, which are of considerable interest because of their pharmacological properties. Much less effort has been devoted to the analysis of volatiles in *Streptomyces* and other bacteria, despite their often obvious smell and the potential that these compounds might act as chemical signals in bacterial ecology.

In recent studies we investigated the volatile profile emitted by different microorganisms as the myxobacteria *Chondromyces crocatus* [1] [2], *Myxococcus xanthus* [3], and *Stigmatella aurantiaca* [4], arctic *Flavobacteriaceae* [5], and marine bacteria from the *Roseobacter* clade [6] by the use of the CLSA technique (closed- loop stripping apparatus) coupled with GC-MS investigations. This technique allows the investigation of small cultures of bacteria grown on agar plates. In the present study we identified the volatiles released from a marine *Streptomyces* sp., strain GWS-BW-H5, isolated from the North Sea and compared the results with analyses of liquid cultures with special attention to differences in the headspace profile obtained by the two methods. Furthermore, the numerous sesquiterpenes can be traced back to few intermediates, and the presence of novel γ- and δ-lactones previously unknown from nature is reported.

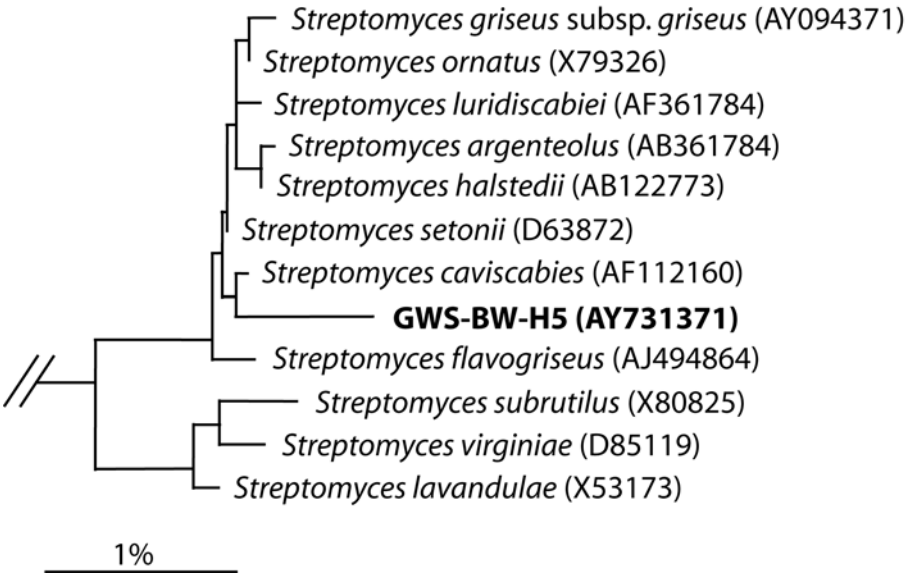


Figure 1. *Maximum likelihood tree based on 16S rRNA gene sequences showing the affiliation of isolate GWS-BW-H5 (boldface) within the genus* Streptomyces. Scale bar indicates 1% sequence divergence.

Results. – Phylogenetic analysis revealed that strain GWS-BW-H5 affiliates with the genus *Streptomyces* (*Figure 1*), with *Streptomyces caviscabies* as closest described relative (99% sequence similarity).

Biomass production of strain GWS-BW-H5 varied for the four tested NaCl concentrations. Most biomass was obtained using 0% NaCl (1.4 mg ml^{-1} dry weight), while values for 1 and 2% NaCl were slightly lower (1.2 and 1.1 mg ml^{-1} dry weight, respectively). Lowest biomass production was observed using 4.5% NaCl (0.7 mg ml^{-1} dry weight). These results indicate that strain GWS-BW-H5 prefers salinities lower than usually found in marine systems, however, the strain still shows good growth at twofold sea salt concentration.

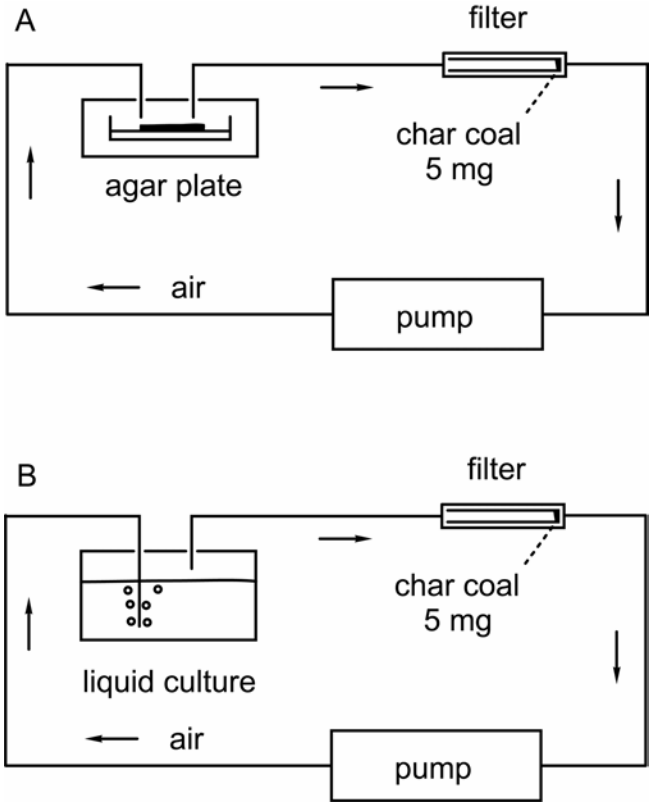


Figure 2. *Closed-loop stripping methods for volatile collection from bacteria grown on agar plates* (A) *and in liquid culture* (B).

Streptomyces strain GWS-BW-H5 was grown on agar plates or as liquid culture. Both cultures were analyzed using the closed-loop stripping technique as shown in *Figure 2*. Agar plate extracts were obtained as described previously [1] [3], while culture extracts (LCE) were

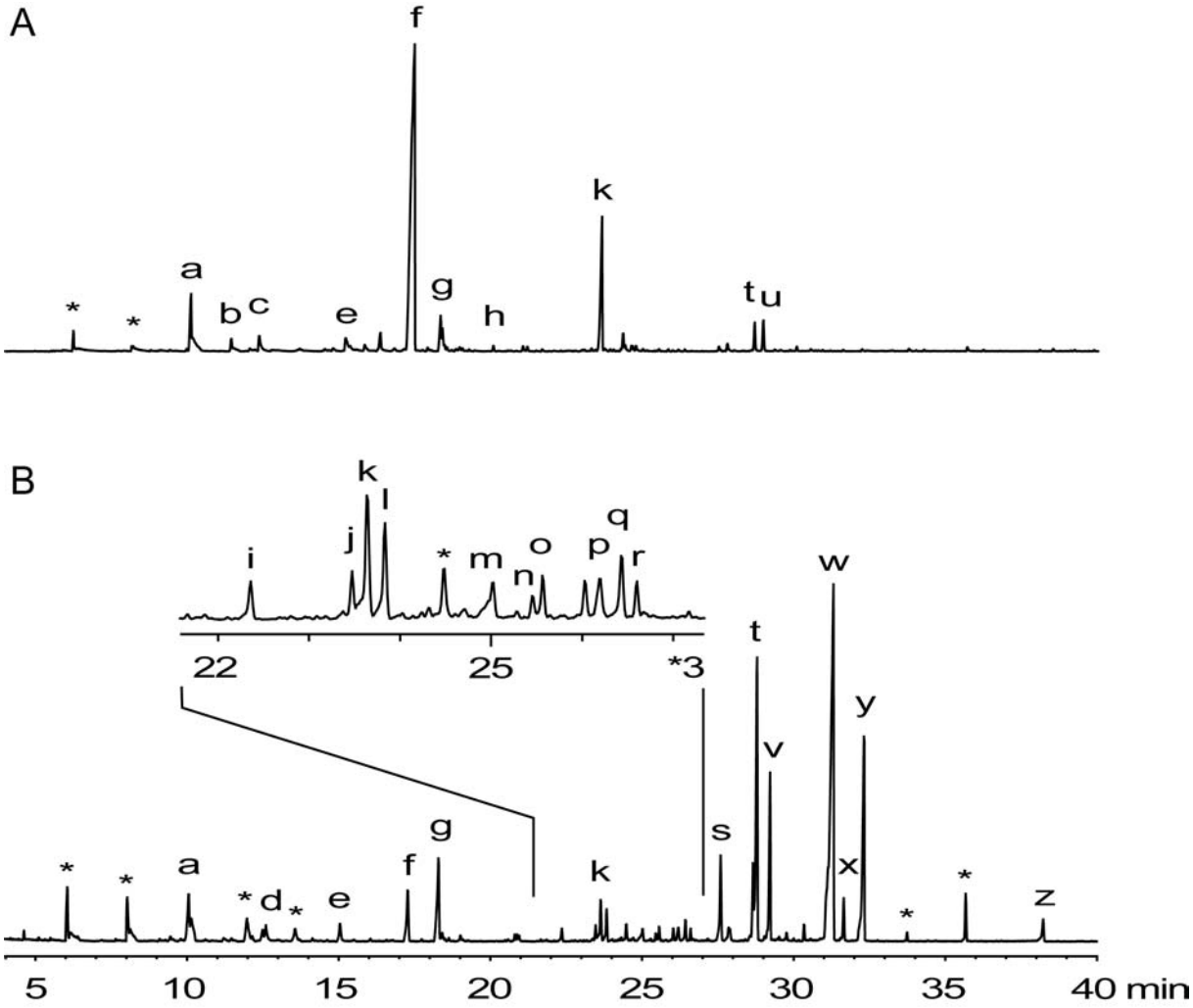


Figure 3. *Total ion chromatograms of volatiles collected from Streptomyces sp. grown on agar plates* (A) *and in liquid culture* (B). Letters refer to compounds in *Table 1*. Artifacts are indicated by asterisks.

obtained by bubbling the circulating air through the aqueous culture. Both extracts were analyzed by GC-MS. Surprisingly, we got two different results from the same strain which can be noticed at a first glance on the total ion chromatograms depicted in *Figure 3*. The identified compounds are summarized in *Table 1*. The identification of the volatiles present in the extracts was based on comparison of mass spectra and retention index data using mass spectra libraries (Wiley 7, Essential Oils) or synthetic standards.

Several terpenoids were identified in the HE as well as the LCE. The main components emitted by the bacteria grown on agar plates were 2-methylisoborneol (**1**) and (-)-geosmin (**4**). The absolute configuration of **4** was elucidated by GC on a chiral

Table 1. *Volatile Compounds Identified in one Liquid Culture Extract and three Headspace Extracts Obtained from Agar Plate Cultures of* Streptomyces *sp. Strain GWS-BW-H5.*

GC[a])	Compound[b])	**I**	*I* [lit]	1.[g])	2.[g])	3.[g])	4.[g])
	dimethyl disulfide	n. d.		xx	xx	xx	x
	ethyl 2-methylpropionate	n. d.			x	xx	
	3-methylbut-3-en-1-ol	n. d.			x	x	
	3-methylbut-2-en-1-ol	804				x	
	butyl acetate	829	812[c])	x			
	methylpyrazine	843	826[c])	x			
	hexan-1-ol	882	867[c])	x			
	heptan-2-one	905	889[c])	x			
	2-acetylfuran	928	910[c])	x	x		x
	4-methylhexan-1-ol	955		x			
	6-methylheptan-2-one	965		x			
	5-methylheptan-2-one	974		x			
a	dimethyl trisulfide	981	983[d])	xx	xx	xx	
	6-methylhept-5-en-2-one	997	985[c])	x			
b	2-methyl-2-bornene (**3**)	1021		x	x	x	x
c	heptane-2,5-dione	1041		x	x	x	x
d	benzyl alcohol	1052	1051[d])	x			
	(*E*)-4,8-dimethylnona-1,3,7-triene (**24**)	1084	1079[e])	x			
	2-phenylpropan-2-ol	1102		x			
	linalool	1109	1098[c])	x	x	x	x
e	2-phenylethanol	1129	1125[d])	x	x	x	x
	1-phenylpropan-2-one	1144		x			
	methyl methylthiomethyl disulfide	1146				x	
	benzyl cyanide	1163		x			
	2-methoxy-3-(1-methylpropyl)pyrazine	1173				x	x
	benzyl acetate	1178	1163[c])	x			
f	2-methylisoborneol (**1**)	1201		xx	xxx	xxx	xxx
	isothujone	1227	1114[c])	x			
g	dimethyl tetrasulfide	1235	1234[d])	xx	xx	x	x
	benzothiazole	1248	1246[d])	x			
	geraniol	1261	1255[c])	x			
	2-phenylethyl acetate	1269	1256[c])	x			
h	(iso)bornyl acetate (**2a** or **2b**)	1296	1285[c])		x	x	x
	S-methyl thiobenzoate	1316		x			
	2-aminoacetophenone	1325	1322[d])	x			

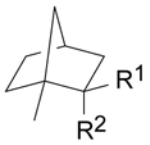
	4,8-dimethylnona-3,7-dien-2-ol	1329		x			
	4-methylquinazoline	1363		x			
i	α-copaene (**20**)	1381	1379[e])	x	x	x	x
	4-methylquinoline	1398	1399[d])	x			
	isolongifolene (**23**)	1402	1393[e])			x	x
	geranyl acetone	1409		x			
	α-gurjunene (**8**)	1413	1413[e])	x			
	kelsoene (**10**)	1420	1416[e])	x			
j	citronellyl acetone	1424		x			
k	(–)-geosmin (**4**)	1431	1430[d])	x	xxx	xx	xx
	bourbon-11-ene (**9**)	1432	1424[e])	x			
l	β-gurjunene (calarene, **7**)	1439	1437[e])	x	x	x	x
	butyl phenylacetate	1446		x			
	eudesma-5,11-diene (**6**)	1454	1444[e])	x			
	cadina-3,5-diene (**22**)	1457	1448[e])	x			
	sesquiterpene (B: 91, M:204)	1462			x	x	x
	γ-muurolene (**15**)	1480	1474[e])	x			
m	dimethyl pentasulfide	1483		x	x	x	
	ε-cadinene (**11**)	1487	1483[e])	x	x	x	
	β-muurolene (**17**)	1493		x	x	x	
n	bicyclosesquiphellandrene (**19**)	1495	1487[e])	x	x	x	x
o	α-muurolene (**16**)	1507	1496[e])	x	x	x	x
	guaioxide	1516			x	x	x
	δ-cadinene (**12**)	1522	1520[e])	x	x	x	x
	zonarene (**18**)	1532	1521[e])	x	x	x	x
p	*cis*-calamenene (**14**)	1533	1521[c])	x	x	x	x
q	cadina-1,4-diene (**13**)	1542	1532[c])	x	x	x	x
r	10-methylundec-3-en-4-olide (**37**)	1550		x			
	11-methyl-2-tridecanone	1573		x			
s	unknown (B: 111, M: 222)	1591		xx	x	x	x
	sesquiterpene alcohol (B: 207, M: 222)	1601		x	x	x	x
	10-methylundec-2-en-4-olide (**35**)	1638		x			
t	1-*epi*-cubenol (**21**)	1643	1623[f])	xxx	x	x	x
u	(1(10)*E*,5*E*)-germacradien-11-ol (**5**)	1654	1655[d])	x	x	x	x
	benzophenone	1655		x			
	10-methylundecan-4-olide (**31**)	1659		x			
v	10-methyldodec-3-en-4-olide (**38**)	1663		xx			
	10-methylundecan-5-olide (**33**)	1687		x			

	dodecan-4-olide	1698	x
w	10-methyldodec-2-en-4-olide (**36**)	1758	xxx
x	10-methyldodecan-4-olide (**32**)	1773	x
y	10-methyldodecan-5-olide (**34**)	1804	xxx
z	cyclooctasulphur	2115	x

[a]) Marker in TIC (*Figure 2*). [b]) Artifacts are not mentioned. [c]) Retention index literature data from reference [11]. [d]) Retention index literature data from reference [3]. [e]) Retention index literature data from reference [67]. [f]) Retention index literature data from reference [68]. [g]) Different samples of *Streptomyces* sp.: 1. LCE, 2.–4. HE. x: 0-2%, xx: 2-8%, xxx: >8% of total area in GC.

cyclodextrin stationary phase connected to a MS detector and comparison of the retention time of natural **4** with those of an enantiomerically enriched sample of **4**. Both **1** and **4** have been shown to be sesquiterpene degradation products [7], and the biosynthesis of **4** is to date controversially discussed in the literature [8–10]. The sesquiterpene alcohol (1(10)*E*,5*E*)-germacradien-11-ol (**5**), also produced in minor amounts, is proposed to be an intermediate en route to **4** [9] [10]. We showed recently that the biosynthetic pathway to **4** that operates in myxobacteria [10] is different from that in the liverwort *Fossombronia pusilla* [8]. The elimination product of **1**, 2-methyl-2-bornene (**3**), might be an artifact and is present in only trace amounts. Structurally related isobornyl acetate (**2a**) was also found in traces, but its identification is tentative, because the epimeric bornyl acetate (**2b**) has a very similar mass spectrum and the retention indices given in the literature are I = 1285 for both isomers [11]. The LCE yielded significantly lower amounts of these volatiles, and **2a/b** was not found.

In addition, a diverse array of sesquiterpenes was identified, which could be classified according to their biogenetic origin. Biosynthetic considerations show that most of the sesquiterpenes produced by this strain are closely related to each other, and therefore the accuracy of the analyses is further corroborated by these reflections. Farnesyl pyrophosphate (**25**), the common precursor for all sesquiterpenes, can be cyclized to the germacradienyl cation (**A**), a central intermediate in sesquiterpene biosynthesis (*Scheme 1*). This process might include the isomerization to the corresponding tertiary allylic nerolidyl pyrophosphate as has been shown for the biosynthesis of other sesquiterpenes [14]. The cationic species **A** is the proposed branching point for the three main pathways to all sesquiterpenes (with few exceptions, see below) emitted by the streptomycete. The first branch is commenced by the attack of water to **A** furnishing the sesquiterpene alcohol hedycaryol (**26**), or the direct loss of



1 (R^1=OH, R^2=Me)
2a (R^1=OAc, R^2=H)
2b (R^1=H, R^2=OAc)

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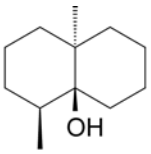

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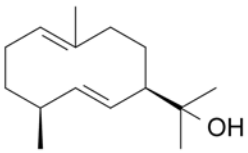

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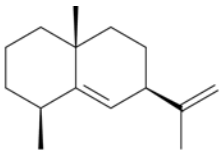

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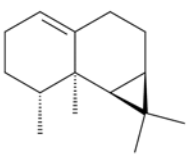

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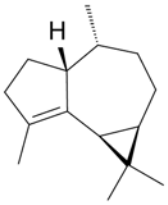

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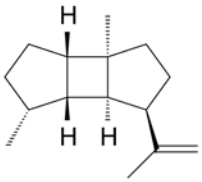

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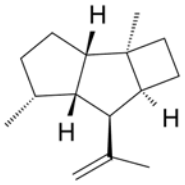

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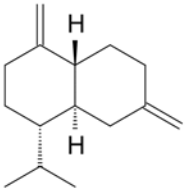

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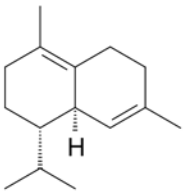

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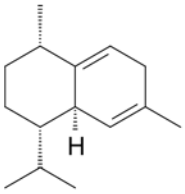

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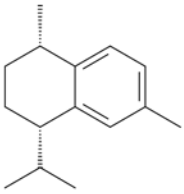

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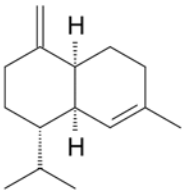

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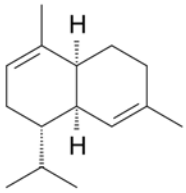

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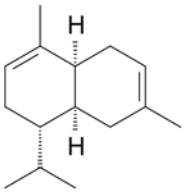

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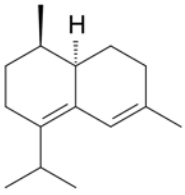

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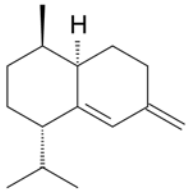

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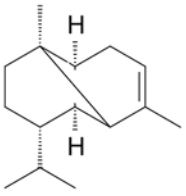

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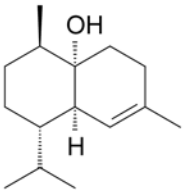

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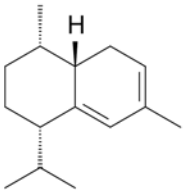

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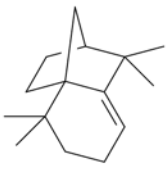

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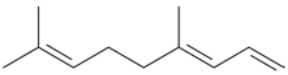

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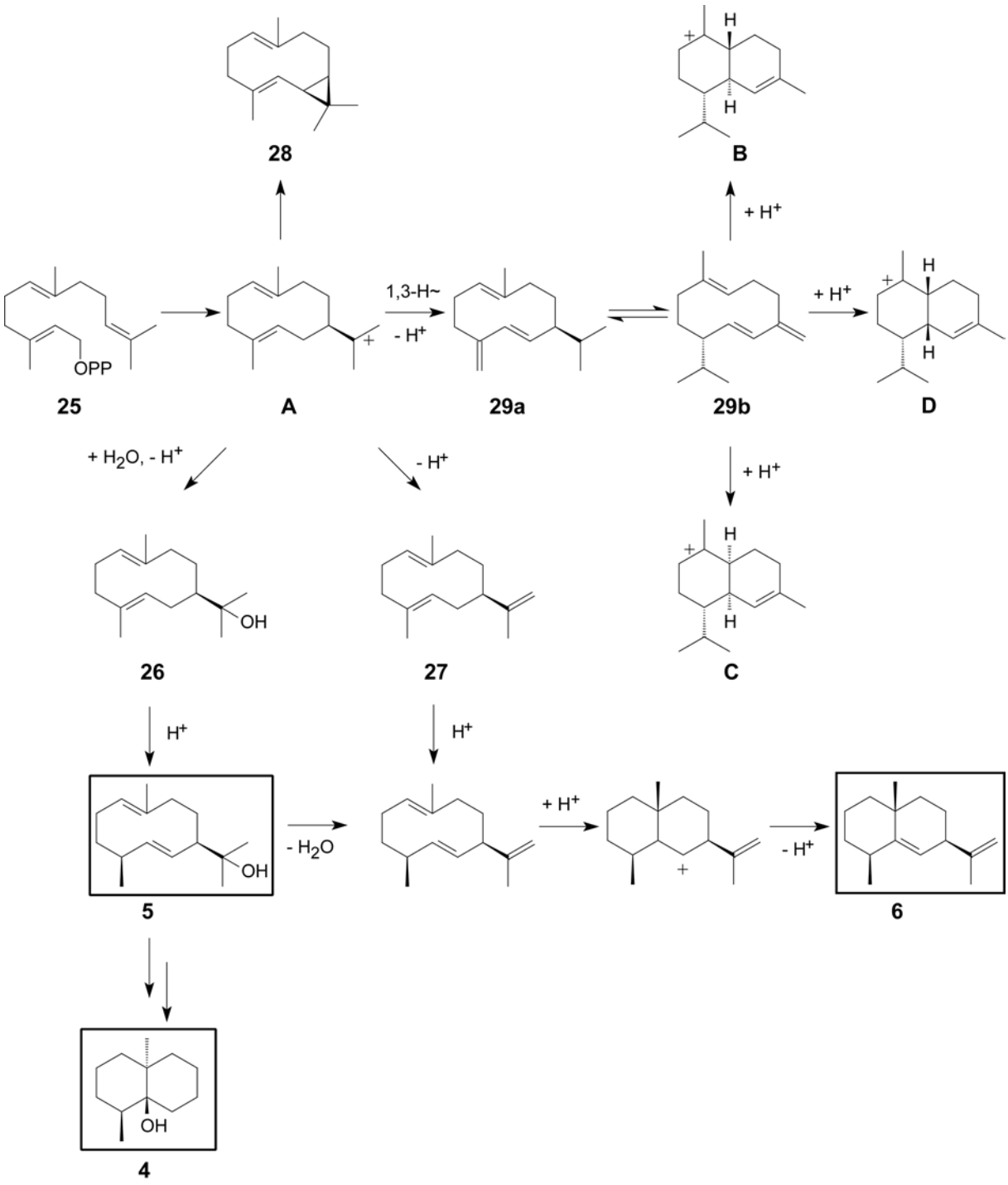
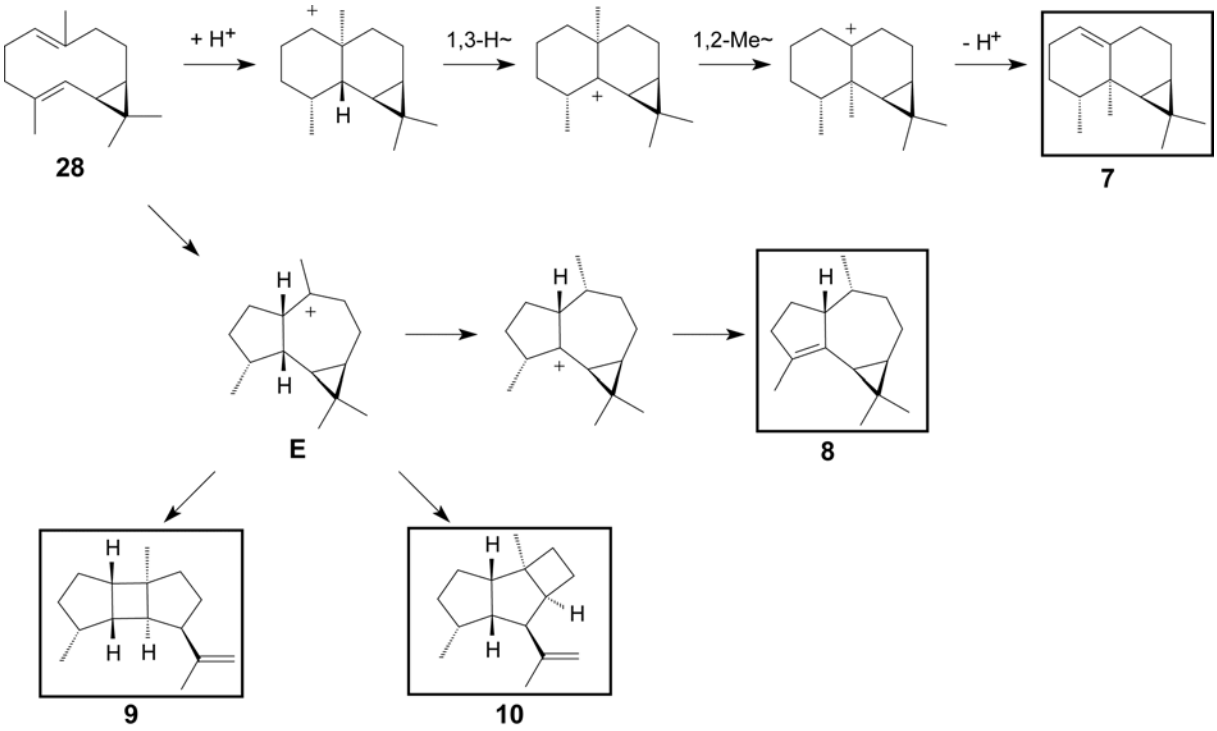


Scheme 1. *First Biosynthetic Steps Proposed to be Involved in the Biosynthesis of Sesquiterpenoid Compounds Emitted by* Streptomyces *sp.*

Compounds present in the extracts are shown in boxes.

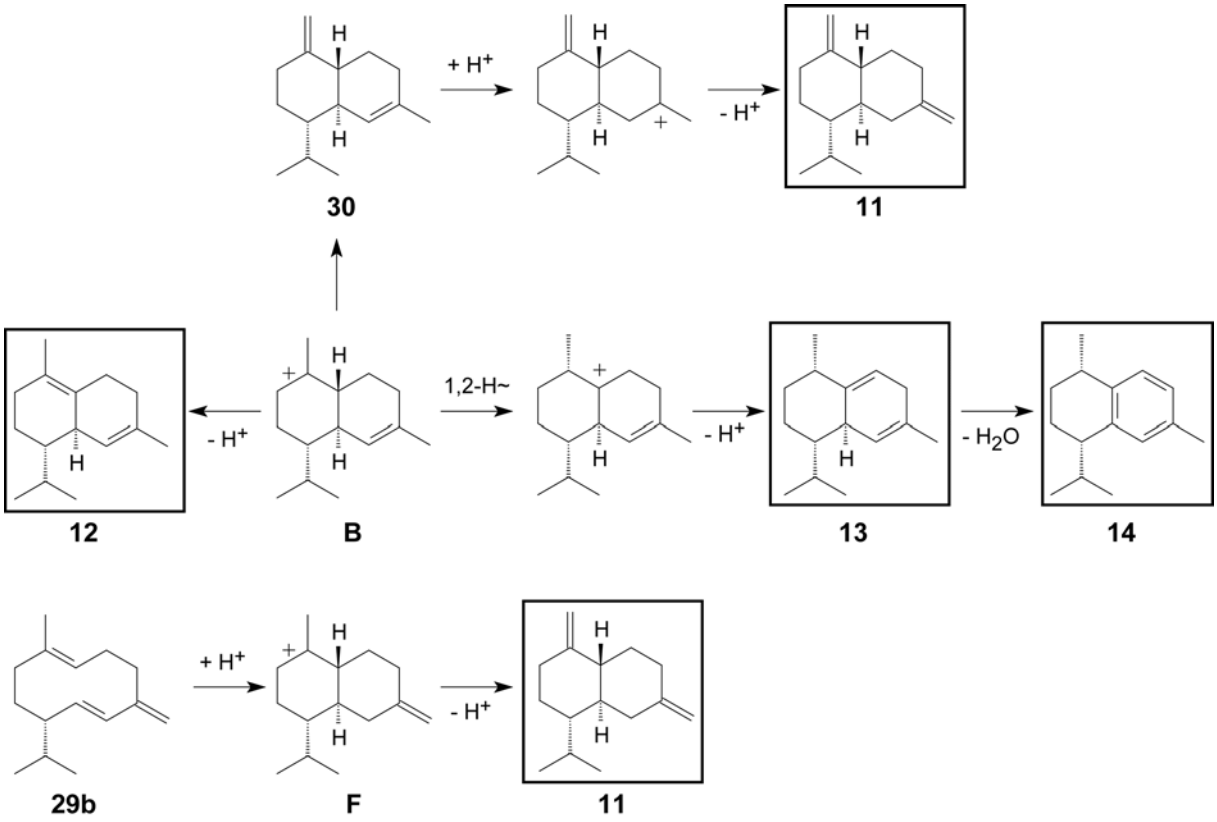
one proton generating germacrene A (**27**). Bicyclogermacrene (**28**) arises from **A** by the loss of one proton under formation of the cyclopropane moiety [12]. Furthermore, a hydride migration and subsequent deprotonation leads to germacrene D in a *cisoid* conformation (**29a**), whereas the formation of the cadinenyl (**B**), muurolenyl (**C**), or amorphenyl (**D**) cations requires a *transoid* germacrene D (**29b**) [13]. More details are given in two excellent reviews [12] [14]. The isomerization of **26** furnishes **5**, and **4** was suggested to be generated either directly from **26** [8] or from **5** [9] [10] (*Scheme 1*). In this proposed biosynthetic scheme the formation of eudesma-5,11-diene (**6**) that was found in the LCE in trace amounts, can be rationalized from **A** by the loss of a proton to give **27**, a proton-mediated isomerization, and subsequent ring closure. A similar cyclization from **5** and elimination of water seems also possible.



Scheme 2. *Proposed Biosynthetic Steps to Gurjunenes, Bourbon-11-ene* (**9**)*, and Kelsoene* (**10**). Compounds present in the extracts are shown in boxes.

Whereas **28** was not found in the extracts, four metabolites derived hereof were emitted by the bacteria (*Scheme 2*). The first, β-gurjunene (**7**), can arise by a proton mediated

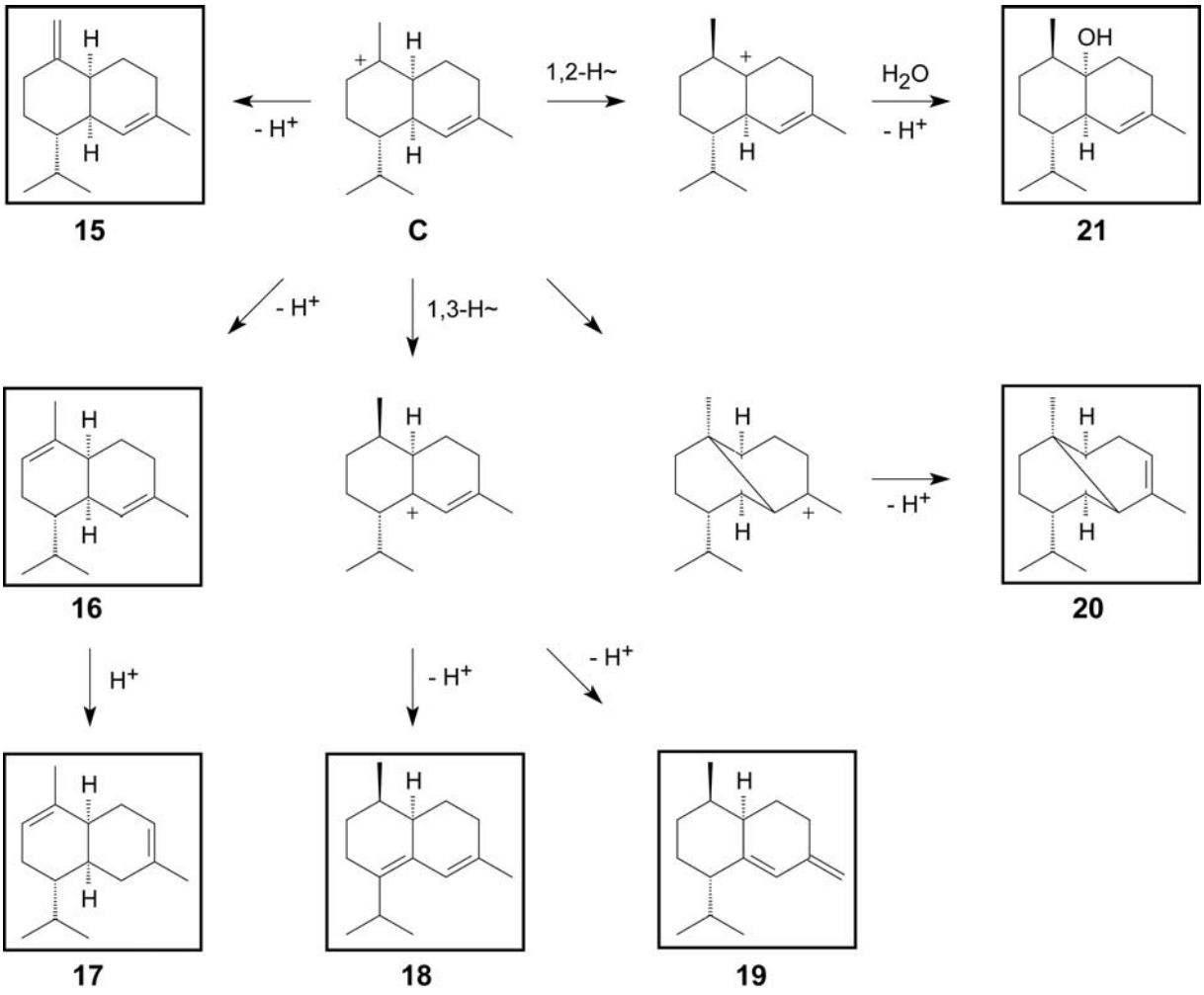
C2-*re*-C7-*re* ring closure, a 1,3-hydride shift, followed by a 1,2-methyl migration, and a final loss of a proton. A C2-*re*-C6-*re* closure leads to the *allo*-aromadendranyl cation (**E**) on the metabolic route to α-gurjunene (**8**) formed by a suprafaciale 1,3-hydride shift and subsequent deprotonation [15]. The proton loss of one of the Me groups attached to the cyclopropyl ring associated with a ring opening/ring closure process furnishes either bourbon-11-ene (**9**) or kelsoene (also called tritomarene, **10**). This biosynthetic pathway to **10** is used by the liverwort *Ptychantus striatus* [15] [16]. Whereas all compounds **7**–**10** occurred in the liquid culture extracts, only **7** was identified in the HE obtained from petri dishes.



Scheme 3. *Proposed Biosynthetic Steps to the Cadinane Type Sesquiterpenes.*
Compounds present in the extracts are shown in boxes.

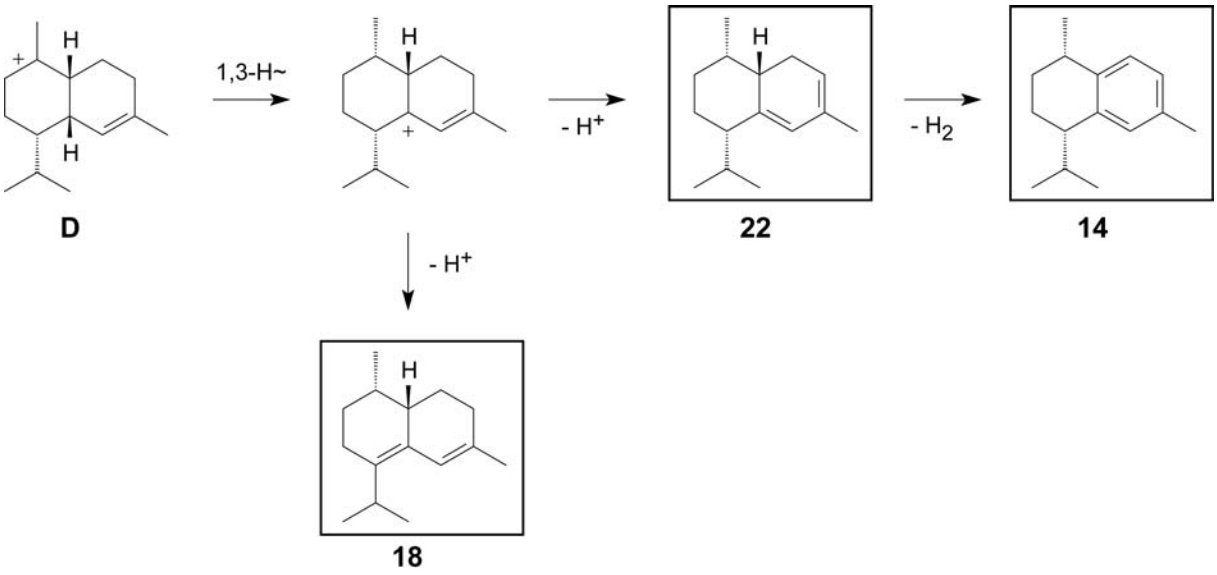
The cadinenyl cation (**B**) arises from **29b** by a proton mediated C1-*re*-C6-*si* closure and is the precursor for the cadinane type sesquiterpenes with *trans*-decaline structure [13] (*Scheme 3*). Deprotonation of the Me group leads to γ-cadinene (**30**), but this compound is not

emitted, whereas the isomer ε-cadinene (**11**) is produced. One possibility for its biosynthesis is the proton catalyzed isomerization. Alternatively, a respective bicyclic cationic intermediate (**F**) with a retained *exo*-methylene group may be formed from **29b**, that would directly give **11** by deprotonation. Furthermore, **B** furnishes δ-cadinene (**12**) by deprotonation, whereas the biosynthesis of cadina-1,4-diene (**13**) first requires a 1,2-hydride shift. The aromatized sesquiterpene hydrocarbon *cis*-calamenene (**14**) may be generated from **13** either enzymatically or by spontaneous oxidation. These cadinane sesquiterpenes are present in the LCE and in the HE.



Scheme 4. *Proposed Biosynthetic Steps to the Muurolane Type Sesquiterpenes.*
Compounds present in the extracts are shown in boxes.

Cyclization of **29b** by a C1-*re*-C6-*re* attack provides the muurolenyl cation (**C**) with *cis*-decaline structure [13]. The muurolanes identified in the headspace extracts are represented by γ-muurolene (**15**) and α-muurolene (**16**). They can directly be formed by deprotonation of **C**. The isomerization of **16** furnishes β-muurolene (**17**). Zonarene (**18**) and bicyclosesquiphellandrene (**19**) can be generated by a 1,3-hydride shift and loss of one proton (*Scheme 4*). A second cyclization step from **C** and deprotonation leads to α-copaene (**20**), and a 1,2-hydride migration with subsequent attack of water to the sterically less hindered side results in 1-*epi*-cubenol (**21**). Two key steps in the biosynthesis of **21**, the 1,3-hydride shift in **A** (*Scheme 1*) as well as the 1,2-hydride shift in **C** (*Scheme 4*), have been established in biosynthetic studies using cell free extracts of *Streptomyces* sp. [17] [18]. Whereas **15** was only present in the LCE, **16**–**21** were also identified in the HE, but **21** was obtained from the liquid culture in significantly higher yields.



Scheme 5. *Proposed Biosynthetic Steps to the Amorphane Type Sesquiterpenes.*
Compounds present in the extracts are shown in boxes.

The amorphenyl cation (**D**) is formed from **29b** by a C1-*si*-C6-*si* ring closure [13] (*Scheme 1*). As outlined in *Scheme 5*, a 1,3-suprafacial hydride shift and subsequent deprotonation generates the sesquiterpenes **18** or cadina-3,5-diene (**22**), respectively. On this pathway arises the other enantiomer of **18** compared to its formation from **C** (*Scheme 4*). It

remains to be elucidated which enantiomer occurs naturally. Oxidation of **22** leads to the same enantiomer of **14** as furnished by the aromatization of **13** (*Scheme 3*). Only the LCE contained trace amounts of **22**.

The only sesquiterpens that do not fit in these biosynthetic schemes are isolongifolene (**23**) and guaioxide, only present in the HE as trace components. Some sesquiterpenes could not be identified from their mass spectra and retention times.

Furthermore, a series of sulphur compounds represented by dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide, and dimethyl pentasulfide in decreasing amounts was present in the extracts. These volatiles have been emitted by both culture types. In addition, the LCE contained cyclooctasulphur (S_8), whereas methyl methylthiomethyl disulfide was only present in the HE. Some additional trace compounds have been identified in the HE and the LCE. Linalool, 2-phenylethanol, and 2,5-heptanedione were found in all extracts. A large number of trace volatiles (28 compounds in total) have only been released by the streptomycetes grown in liquid cultures. Their identification is based on their mass spectra and comparison to library spectra, and (in some cases) on literature data for their retention indices. Among these compounds monoterpenes (geraniol, isothujone), homomonoterpenes ((*E*)-4,8-dimethylnona-1,3,7-triene (**24**) and related 4,8-dimethylnona-3,7-dien-2-ol), terpenoid ketones (6-methylheptan-2-one, 6-methylhept-5-en-2-one, and the higher homologues geranyl acetone and citronellyl acetone), oxygenated compounds (butyl acetate, hexan-1-ol, heptan-2-one, 4-methylhexan-1-ol, and 5-methylheptan-2-one), different aromatic compounds (benzyl alcohol, 2-phenylpropan-2-ol, 1-phenylpropan-2-one, benzyl cyanide, benzyl acetate, 2-phenylethyl acetate, *S*-methyl thiobenzoate, 2-aminoacetophenone, butyl phenylacetate, and benzophenone), and aromatic heterocycles (methylpyrazine, benzothiazole, 4-methylquinazoline, and 4-methylquinoline) were identified. Furthermore, only a few additional compounds represented by ethyl 2-methylpropionate, 3-methylbut-3-en-1-ol, 3-methylbut-2-en-1-ol, and 2-methoxy-3-(1-methylpropyl)pyrazine not present in the LCE have been found in the HE.

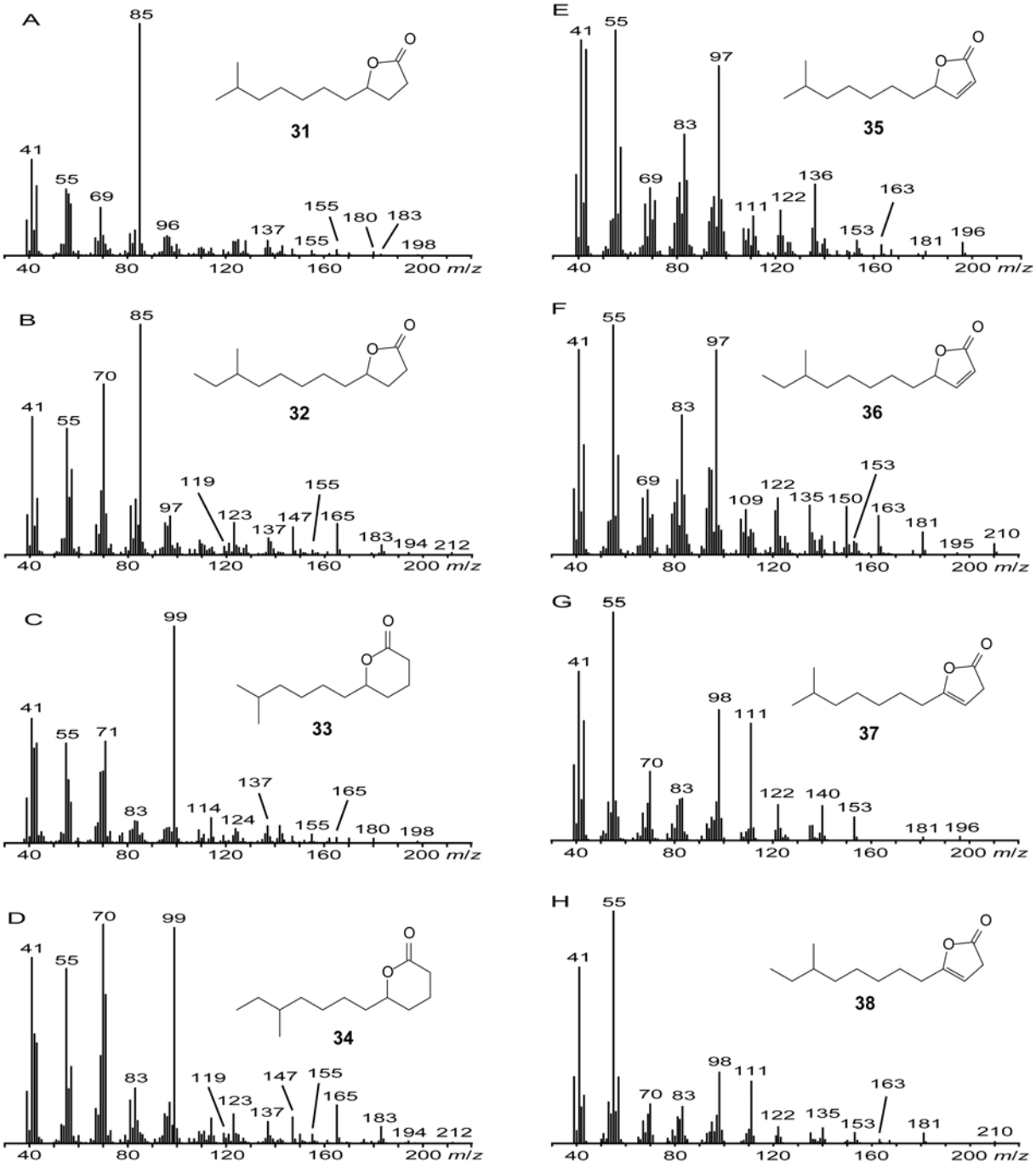


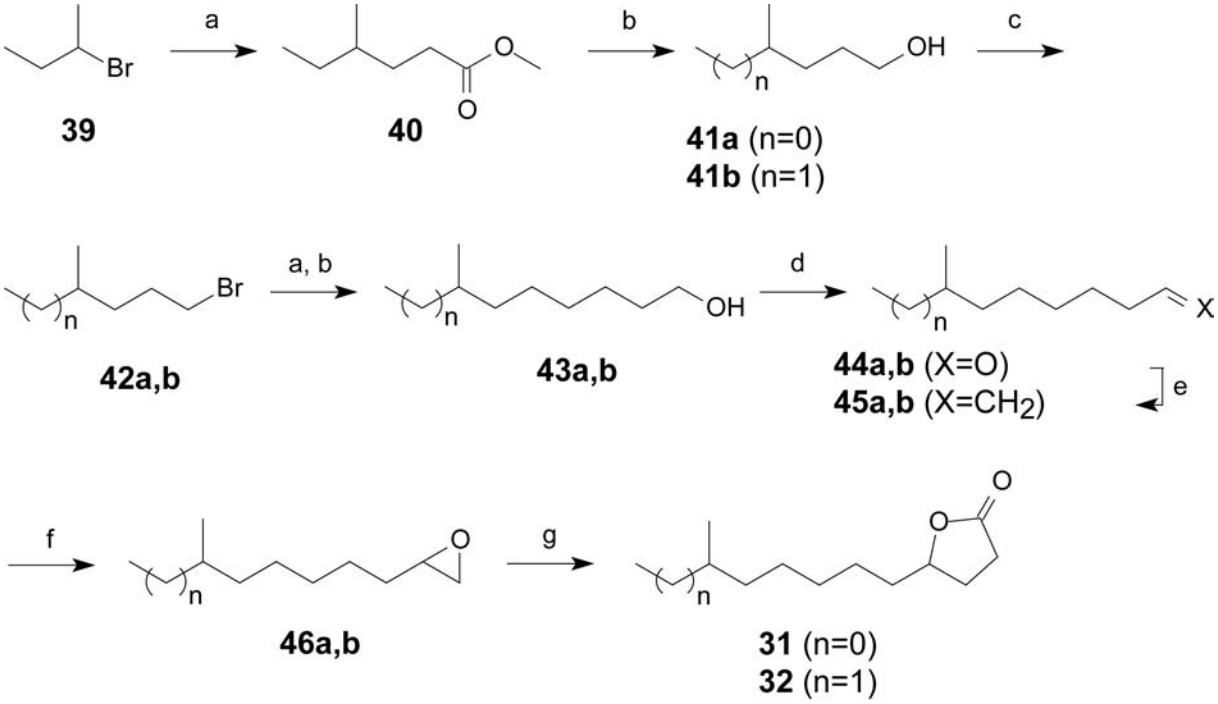
Figure 4. *Structures and mass spectra of new lactones present in the liquid culture extracts of* Streptomyces *sp.*

The most intriguing difference between the bacteria grown on agar plates and in liquid culture was the occurrence of some late eluting compounds only present in the LCE. Although some of these volatiles belonged to the main components obtained from the liquid

culture, they were totally absent in the HE. One minor compound was readily identified by comparison with a synthetic standard as dodecan-4-olide. The other volatiles were previously unknown, and their structures and mass spectra are depicted in *Figure 4*. Structural proposals for these compounds were derived from their mass spectra and GC retention indices and verified by comparison with synthetic compounds. Two of the volatiles showed a mass spectrum very similar to that of dodecan-4-olide dominated by *m*/*z* = 85 as is expected for alkan-4-olides. The first volatile (*Figure 4A*) had the same molecular mass as dodecan-4-olide (*m*/*z* = 198) and eluted with slightly shorter retention time (*I* = 1659 vs. 1698). Therefore it was a branched compound. In addition, the mass spectrum showed small fragment ions at *m*/*z* = 180 ($[M - H_2O]^+$), 155 ($[M - C_3H_7]^+$), and 137 ($[M - H_2O - C_3H_7]^+$), respectively. The loss of a Me group was indicated by fragment ions at *m*/*z* = 183 ($[M - CH_3]^+$) and 165 ($[M - H_2O - CH_3]^+$), whereas no fragment ions showing the loss of an Et group were present. This fragmentation pattern is typical for ω–1 methyl branched lactones [19]. A second alkan-4-olide showed a molecular ion at *m*/*z* = 212 and was thus a higher homologue with an additional methylene group (*Figure 4B*). This compound was also branched (*I* = 1773), and fragment ions at *m*/*z* = 194 ($[M - H_2O]^+$), 183 ($[M - C_2H_5]^+$), 165 ($[M - H_2O - C_2H_5]^+$), 155 ($[M - C_4H_9]^+$), and 137 ($[M - H_2O - C_4H_9]^+$) indicated a ω–2 Me branch. Fragment ions arising from the double loss of water at *m*/*z* = 147 ($[M - 2H_2O - C_2H_5]^+$) and 119 ($[M - 2H_2O - C_4H_9]^+$) further corroborated the structure of a ω–2 Me-branched lactone [19]. Conclusively, the unknown alkan-4-olides were 10-methylundecan-4-olide **31** and 10-methyldodecan-4-olide **32**.

These two compounds were synthesized according to *Scheme 6*. In the first step a copper-catalyzed 1,4-addition of the Grignard reagent obtained from *sec*-butyl bromide **39** to methyl acrylate furnished the methyl ester **40** [20]. Standard $LiAlH_4$ reduction and bromination with triphenylphosphane and bromine gave the respective alkyl bromide **42b**. Accordingly, **42a** was generated from the starting compound **41a**. The 1,4-addition and reduction procedure was repeated with both alkyl bromides **42a,b** to obtain the elongated alcohols **43a,b**. Subsequent PCC oxidation and Wittig olefination yielded the alkenes **45a,b** that were transformed into the epoxides **46a,b** using *m*-CPBA. The epoxides were reacted with the anion of diethyl malonate, and the intermediate alkoxide spontanously cyclized to the ester lactone. Subsequent addition of $MgCl_2$ to the reaction mixture and heating to 160°C directly gave the lactones **31** and **32** by deethoxycarbonylation [21], but the last step

proceeded with significantly lower yields than noted in the literature. Comparison of the MS data and retention indices confirmed the identity of the natural alkan-4-olides.

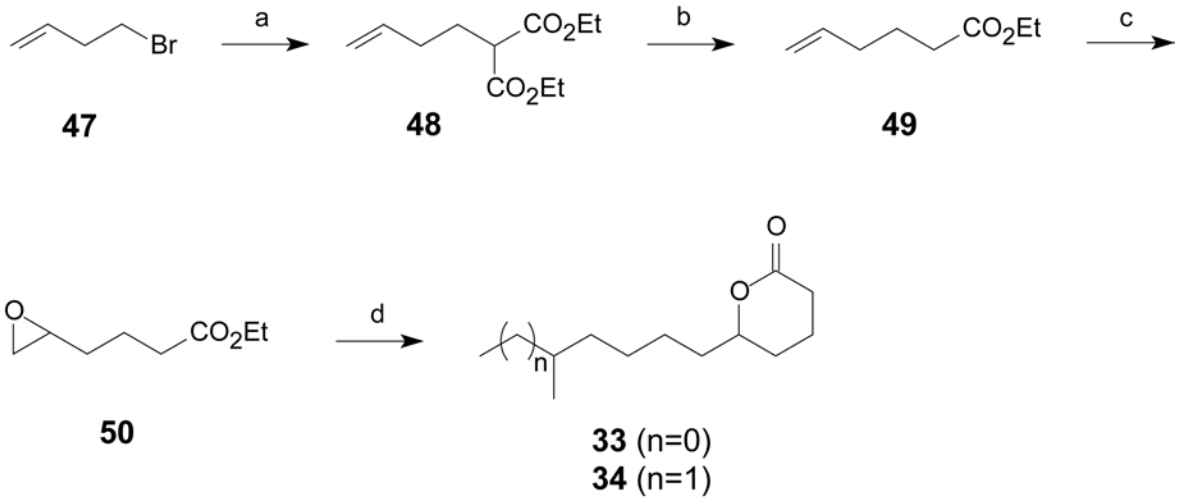


Scheme 6. *Synthesis of γ-Lactones.*

a) 1. Mg, THF, 2. CuBr, Me_2S, DMAP, TMSCl, methyl acrylate; 55–73%. b) $LiAlH_4$, THF; 89–97%. c) PPh_3, Br_2, CH_2Cl_2; 82–85%. d) PCC, CH_2Cl_2; 65–85%. e) $MePPh_3Br$, BuLi; 54–61%. f) *m*-CPBA, CH_2Cl_2; 84–94%. g) Diethyl malonate, NaH, $AcNMe_2$, then **46a,b**; 16–17%.

In addition, two compounds showing a base peak at *m*/*z* = 99 characteristic for alkan-5-olides with the same molecular ions as **31** and **32**, respectively, were emitted by the bacteria (for mass spectra see *Figures 4C* and *D*). They showed the same fragmentation pattern in the high mass region as the alkan-4-olides, and therefore these volatiles were suggested to be 10-methylundecan-5-olide **33** and 10-methyldodecan-5-olide **34**. The designated structures were verified by a synthesis starting from 1-bromobut-3-ene **47** (*Scheme 7*). Alkylation of diethyl malonate and subsequent deethyoxycarbonylation [22] [23] leads to ethyl hex-5-enoate **49**. Its epoxide **50** was obtained by *m*-CPBA oxidation, and a copper-catalyzed ring opening reaction applying the alkylmagnesium bromides generated from **42a,b** gave the respective alkoxides that spontanously furnished the alkan-5-olides **33** and **34** by cyclization, albeit in low yields.

These synthetic compounds confirmed the identity of the natural volatiles emitted by *Streptomyces*.

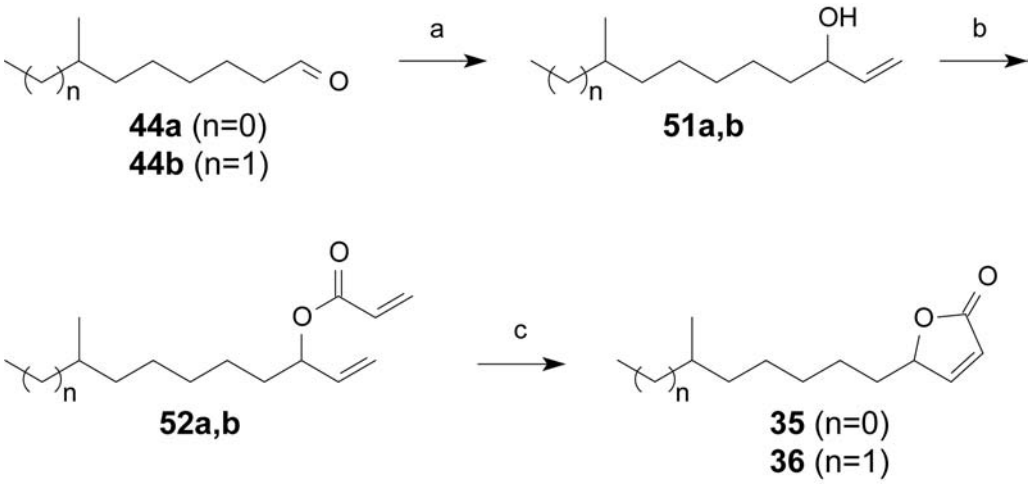


Scheme 7. *Synthesis of* δ*-Lactones.*

a) Diethyl malonate, NaH, DME, then **47**, Bu_4NI; 64%. b) NaCl, DMSO, H_2O; 82%. c) *m*-CPBA, CH_2Cl_2; 97%. d) 1. **42a,b**, Mg, THF, 2. CuBr, Me_2S; 12–16%.

The LCE contained two additional unknown compounds, each shortly eluting before **31** (*I* = 1638) or **32** (*I* = 1758) that exhibited mass spectra with the fragment ions *m*/*z* = 83 and 97 (*Figures 4E* and *F*). The molecular ions were found at *m*/*z* = 196 and 210. In conclusion, these compounds are unsaturated counterparts of **31** and **32**. The strong formation of a fragment ion at *m*/*z* = 83 is explainable by a double bond in the lactone ring (*α*-fragmentation, see *m*/*z* = 85 for saturated **31** and **32**), whereas *m*/*z* = 97 might be formed by cleavage between C-5 and C-6. Two other unknown compounds eluting earlier (*I* = 1550 and 1663) showed mass spectra also containing the fragment ion *m*/*z* = 83 and molecular ions at *m*/*z* = 196 and 210 (*Figures 4G* and *H*). These compounds seemed also to be unsaturated counterparts of **31** and **32** bearing a unsaturated lactone ring. In addition, strong formation of fragment ions at *m*/*z* = 98 and 111 was observed. These ions can arise by a McLafferty rearrangement with bond breaking between C-5 and C-6, and by cleavage between C-6 and C-7, respectively. In the higher mass region two of these compounds (*Figures 4E* and *G*) showed the loss of a neutral CH_3 and C_3H_7 fragment (*m*/*z* = 181 and 153, respectively), whereas the other two

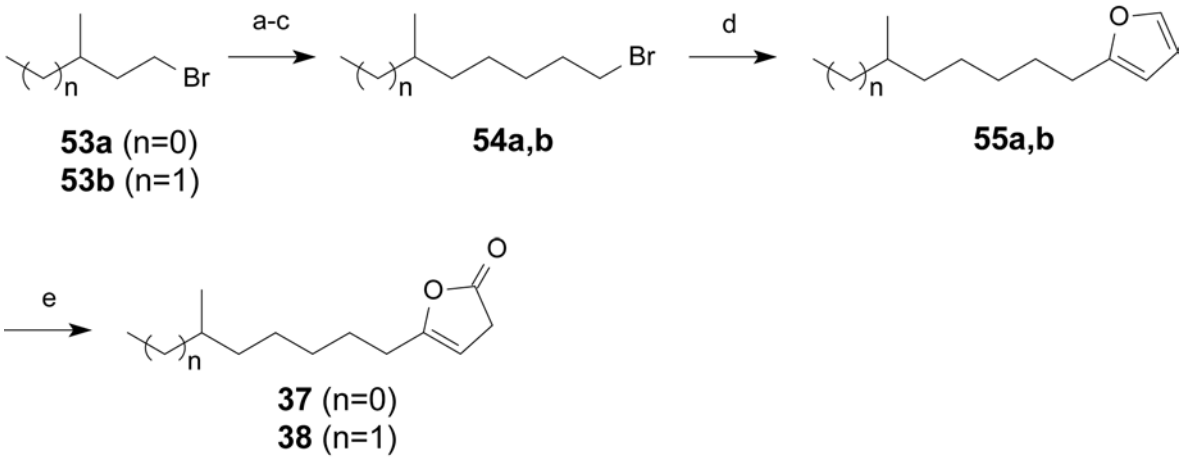
volatiles (*Figures 4F* and *H*) were characterized by the loss of C_2H_5 and subsequent loss of water (*m*/*z* = 181 → 163), and the loss of C_4H_9 and subsequent loss of water (*m*/*z* = 153 → 135). These data corroborated the presence of Me branches in ω–1 and ω–2 position, respectively. Consequently, the unknown compounds were 10-methylundec-2-en-4-olide (**35**), 10-methyldodec-2-en-4-olide (**36**), and the isomeric 10-methylundec-3-en-4-olide (**37**), and 10-methyldodec-2-en-4-olide (**38**). The double bond in the lactone ring could be responsible for the preferred bond breaking in the homoallylic position. Thus, the α,β-unsaturated lactones **35** and **36** would form a major fragment ion at *m*/*z* = 97, whereas the β,γ-unsaturated lactones **37** and **38** might similarly furnish the fragment ion *m*/*z* = 111.



Scheme 8. *Synthesis of* α,β-*Unsaturated* γ-*Lactones.*

a) Vinylmagnesium bromide, THF; 69–81%. b) Acryloyl chloride, NEt_3; 61–76%. c) Grubbs catalyst (2nd generation), toluene; 80–83%.

A synthesis of **35** and **36** was carried out starting with the aldehydes **44a,b** (*Scheme 8*). Treatment with vinylmagnesium bromide gave the allyl alcohols **51a,b**. Reaction with acryloyl chloride in triethylamine furnished the acrylates **52a,b** which were subsequently cyclyzed by a ring closing metathesis (RCM) with Grubb´s catalyst of the second generation yielding **35** and **36** in high yields, using a procedure developed by Marco et al. [24]. The synthetic compounds **35** and **36** both proved to be identical to the bacterial volatiles.



Scheme 9. *Synthesis of* β,γ*-Unsaturated* γ*-Lactones.*

a) 1. Mg, THF, 2. CuBr, Me_2S, DMAP, TMSCl, methyl acrylate; 84–85%. b) $LiAlH_4$, THF; 87–95%. c) PPh_3, Br_2, CH_2Cl_2; 88–95%. d) Furan, BuLi, THF, then **54a,b**; 87–99%. e) 1. BuLi, Et_2O, 2. $B(OMe)_3$, BCl_3, 3. *m*-CPBA, K_2CO_3; 61–63%.

The lactones **37** and **38** were synthesized starting with the alkyl bromides **53a,b** (*Scheme 9*). Copper-catalyzed 1,4-addition to methyl acrylate, standard reduction, and bromination yielded the elongated alkyl bromides **54a,b**. Alkylation of furan furnished the precursors **55a,b**. The *β,γ*-unsaturated *γ*-lactones were then obtained in a one-pot-procedure by Pelter and Rowlands [25]. The furans **55a,b** were lithiated with BuLi and the resulting solution was added to $ClB(OMe)_2$. Oxidative workup of the obtained dimethylboronates led to the desired lactones **37** and **38** that confirmed the identity of the lactones present in the LCE.

With the exception of **37** all lactones contained at least one stereogenic center. However, the absolute configuration of these volatiles remained unknown and is subject of further investigations.

Knowing the structures of several lactones and having the synthetic compounds in hand some investigations regarding their ecological function were carried out. Strain GWS-BW-H5 was able to grow and form aerial mycelium on all media tested, but the differentiation was much slower and less distinct on yeast-malt-agar (YMA). However, no stimulating effect by addition of the lactone compounds on the aerial mycelium differentiation could be observed (data not shown). Nevertheless, in the experiment with compound **36**

growth inhibition of strain GWS-BW-H5 was observed. Tests for antagonistic activity carried out with twelve target strains isolated from the same habitat as the streptomycete revealed that strain GWS-BW-H5 was able to inhibit growth of four of the test strains (*Table 2*, *Exp. 1*). Growth of four target strains, three affiliated with *Actinobacteria* and one affiliated with *Flavobacteria*, was inhibited. In order to check if the production of bioactive compounds in strain GWS-BW-H5 is stimulated by the γ-lactones (**31**–**38**), liquid cultures were supplemented with the compounds at concentrations of 1 mM (*Table 2*, *Exp. 2*). Growth of the same target strains as in the experiment with the culture broth only was inhibited, and the observed growth inhibition against all four strains was in the same range.

The results of the agar diffusion assays with the different γ-lactones revealed no inhibitory effect of any lactone tested with amounts of 2 μg (1 mM) against the target-organisms (data not shown). Since it was observed that compound **36** inhibited growth of strain GWS-BW-H5 in the mycelium induction assay, amounts of 2.5, 5.0, 12.5, and 25.0 μg (1.25, 2.5, 6.0, and 12 mM) of this compound were tested against all target strains (*Table 2*, *Exp. 3*). Hexane (10 μl) and penicillin G (3.6 μg, 1 mM) were tested as controls. Compound **36** inhibited growth of one target strain (T15) when applied in an amount of 2.5 μg (1.25 mM). With increased amounts (5 μg, 2.5 mM) growth of two target strains (T15 and TN) was inhibited, while amounts of 25 μg (12 mM) showed inhibitory effects against four target strains.

Discussion. – *Streptomyces* strain GWS-BW-H5 was isolated from a marine habitat and grew well with a marine medium and thus could be defined as marine, like other authors proposed [26] [27]. Growth experiments with different salinities demonstrated an increased osmotolerance, but slightly enhanced growth with salinities lower than normal sea salt concentrations. Salt tolerance up to 7% NaCl is a common phenomenon among *Streptomyces* species [28], proving the impossibility to distinguish clearly between marine and terrestrial origin on this particular feature.

The profile of volatiles released by *Streptomyces* sp. grown on agar plates and in liquid culture was investigated. Both types of cultures yielded a large number of compounds, but the results differed. The LCE contained significantly more volatiles. Whereas **1** was the main component present in the HE, the LCE contained mainly **36**. All experiments yielded a

Table 2. *Phylogenetic Affilations of Bacterial Isolates Used for Agar Diffusion Assays and Results of Inhibition Tests.*

Target strain	Phylogenetic group (class)	Closest described relative[a]) (accession no.)	16S sRNA similarity (%)	Activity / mm[b]), [c])						
				Exp. 1	Exp. 2	Exp. 3				
				GWS-BW-H5 culture broth	GWS-BW-H5 culture broth + lactone[d])	**36** 2.5 µg 1.25 mM	**36** 5 µg 2.5 mM	**36** 12.5 µg 6.0 mM	**36** 25 µg 12 mM	Penicillin G 3.6 µg 1 mM
T3	α-Proteobacteria	*Erythrobacter citreus* (AF118020)	98	–	–	–	–	–	–	14[e])
TK	α-Proteobacteria	*Mesorhizobium tianshanense* (AF041447)	97	–	–	–	–	–	9	32[e]) [f])
TL	α-Proteobacteria	*Thalassobacter stenotrophicus* (AJ631302)	95	–	–	–	–	–	–	76 [f])
T1	γ-Proteobacteria	*Shewanella colwelliana* (AY653177)	98	–	–	–	–	–	–	–
T8	γ-Proteobacteria	*Pseudoalteromonas tetraodonis* (AF214730)	99	–	–	–	–	–	–	–
T16	γ-Proteobacteria	*Vibrio pacinii* (AJ316194)	97	–	–	–	–	–	–	12[e])
BIA	Flavobacteria	*Algibacter lectus* (AY187689)	93	–	–	–	–	–	–	40
T15	Flavobacteria	*Tenacibaculum mesophilum* (AB032504)	93	16	12-16	11	15	>30	>40	30 [f])
TN	Flavobacteria	*Zobellia russellii* (AB121976)	91	–	–	–	13	16	24	–
T2	Actinobacteria	= *Aeromicrobium marinum* (AY166703)	–	14	12-22	–	–	–	8	46
T4	Actinobacteria	*Pseudonocardia alni* (Y08535)	99	8	8-10	–	–	–	–	14[e])
H145	Actinobacteria	*Microbacterium phyllosphaerae* (AJ277840)	99	10	8-10	–	–	–	–	–

[a]) By BLAST analysis (http://www.ncbi.nlm.nih.gov/blast). Only validly published organisms were taken into account. [b]) Effective diameter of the inhibition zone. [c]) –: No growth inhibition. [d]) GWS-BW-H5 liquid culture supplemented with compounds **31**–**38** (1 mM). Values give the range of the obtained results of the experiments using the eight different lactones. [e]) No clear inhibition, but areas with reduced growth were observed. [f]) Data from reference [67].

large number of sesquiterpenes, but the LCE comprised some additional sesquiterpenes in trace amounts. 2-Methylisoborneol (**1**) and (–)-geosmin (**4**) were more intensively produced on agar plates compared to the liquid culture. This might reflect the slower differentiation in liquid media, since previous studies revealed a striking coincidence between the secretion of terpenoids and the presence of aerial mycelium and spores [7] [29].

The most interesting substance class present in the LCE were the lactones **31**–**38** representing more than 50% of the volatiles (based on peak areas in the total ion chromatogram). In contrast, the lactones were totally absent in the HE. A common biosynthetic pathway to the lactones is advised by their structural similarities. Whereas **31**, **33**, **35**, and **37** are ω–1 Me-branched, the homologues **32**, **34**, **36**, and **38** with one additional carbon are ω–2 Me-branched. This pattern is explainable by a biosynthesis from valine and isoleucine, respectively. The leucine-analogues (e. g. 11-methyldodecan-4-olide) were not found. To the best of our knowledge all lactones **31**–**38** identified in *Streptomyces* strain GWS-BW-H5 have not been reported from nature before.

Production of structurally more complex butyrolactones as diffusible signaling molecules that control secondary metabolism and/or morphological differentiation is widespread among actinomycetes [30]. In some cases the butyrolactones directly posses antibiotic activity [31–33], but more often they are involved in quorum sensing signaling systems. The so called A-factor triggers streptomycin production and sporulation in *Streptomyces griseus*. Other examples are the regulation of virginiamycin production in *Streptomyces virginiae* [34], or tylosin production in *Streptomyces fradiae* [35]. The phylogenetic analysis revealed that almost all *Streptomyces* species related to GWS-BW-H5 are known to produce bioactive compounds with antibacterial or antifungal activities. No effects of the lactones **31**–**38** on colony differentiation could be observed. Strain GWS-BW-H5 showed antibacterial activity against four out of twelve tested target strains. When cultured with the lactones (1 mM), growth inhibition against the same four strains and in the same range was observed, indicating that the lactones **31**–**38** do not stimulate the production of antibiotics in strain GWS-BW-H5. Previous studies revealed effective butyrolactone doses even smaller as used in this study [36–38].

Compound **36** showed growth inhibition against strain GWS-BW-H5 itself. Therefore this compound was used in growth inhibition tests against twelve target strains isolated from the same habitat as *Streptomyces* sp. Since different species were affected in these

experiments than in the assay with the culture broth of strain GWS-BW-H5, the antagonistic effects of the liquid culture broth of GWS-BW-H5 cannot be caused by **36** only, but a participation of **36** in a mixture of compounds cannot be ruled out.

The strong biosynthetic relationship between most of the sesquiterpenes present in the extracts gives rise to the suspicion, that only one or a few enzymes catalyze the formation of these volatiles. The ability of terpene cyclases to generate a diverse array of products has been well documented in several studies, e. g. on the germacrene C synthase in *Lycopersicon esculentum* [39] or the δ-selinene synthase and γ-humulene synthase in *Abies grandis* [40]. The production of a high number of different terpenes using a minimal number of genes and enzymes seems to be favourable for all these organisms. It has been shown in studies on monoterpene cyclases that monoterpene products with related stereochemistry are generated via common cationic intermediates on the same catalytic site of the same cyclase [41] [42]. The absolute configuration of **4** emitted by *Streptomyces* has been determined by GC on a chiral cyclodextrin stationary phase (only the (–)-enantiomer was found), and the shown absolute configuration of all sesquiterpenes (**5**–**23**) is related to that of **4**, as can be deduced from the biosynthetic pathways depicted in *Schemes 1–5*. Whether these suggested absolute configurations are right and the sesquiterpenes occur only in a single enantiomeric form remains to be elucidated.

Some of the terpenes identified in the extracts are widespread in nature, whereas others have only rarely been found. Only a few reports deal with the occurrence of terpenes in extracts of bacterial cultures. Most work has been carried out on terpenes from plant sources. Nevertheless, this is not the case for **4** that was first isolated from *Streptomyces griseus* [43–45]. In addition, **4** has been identified from the myxobacteria *Nannocystis exedens* [46], *Myxococcus xanthus* [3], *Chondromyces crocatus* [1], and *Stigmatella aurantiaca* [4].

The unusual sesquiterpene **10** was first isolated from the marine sponge *Cymbastela hooperi* and co-occurs in this species with **9**, γ-gurjunene and *epi*-γ-gurjunene [47]. The co-occurrence of **9** and **10** has been reported from a number of liverwort species as *Tritomaria quinquedentata* [48], *Calypogeia muelleriana* [49], as well as *Mylia taylorii* and *Mylia nuda* [50], but both sesquiterpenes have never been identified in bacteria. Furthermore, **7** is produced by *Streptomyces citreus* [51].

The isoprenoids **12**, **13**, and **14** are widespread especially in the plant kingdom, and **13** was also identified in headspace extracts of the myxobacterium *Chondromyces crocatus* [1].

In contrast, **11** has only rarely been found in nature, and this might be due to the fact, that **11** can not arise directly from **B** by a hydride shift and/or deprotonation as **12** and **13**, but requires an isomerization step with intermediate formation of a second cationic species. The second possibility, cyclization to **F** requires an unusual sesquiterpene cyclase activity. One of the few examples for the occurrence of **11** is the essential oil of the ylang-ylang tree *Cananga odorata* [52].

The sesquiterpene hydrocarbons **15** and **20** are widespread natural compounds, but have never been identified in bacteria, whereas **16** and **18** are also present in *Chondromyces crocatus* [1]. Zonarene (**18**) occurs only rarely in nature and was first isolated from the brown seaweed *Dictyopteris zonarioides* [53]. The sesquiterpene alcohol **21** and its epimer cubenol are present in cubeb oil [54], and have recently been identified in hydrodistillates from the liverwort *Bazzania japonica* [55]. Furthermore, **21** was isolated from different species of the *Streptomyces* genus [56]. Whereas cubeb oil contains the (–)-enantiomer, the isolate from *Streptomyces* was shown to be the (+)-enantiomer.

The degraded sesquiterpene **24** occurs in many plants and plays a role in tritrophic interactions. It is induced often after herbivore attack [57] [58] and used by some parasitoids of the herbivores to locate them [59]. To the best of our knowledge it has not been previously reported to occur in microorganisms.

In summary, the liquid culture produced more volatiles than the agar plate culture. This may be related to the higher numbers of cells obtained during liquid culture, but the additional occurrence of the lactones also might point to more fundamental differences between the two culture types.

Experimental Part

General methods. Chemicals were purchased from Fluka (Buchs, Switzerland) or Sigma-Aldrich (Steinheim, Germany). Solvents were purified by distillation and dried according to standard methods. The reaction progress was monitored by thin layer chromatography that was carried out using 0.2 mm pre-coated plastic sheets Polygram Sil G/UV_{254} (Marcherey-Nagel). Compounds were detected by the use of a molybdatophosphoric acid solution (10% in ethanol) and heating with a heat gun or by UV (254 nm). Column chromatography (CC) was carried out using Merck Kieselgel 60. Solvent mixtures are given in volume ratios. ^{1}H-NMR spectra were obtained on a Bruker AMX400 (400 MHz) spectrometer. ^{13}C-NMR spectra were recorded using a Bruker AMX400 (100 MHz). Chemical shifts are given in ppm relative to TMS as an internal standard. Coupling constants *J* are given in Hz as *J*(H,H) coupling constants.

Isolation of strain GWS-BW-H5. Strain GWS-BW-H5 was obtained from a most probable number (MPN) dilution series [60] with artificial seawater as medium [61], modified as described by Stevens [62], and chitin (1 g l^{-1}) as substrate. Bulk water (1 ml) sampled from the German Wadden Sea (53° 42' 20" N, 07° 43' 11" E, southern North Sea), was used as inoculum for 10-fold dilution series. Growth was checked by turbidity and microscopically. Portions of the liquid culture (20 µl) were transferred to agar plates with the same medium and streaked out to single colonies. Colonies were transferred five times until considered as pure. The isolate was checked for purity by denaturing gradient electrophoresis (DGE) of PCR-amplified 16S rRNA gene fragments as described by Teske et al. [63]. Maintenance of the pure culture was carried out with marine broth 2216 (denoted as MB 2216, Difco, Germany).

Cultivation in liquid culture for chemical analysis was carried out in 250 ml shake flasks containing the culture medium MB 2216 (50 ml). The culture was incubated for 48 h at 20°C while shaken at 100 rpm.

Phylogenetic analysis. PCR amplification and sequencing of the 16S rRNA gene of strain GWS-BW-H5 were performed according to the methods described by Brinkhoff and Muyzer [64]. The sequence of strain GWS-BW-H5 was compared to similar sequences of reference organisms by BLAST search [65] (http://www.ncbi.nlm.nih.gov/blast). The sequence obtained from strain GWS-BW-H5 is available from GenBank under accession no. AY731371. A phylogenetic tree was constructed using the maximum-likelihood method included in the ARB software package [66] (http://www.arb-home.de).

Growth experiments. The osmotollerance with different salt concentrations of strain GWS-BW-H5 was determined as follows. Liquid MB 2216 medium was modified with NaCl concentrations of 0, 1, 2 and 4.5% to adjust salinity values between 11.7 and 56.7 g l^{-1}. The experiments were carried out with 5 parallels in 100 ml shake flasks at 140 rpm, 20°C and a culture volume of 20 ml. The cultures were inoculated with 1 ml of a well grown MB 2216 preculture and harvested after 17 h. Since strain GWS-BW-H5 forms aggregates in liquid media, growth observation by measuring optical density was not possible. Instead we used dried 0.2 µm membrane filters (ME24, Schleicher & Schuell) to separate the cell flocks from the media supernatant. The cell dry weight of the complete culture was determined after 18 h incubation of the filters at 105°C.

Screening for inhibitory effects. Agar diffusion assays were performed to test whether strain GWS-BW-H5 or the γ-lactones exhibit antagonistic activities. Twelve different bacterial isolates also obtained from the German Wadden Sea and belonging to four different phylogenetic classes were used as target strains (*Table 2*) and grown in 5 ml of liquid MB 2216 medium to OD_{600} ~ 0.6. In case of strains T4 and T15 an OD_{600} of only ~ 0.07 was obtained because of weaker growth. Culture broth (100 µl) of the target organisms was spread on MB 2216 agar plates. Agar diffusion assays were performed as described by Brinkhoff et al. [67].

Strain GWS-BW-H5 was grown in 100 ml shake flasks with 20 ml MB 2216 medium for 60 h at 20°C. Subsequently 10 µl of the culture broth were tested against the target strains (*Table 2, Exp. 1*).

Liquid cultures of strain GWS-BW-H5 grown in MB 2216 medium for 48 h at 20°C were supplemented with the compounds **31**–**38** at concentrations of 1 mM culture broth (*Table 2, Exp. 2*), comparable to those estimated in other *Streptomyces* species [36]. The cultures were incubated for 24 h and subsequently the agar diffusion assay was performed with the target strains.

To screen the γ-lactones (**31**–**38**) produced by strain GWS-BW-H5 for inhibitory effects, a solution (10 µl, 1 mM, 2 µg) of the compounds **31**–**38** dissolved in hexane was applied on sterile antibiotics assay discs which were put on the agar plates after the hexane was volatilized. Amounts of 2.5, 5.0, 12.5, and 25.0 µg (1.25, 2.5, 6.0, and 12 mM) were tested against all target strains. Hexane (10 µl) and penicillin G (1 mM, 3.6 µg) were tested as controls.

In all experiments plates were incubated for 5 days at 20°C and inspected daily for zones of inhibition. The experiments were performed in three parallels. A result was considered as positive if inhibition was observed for at least two parallels and when the diameter of the zone of inhibition was at least 2 mm greater than the diameter of the antibiotic assay disc.

Sampling of volatiles. Volatile organic compounds emitted by cell cultures of *Streptomyces* sp. grown on Petri dishes were collected using the CLSA technique [1] [3]. For sampling of volatiles from *Streptomyces* sp. grown in liquid cultures a modified CLSA with a circulating air stream bubbling through the liquid culture was used. The volatiles were adsorbed on charcoal (Chromtech, Precision Charcoal Filter, 5 mg) for 24 h, and then eluted with 30 µl of dichloromethane. The obtained solutions were analysed by GC-MS immediately and stored at –70°C.

GC-MS. GC-MS analyses were carried out on a HP 6890 Series GC System connected to a HP 5973 Mass Selective Detector (Hewlett-Packard) fitted with a BPX5 fused-silica capillary column (25 m x 0.22 mm i. d., 0.25 µm film, SGE). Conditions were as follows: inlet pressure: 77.1 kPa, He 23.3 ml min^{-1}; injection volume: 1 µl; transfer line: 300°C; electron energy: 70 eV. The GC was programmed as follows: 5 min at 50°C increasing at 5°C min^{-1} to 320°C, and operated in splitless mode (60 s valve time). The carrier gas was He at 1 ml min^{-1}. Retention indices *I* were determined from a homologous series of *n*-alkanes (C_8 – C_{25}). Identification of compounds was performed by comparison of mass spectra to the Wiley 6 Library and the Essential Oils Library (Massfinder), by comparison with synthetic standards, or retention index data from the literature [3] [11] [68] [69].

Preparation of methylesters. As described by Horiguchi et al. [20], methylesters were prepared by the CuI catalyzed 1,4-addition of suitable Grignard reagents to methyl acrylate.

Methyl 4-methylhexanoate (**40**). Yield: 55% (5.15 g, 35.8 mmol). TLC (pentane/Et_2O 10:1): R_f 0.32. GC: *I* 1000. ^{1}H-NMR ($CDCl_3$): 0.80 (*d*, *J* = 6.5, 3 H); 0.81 (*t*, *J* = 7.2, 3 H); 1.04–1.42 (*m*, 4 H); 1.56–1.65 (*m*, 1 H); 2.18–2.32 (*m*, 2 H); 3.60 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$): 11.2 (Me); 18.8 (Me); 29.1 (CH_2); 31.5 (CH_2); 31.9 (CH_2); 34.0 (CH); 51.4 (Me); 174.6 (C). EI-MS: *m*/*z* 115 (18) $[M–29]^+$, 95 (13), 87 (58), 74 (63), 55 (82), 41 (100).

Methyl 7-methyloctanoate. Physical and spectroscopic data are reported in [3].

Methyl 7-methylnonanoate. Yield: 73% (2.25 g, 12.1 mmol). TLC (pentane/Et_2O 10:1): R_f 0.63. GC: *I* 1300. ^{1}H-NMR ($CDCl_3$): 0.84 (*d*, *J* = 6.3, 3 H); 0.85 (*t*, *J* = 7.3, 3 H); 1.05–1.18 (*m*, 2 H); 1.21–1.36 (*m*, 7 H); 1.59–1.68 (*m*, 2 H); 2.30 (*t*, *J* = 7.6, 2 H); 3.67 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$): 11.4 (Me); 19.1 (Me); 25.0 (CH_2); 26.7 (CH_2); 29.4 (CH_2); 29.5 (CH_2); 34.1 (CH_2); 34.3 (CH); 36.4 (CH_2); 51.4 (Me); 174.3 (C). EI-MS: *m*/*z* 157 (31) $[M–29]^+$, 137 (10), 129 (17), 125 (14), 107 (4), 97 (28), 87 (82), 74 (100), 55 (66), 41 (62).

Methyl 6-methylheptanoate. Physical and spectroscopic data are reported in [5].

Methyl 6-methyloctanoate. Yield: 84% (8.72 g, 50.7 mmol). TLC (pentane/Et_2O 5:1): R_f 0.95. GC: *I* 1198. ^{1}H-NMR ($CDCl_3$): 0.84 (*d*, *J* = 6.2, 3 H); 0.85 (*t*, *J* = 7.4, 3 H); 1.05–1.16 (*m*, 2 H); 1.20–1.38 (*m*, 5 H); 1.49–1.59 (*m*, 2 H); 2.30 (*t*, *J* = 7.6, 2 H); 3.66 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$): 11.3 (Me); 19.1 (Me); 25.3 (CH_2); 26.6 (CH_2); 29.4 (CH_2); 34.1 (CH_2); 34.2 (CH); 36.2 (CH_2); 51.4 (Me); 174.3 (C). EI-MS: *m*/*z* 143 (4) $[M–29]^+$, 123 (5), 115 (10), 111 (9), 96 (18), 87 (45), 83 (30), 74 (69), 69 (21), 59 (46), 55 (77), 41 (100).

Preparation of alkan-1-ols. The alkan-1-ols were prepared by standard reduction with $LiAlH_4$ in dry diethyl ether or THF [3].

4-Methylhexan-1-ol (**41b**). Yield: 94% (3.40 g, 33.7 mmol). TLC (pentane/Et_2O 2:1): R_f 0.30. GC: *I* 950. ^{1}H-NMR ($CDCl_3$): 0.87 (*t*, *J* = 7.3, 3 H); 0.87 (*d*, *J* = 6.5, 3 H); 1.10–1.20 (*m*, 2 H); 1.29–1.41 (*m*, 3 H); 1.47–1.66 (*m*, 3 H); 3.63 (*t*, *J* =

6.7, 2 H). ^{13}C-NMR ($CDCl_3$): 11.3 (Me); 19.1 (Me); 29.4 (CH_2); 30.4 (CH_2); 32.5 (CH_2); 34.2 (CH); 63.4 (CH_2). EI-MS: *m*/*z* 98 (4) $[M–18]^+$, 83 (7), 70 (100), 55 (45), 41 (98).

7-Methyloctan-1-ol (**43a**). Yield: 97% (4.82 g, 33.5 mmol). TLC (pentane/Et_2O 2:1): R_f 0.32. GC: *I* 1144. ^{1}H-NMR ($CDCl_3$): 0.86 (*d*, *J* = 6.6, 6 H); 1.13–1.19 (*m*, 2 H); 1.24–1.39 (*m*, 6 H); 1.52 (*non*, *J* = 6.6, 1 H); 1.52–1.60 (*m*, 2 H); 1.87 (br. *s*, 1 H); 3.62 (*t*, *J* = 6.7, 2 H). ^{13}C-NMR ($CDCl_3$): 22.6 (2 Me); 25.8 (CH_2); 27.3 (CH_2); 27.9 (CH); 29.7 (CH_2); 32.8 (CH_2); 38.9 (CH_2); 62.9 (CH_2). EI-MS: *m*/*z* 111 (9) $[M–33]^+$, 98 (7), 83 (33), 69 (77), 56 (100), 41 (89).

7-Methylnonan-1-ol (**43b**). Yield: 89% (1.47 g, 9.30 mmol). TLC (pentane/Et_2O 3:1): R_f 0.17. GC: *I* 1254. ^{1}H-NMR ($CDCl_3$): 0.84 (*d*, *J* = 6.3, 3 H); 0.85 (*t*, *J* = 7.3, 3 H); 1.07–1.20 (*m*, 2 H); 1.24–1.39 (*m*, 9 H); 1.47 (br. *s*, 1 H); 1.53–1.60 (*m*, 2 H); 3.64 (*t*, *J* = 6.6, 2 H). ^{13}C-NMR ($CDCl_3$): 11.4 (Me); 19.2 (Me); 25.8 (CH_2); 27.0 (CH_2); 29.5 (CH_2); 29.8 (CH_2); 32.8 (CH_2); 34.4 (CH); 36.5 (CH_2); 63.1 (CH_2). EI-MS: *m*/*z* 111 (37) $[M–47]^+$, 97 (4), 83 (36), 69 (100), 55 (79), 41 (78).

6-Methylheptan-1-ol. Physical and spectroscopic data are reported in [5].

6-Methyloctan-1-ol. Yield: 87% (4.88 g, 33.9 mmol). TLC (pentane/Et_2O 2:1): R_f 0.31. GC: *I* 1154. ^{1}H-NMR ($CDCl_3$): 0.84–0.87 (*m*, 6 H); 1.06–1.18 (*m*, 2 H); 1.23–1.38 (*m*, 7 H); 1.52–1.61 (*m*, 3 H); 3.64 (*t*, *J* = 6.6, 2 H). ^{13}C-NMR ($CDCl_3$): 11.4 (Me); 19.2 (Me); 26.1 (CH_2); 26.9 (CH_2); 29.5 (CH_2); 32.8 (CH_2); 34.3 (CH); 36.5 (CH_2); 63.0 (CH_2). EI-MS: *m*/*z* 97 (45) $[M–47]^+$, 83 (8), 69 (35), 55 (95), 41 (100).

Preparation of alkyl bromides. The alkyl bromides were prepared from the respective alcohols with triphenylphosphane and bromine in CH_2Cl_2 as described in [3].

1-Bromo-4-methylpentane (**42a**). Physical and spectroscopic data are reported in [3].

1-Bromo-4-methylhexane (**42b**). Yield: 82% (4.50 g, 25.1 mmol). TLC (pentane/Et_2O 10:1): R_f 0.92. GC: *I* 1013. ^{1}H-NMR ($CDCl_3$): 0.87 (*t*, *J* = 7.3, 3 H); 0.87 (*d*, *J* = 6.5, 3 H); 1.11–1.28 (*m*, 2 H); 1.29–1.48 (*m*, 3 H); 1.77–1.94 (*m*, 2 H); 3.389 (*t*, *J* = 6.9, 1 H); 3.391 (*t*, *J* = 6.9, 1 H). ^{13}C-NMR ($CDCl_3$): 11.3 (Me); 19.1 (Me); 29.3 (CH_2); 30.6 (CH_2); 33.8 (CH); 34.2 (CH_2); 35.0 (CH_2). EI-MS: *m*/*z* 149 (28) $[M–29]^+$, 107 (5), 93 (6), 83 (4), 69 (60), 57 (41), 41 (100).

1-Bromo-6-methylheptane (**54a**). Physical and spectroscopic data are reported in [5].

1-Bromo-6-methyloctane (**54b**). Yield: 95%. (3.11 g, 15.0 mmol) TLC (pentane/Et_2O 10:1): R_f 0.95. GC: *I* 1225. ^{1}H-NMR ($CDCl_3$): 0.85 (*d*, *J* = 6.2, 3 H); 0.86 (*t*, *J* = 7.4, 3 H); 1.07–1.21 (*m*, 2 H); 1.22–1.45 (*m*, 7 H); 1.87 (*quin*, *J* = 7.2, 2 H); 3.41 (*t*, *J* = 6.9, 2 H). ^{13}C-NMR ($CDCl_3$): 11.4 (Me); 19.2 (Me); 26.2 (CH_2); 28.5 (CH_2); 29.4 (CH_2); 32.9 (CH_2); 34.0 (CH_2); 34.3 (CH); 36.4 (CH_2). EI-MS: *m*/*z* 177 (2) $[M–29]^+$, 149 (20), 135 (4), 107 (5), 97 (38), 81 (3), 69 (22), 55 (72), 41 (100).

Preparation of aldehydes. Aldehydes were prepared by standard methods using PCC in dry CH_2Cl_2 [5].

7-Methyloctanal (**44a**). Physical and spectroscopic data are reported in [3].

7-Methylnonanal (**44b**). Yield: 65% (910 mg, 5.83 mmol). TLC (pentane/Et_2O 2:1): R_f 0.76. GC: *I* 1185. ^{1}H-NMR ($CDCl_3$): 0.84 (*d*, *J* = 6.2, 3 H); 0.85 (*t*, *J* = 7.3, 3 H); 1.07–1.18 (*m*, 2 H); 1.23–1.37 (*m*, 7 H); 1.64 (*quin*, *J* = 7.3, 2 H); 2.42 (*dt*, *J* = 1.9, 7.3, 2 H); 9.77 (*t*, *J* = 1.9, 1 H). ^{13}C-NMR ($CDCl_3$): 11.7 (Me); 19.5 (Me); 22.4 (CH_2); 27.1 (CH_2); 29.7 (CH_2); 29.8 (CH_2); 34.6 (CH); 36.7 (CH_2); 44.2 (CH_2); 202.8 (C). EI-MS: *m*/*z* 138 (1) $[M–18]^+$, 128 (4), 123 (3), 109 (77), 96 (8), 81 (30), 70 (51), 57 (81), 41 (100).

Preparation of alkenes by Wittig reaction. A solution of BuLi in hexane (1 eq., 1.6 mol l^{-1}) was added to a suspension of methyltriphenylphosphonium bromide (1 eq.) in dry THF (0.2 mol l^{-1}). The mixture was stirred at room temperature for 30 min. The aldehyde **44a,b** (1 eq.) was added dropwise. The reaction mixture was stirred at room temperature over night and then concentrated. Triphenylphosphane oxide was precipitated by the addition of pentane. The

mixture was filtered and the filtrate was concentrated to dryness. The residue was purified by CC on silica gel with pentane/Et_2O 20:1 to give the pure alkenes **45a,b** as colorless liquids.

8-Methylnon-1-ene (**45a**). Yield: 54% (1.46 g, 10.4 mmol). TLC (pentane/Et_2O 20:1): R_f 1.00. GC: *I* 954. ^{1}H-NMR ($CDCl_3$): 0.86 (*d*, *J* = 6.6, 6 H); 1.12–1.18 (*m*, 2 H); 1.24–1.32 (*m*, 4 H); 1.34–1.43 (*m*, 2 H); 1.52 (*non*, *J* = 6.6, 1 H); 2.01–2.07 (*m*, 2 H); 4.93 (*ddt*, *J* = 1.2, 2.2, 10.3, 1 H); 4.99 (*ddt*, *J* = 1.6, 2.2, 17.1, 1 H); 5.81 (*ddt*, *J* = 6.7, 10.3, 17.1, 1 H). ^{13}C-NMR ($CDCl_3$): 23.0 (2 Me); 27.6 (CH_2); 28.3 (CH); 29.3 (CH_2); 29.8 (CH_2); 34.2 (CH_2); 39.3 (CH_2); 114.4 (CH_2); 139.6 (CH). EI-MS: *m*/*z* 140 (1) $[M]^+$, 125 (5), 112 (6), 97 (12), 83 (18), 69 (59), 56 (87), 41 (100).

8-Methyldec-1-ene (**45b**). Yield: 61% (0.45 g, 2.92 mmol). TLC (pentane/Et_2O 20:1): R_f 1.00. GC: *I* 1056. ^{1}H-NMR ($CDCl_3$): 0.84 (*d*, *J* = 6.3, 3 H); 0.85 (*t*, *J* = 7.3, 3 H); 1.05–1.19 (*m*, 2 H); 1.20–1.43 (*m*, 9 H); 2.01–2.07 (*m*, 2 H); 4.93 (*ddt*, *J* = 1.2, 2.2, 10.3, 1 H); 4.99 (*ddt*, *J* = 1.7, 1.9, 17.1, 1 H); 5.81 (*ddt*, *J* = 6.7, 10.3, 17.1, 1 H). ^{13}C-NMR ($CDCl_3$): 11.4 (Me); 19.2 (Me); 27.0 (CH_2); 29.0 (CH_2); 29.5 (CH_2); 29.5 (CH_2); 33.9 (CH_2); 34.4 (CH); 36.6 (CH_2); 114.1 (CH_2); 139.3 (CH). EI-MS: *m*/*z* 138 (1) $[M–16]^+$, 128 (4), 123 (3), 109 (77), 96 (8), 81 (29), 70 (50), 57 (79), 41 (100).

Preparation of epoxides. A solution of the alkenes **45a,b** or **49**, respectively, (1 eq.) in CH_2Cl_2 (2 mol l^{-1}) was added dropwise to an ice-cooled suspension of *m*-CPBA (*meta*-chloroperoxybenzoic acid, 70%, 1.2 eq.) in CH_2Cl_2 (2 mol l^{-1}). The reaction mixture was stirred over night and then washed with saturated $NaHCO_3$. The aqueous layer was separated and extracted three times with Et_2O. The combined organic layers were dried ($MgSO_4$) and concentrated. The crude product was purified by CC (silica gel; pentane/Et_2O 20:1) to obtain the epoxides as colorless liquids.

1,2-Epoxy-8-methylnonane (**46a**). Yield: 84% (1.25 g, 8.01 mmol). TLC (pentane/Et_2O 20:1): R_f 0.29. GC: *I* 1177. ^{1}H-NMR ($CDCl_3$): 0.87 (*d*, *J* = 6.6, 6 H); 1.13–1.21 (*m*, 2 H); 1.25–1.37 (*m*, 4 H); 1.41–1.57 (*m*, 5 H); 2.46 (*dd*, *J* = 2.7, 5.1, 1 H); 2.74 (*dd*, *J* = 4.0, 5.0 Hz, 1 H); 2.88–2.92 (*m*, 1 H). ^{13}C-NMR ($CDCl_3$): 22.3 (2 Me); 25.7 (CH_2); 27.0 (CH_2); 27.6 (CH); 29.4 (CH_2); 32.2 (CH_2); 38.6 (CH_2); 46.8 (CH_2); 52.0 (CH). EI-MS: *m*/*z* 123 (2) $[M–33]^+$, 109 (18), 95 (22), 81 (35), 71 (44), 55 (58), 41 (100).

1,2-Epoxy-8-methyldecane (**46b**). Yield: 94% (468 mg, 2.75 mmol). TLC (pentane/Et_2O 20:1): R_f 0.33. GC: *I* 1283. ^{1}H-NMR ($CDCl_3$): 0.84–0.90 (*m*, 6 H); 1.08–1.19 (*m*, 2 H); 1.21–1.39 (*m*, 6 H); 1.41–1.56 (*m*, 5 H); 2.46 (*dd*, *J* = 2.7, 5.1, 1 H); 2.74 (*dd*, *J* = 4.0, 5.0, 1 H); 2.88–2.92 (*m*, 1 H). ^{13}C-NMR ($CDCl_3$): 11.3 (Me); 19.2 (Me); 26.0 (CH_2); 27.0 (CH_2); 29.4 (CH_2); 29.8 (CH_2); 32.5 (CH_2); 34.3 (CH); 36.5 (CH_2); 47.1 (CH_2); 52.3 (CH). EI-MS: *m*/*z* 141 (1) $[M–29]^+$, 123 (9), 109 (33), 95 (22), 81 (47), 70 (51), 55 (88), 41 (100).

Ethyl 5,6-epoxyhexanoate (**50**). Yield: 97% (3.30 g, 20.9 mmol). TLC (pentane/Et_2O 3:1): R_f 0.40. GC: *I* 1214. ^{1}H-NMR ($CDCl_3$): 1.26 (*t*, *J* = 7.1, 3 H); 1.50–1.67 (*m*, 2 H); 1.72–1.89 (*m*, 2 H); 2.38 (*dt*, *J* = 1.8, 7.6, 2 H); 2.48 (*dd*, *J* = 2.7, 4.9, 1 H); 2.76 (*t*, *J* = 4.5, 1 H); 2.90–2.95 (*m*, 1 H); 4.14 (*q*, *J* = 7.1, 2 H). ^{13}C-NMR ($CDCl_3$): 14.2 (Me); 21.4 (CH_2); 31.8 (CH_2); 33.8 (CH_2); 46.9 (CH_2); 51.8 (CH); 60.3 (CH_2); 173.3 (C). EI-MS: *m*/*z* 128 (7) $[M–28]^+$, 113 (12), 99 (17), 84 (45), 69 (54), 55 (98), 41 (100).

Preparation of γ-lactones. As described by Chattopadhyay et al. [21], diethyl malonate (3 eq.) was added dropwise to a suspension NaH (3 eq.) in dry *N*,*N*-dimethylacetamide (1 mol l^{-1}). The mixture was stirred until no more H_2 evolved (1 h). Then a solution of the epoxide **46a,b** (1 eq.) in dry *N*,*N*-dimethylacetamide (0.5 mol l^{-1}) was added. The reaction mixture was stirred for 3 h at 160°C. $MgCl_2$ (1 eq.) was added, stirring at 160°C was continued for 48 h, and then the reaction mixture was quenched by the addition of 2N HCl (50 ml). The mixture was extracted three times with Et_2O. The combined organic layers were dried ($MgSO_4$) and concentrated. Purification of the residue by CC (silica gel; pentane/Et_2O 2:1) furnished the γ-lactones **31** and **32** as colorless liquids.

10-Methylundecan-4-olide **31**. Yield: 17% (260 mg, 1.31. mmol). TLC (pentane/Et_2O 2:1): R_f 0.29. GC: *I* 1659. ^{1}H-NMR ($CDCl_3$): 0.87 (*d*, *J* = 6.6, 6 H); 1.13–1.19 (*m*, 2 H); 1.25–1.64 (*m*, 8 H); 1.70–1.76 (*m*, 1 H); 1.77–1.90 (*m*, 1 H); 2.28–

2.36 (*m*, 1 H); 2.51–2.55 (*m*, 2 H); 4.45–4.52 (*m*, 1 H). ^{13}C-NMR ($CDCl_3$): 22.6 (2 Me); 25.2 (CH_2); 27.1 (CH_2); 27.9 (CH); 28.0 (CH_2); 28.8 (CH_2); 29.5 (CH_2); 35.5 (CH_2); 38.8 (CH_2); 81.0 (CH); 177.2 (C). EI-MS: *m*/*z* 180 (1) $[M–18]^+$, 165 (2), 155 (2), 143 (4), 137 (6), 125 (7), 110 (3), 96 (8), 85 (100), 69 (21), 55 (29), 41 (43).

10-Methyldodecan-4-olide **32**. Yield: 16% (89 mg, 0.42 mmol). TLC (pentane/Et_2O 2:1): R_f 0.24. GC: *I* 1773. ^{1}H-NMR ($CDCl_3$): 0.83–0.87 (*m*, 6 H); 1.07–1.17 (*m*, 2 H); 1.19–1.52 (*m*, 9 H); 1.55–1.64 (*m*, 1 H); 1.70–1.79 (*m*, 1 H); 1.80–1.90 (*m*, 1 H); 2.28–2.36 (*m*, 1 H); 2.53 (*dd*, *J* = 7.1, 9.3, 2 H); 4.45–4.52 (*m*, 1 H). ^{13}C-NMR ($CDCl_3$): 11.3 (Me); 19.1 (Me); 25.2 (CH_2); 26.8 (CH_2); 27.9 (CH_2); 28.8 (CH_2); 29.4 (CH_2); 29.6 (CH_2); 34.3 (CH); 35.5 (CH_2); 36.4 (CH_2); 81.0 (CH); 177.2 (C). EI-MS: *m*/*z* 194 (1) $[M–18]^+$, 183 (4), 165 (13), 147 (12), 137 (7), 123 (14), 109 (6), 97 (17), 85 (100), 70 (74), 55 (55), 41 (60).

Alkylation of diethyl malonate. Diethyl malonate (13.6 g, 85.2 mmol) was added dropwise to an ice-cooled suspension of NaH (60% in mineral oil, 3.41 g, 85.2 mmol) in dry DME (40 ml). The mixture was stirred until no more H_2 evolved (1 h). A catalytic amount of $Bu_4N^+ I^-$ (0.91 g, 4.3 mmol) was added. Then **47** (5.76 g, 42.6 mmol) was added dropwise. The reaction mixture was stirred at 80°C and then quenched by the addition of 2N HCl. The mixture was extracted three times with Et_2O. The combined organic layers were dried ($MgSO_4$) and concentrated. The excess diethyl malonate was removed by distillation (bp. 117°C, 50 mbar). The residue was purified by CC (silica gel; pentane/Et_2O 10:1) to give **48** (5.80 g, 27.1 mmol, 64%) as a colorless liquid.

TLC (pentane/Et_2O 10:1): R_f 0.61. GC: *I* 1338. ^{1}H-NMR ($CDCl_3$): 1.27 (*t*, *J* = 7.1, 6 H); 1.97–2.03 (*m*, 2 H); 2.08–2.14 (*m*, 2 H); 3.36 (*t*, *J* = 7.4, 1 H); 4.20 (*q*, *J* = 7.1, 2 H); 4.20 (*q*, *J* = 7.1, 2 H); 4.99–5.07 (*m*, 2 H); 5.77 (*ddt*, *J* = 6.5, 10.3, 17.0, 1 H). ^{13}C-NMR ($CDCl_3$): 14.1 (2 Me); 27.8 (CH_2); 31.3 (CH_2); 51.2 (CH_2); 61.3 (2 CH_2); 115.9 (CH_2); 136.9 (CH); 169.4 (2 C). EI-MS: *m*/*z* 169 (8) $[M–45]^+$, 160 (100), 140 (4), 133 (42), 123 (56), 114 (18), 104 (12), 95 (45), 86 (31), 73 (17), 67 (41), 55 (92), 39 (56).

Preparation of ethyl hex-5-enoate. Similar to the method of Krapcho and Lovey [23], a mixture of **48** (5.80 g, 27.1 mmol), NaCl (3.37 g, 70 mmol), DMSO (30 ml) and H_2O (3 ml) was heated to 150°C for 48 h. The reaction mixture was cooled to room temperature and H_2O (300 ml) was added. The aqueous layer was extracted three times with Et_2O. The combined organic layers were dried ($MgSO_4$) and concentrated. Pure **49** (3.15 g, 22.2 mmol, 82%) was obtained by CC (silica gel; pentane/Et_2O 10:1) as a colorless liquid.

TLC (pentane/Et_2O 10:1): R_f 0.50. GC: *I* 993. ^{1}H-NMR ($CDCl_3$): 1.26 (*t*, *J* = 7.1, 3 H); 1.73 (*quin*, *J* = 7.5, 2 H); 2.06–2.12 (*m*, 2 H); 2.31 (*t*, *J* = 7.5, 2 H); 4.13 (*q*, *J* = 7.1, 2 H); 4.97–5.06 (*m*, 2 H); 5.78 (*ddt*, *J* = 6.7, 10.3, 17.1, 1 H). ^{13}C-NMR ($CDCl_3$): 14.5 (Me); 24.4 (CH_2); 33.4 (CH_2); 33.9 (CH_2); 60.5 (CH_2); 115.6 (CH_2); 138.0 (CH); 173.9 (C). EI-MS: *m*/*z* 114 (2) $[M–28]^+$, 97 (23), 88 (26), 68 (48), 60 (37), 55 (39), 41 (91), 39 (100).

Preparation of δ*-lactones*. Similar to the method of Ishigami and Kitahara [70], a solution of the alkylmagnesium bromide prepared from **42a,b** (2.5 eq.) and Mg (2.5 eq.) in dry THF (0.5 mol l^{-1}) was added at –78°C to a solution of CuBr (0.1 eq.) and Me_2S (0.1 eq.) in dry THF (0.05 mol l^{-1}). A solution of **50** (1 eq.) in dry THF (0.5 mol l^{-1}) was added dropwise. The reaction mixture was stirred over night and allowed to reach room temperature slowly. H_2O and then 2N HCl were added. The aqueous layer was separated and extracted three times with Et_2O. The combined organic layers were dried ($MgSO_4$). The solvents were removed under reduced pressure. Purification of the residue by CC (silica gel; pentane/Et_2O 3:1) yielded the δ-lactones **33** and **34** as colorless liquids.

10-Methylundecan-5-olide (**33**). Yield: 16% (340 mg, 1.60 mmol). TLC (pentane/Et_2O 3:1): R_f 0.14. GC: *I* 1687. ^{1}H-NMR ($CDCl_3$): 0.86 (*d*, *J* = 6.6, 6 H); 1.14–1.23 (*m*, 2 H); 1.24–1.40 (*m*, 4 H); 1.43–1.62 (*m*, 4 H); 1.67–1.76 (*m*, 1 H); 1.84–1.95 (*m*, 2 H); 2.40–2.50 (*m*, 1 H); 2.54–2.63 (*m*, 1 H); 4.24–4.31 (*m*, 1 H). ^{13}C-NMR ($CDCl_3$): 18.4 (CH_2); 22.5 (2

Me); 25.1 (CH_2); 27.1 (CH_2); 27.7 (CH_2); 27.8 (CH); 29.4 (CH_2); 35.8 (CH_2); 38.7 (CH_2); 80.6 (CH); 172.0 (C). EI-MS: *m*/*z* 198 (1) $[M]^+$, 183 (1), 180 (2), 165 (3), 155 (4), 143 (4), 137 (8), 125 (6), 114 (12), 99 (100), 83 (11), 71 (57), 55 (52), 41 (73).

10-Methyldodecan-5-olide (**34**). Yield: 12% (36 mg, 0.17 mmol). TLC (pentane/Et_2O 3:1): R_f 0.14. GC: *I* 1804. ^{1}H-NMR ($CDCl_3$): 0.83–0.89 (*m*, 6 H); 1.05–1.18 (*m*, 2 H); 1.22–1.41 (*m*, 6 H); 1.42–1.93 (*m*, 7 H); 2.38–2.48 (*m*, 1 H); 2.55–2.62 (*m*, 1 H); 4.24–4.31 (*m*, 1 H). ^{13}C-NMR ($CDCl_3$): 11.4 (Me); 18.5 (CH_2); 19.2 (Me); 25.2 (CH_2); 26.7 (CH_2); 27.8 (CH_2); 29.4 (CH_2); 29.5 (CH_2); 34.3 (CH); 35.9 (CH_2); 36.4 (CH_2); 80.6 (CH); 172.0 (C). EI-MS: *m*/*z* 194 (1) $[M–18]^+$, 183 (7), 165 (16), 147 (12), 137 (10), 123 (13), 114 (11), 99 (97), 83 (25), 70 (100), 55 (81), 41 (87).

Preparation of allyl alcohols by Grignard reaction. The following sequence of vinylation, acylation, and ring closing metathesis was carried out similar to the protocol of Marco et al. [24]. A solution of vinylmagnesium bromide in THF (1 mol l^{-1}, 1.2 eq.) was added dropwise to an ice-cooled solution of the aldehyde **44a,b** (1 eq.) in dry THF (0.25 mol l^{-1}). The reaction mixture was stirred over night at room temperature and then quenched by the addition of 2N HCl. The aqueous layer was separated and extracted three times with Et_2O. The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The crude product was purified by CC (silica gel; pentane/Et_2O 5:1) to yield the allyl alcohols **51a,b** as colorless liquids.

9-Methyldec-1-en-3-ol (**51a**). Yield: 69% (4.12 g, 24.2 mmol). TLC (pentane/Et_2O 5:1): R_f 0.29. GC: *I* 1255. ^{1}H-NMR ($CDCl_3$): 0.86 (*d*, *J* = 6.6, 6 H); 1.13–1.19 (*m*, 2 H); 1.23–1.43 (*m*, 7 H); 1.45–1.58 (*m*, 2 H); 2.07 (br. *s*, 1 H); 4.07 (*q*, *J* = 6.4, 1 H); 5.08 (*dd*, *J* = 1.4, 10.4, 1 H); 5.20 (*dd*, *J* = 1.4, 17.2, 1 H); 5.86 (*ddd*, *J* = 6.3, 10.4, 17.0, 1 H). ^{13}C-NMR ($CDCl_3$): 22.6 (2 Me); 25.3 (CH_2); 27.3 (CH_2); 27.9 (CH); 29.8 (CH_2); 37.0 (CH_2); 38.9 (CH_2); 73.1 (CH); 114.3 (CH_2); 141.4 (CH). EI-MS: *m*/*z* 123 (4) $[M–47]^+$, 109 (6), 96 (10), 85 (12), 72 (19), 57 (100), 43 (34).

9-Methylundec-1-en-3-ol (**51b**). Yield: 81% (830 mg, 4.51 mmol). TLC (pentane/Et_2O 5:1): R_f 0.31. GC: *I* 1362. ^{1}H-NMR ($CDCl_3$): 0.84 (*d*, *J* = 6.4, 3 H); 0.85 (*t*, *J* = 7.3, 3 H); 1.07–1.17 (*m*, 2 H); 1.22–1.43 (*m*, 9 H); 1.46–1.56 (*m*, 2 H); 1.68 (br. *s*, 1 H); 4.09 (*q*, *J* = 6.4, 1 H); 5.10 (*dd*, *J* = 1.4, 10.4, 1 H); 5.21 (*dd*, *J* = 1.4, 17.2, 1 H); 5.87 (*ddd*, *J* = 6.3, 10.4, 17.0, 1 H). ^{13}C-NMR ($CDCl_3$): 11.4 (Me); 19.2 (Me); 25.4 (CH_2); 27.0 (CH_2); 29.5 (CH_2); 29.9 (CH_2); 34.4 (CH); 36.5 (CH_2); 37.0 (CH_2); 73.3 (CH); 114.5 (CH_2); 141.3 (CH). EI-MS: *m*/*z* 137 (5) $[M–47]^+$, 109 (6), 95 (10), 81 (13), 72 (18), 57 (100), 41 (33).

Preparation of acrylates. Acryloyl chloride (2.5 eq.) was added dropwise to an ice-cooled colution of the allyl alcohol **51a,b** in dry NEt_3 (1 mol l^{-1}). The reaction mixture was stirred 30 min at room temperature, poured on ice, and acidified by the addition of 2N HCl. The aqueous layer was extracted three times with Et_2O. The combined extracts were dried ($MgSO_4$). The Et_2O was removed and the residue was purified by CC (silica gel; pentane/Et_2O 10:1) to give the acrylates **52a,b** as colorless liquids.

9-Methyldec-1-en-3-yl acrylate (**52a**). Yield: 76% (1.82 g, 10.7 mmol). TLC (pentane/Et_2O 10:1): R_f 0.47. GC: *I* 1443. ^{1}H-NMR ($CDCl_3$): 0.86 (*d*, *J* = 6.6, 6 H); 1.14–1.19 (*m*, 2 H); 1.23–1.37 (*m*, 6 H); 1.51 (*non*, *J* = 6.6, 1 H); 1.57–1.74 (*m*, 2 H); 5.16 (*dt*, *J* = 1.2, 10.5, 1 H); 5.25 (*dt*, *J* = 1.3, 17.2, 1 H); 5.31 (*q*, *J* = 6.5, 1 H); 5.81 (*ddd*, *J* = 6.4, 10.5, 17.1, 1 H); 5.81 (*dd*, *J* = 1.6, 10.4, 1 H); 6.13 (*dd*, *J* = 10.4, 17.4, 1 H); 6.41 (*dd*, *J* = 1.5, 17.3, 1 H). ^{13}C-NMR ($CDCl_3$): 22.6 (2 Me); 25.0 (CH_2); 27.2 (CH_2); 27.9 (CH); 29.6 (CH_2); 34.2 (CH_2); 38.9 (CH_2); 74.9 (CH); 116.5 (CH_2); 128.8 (CH); 130.4 (CH_2); 136.5 (CH); 165.4 (C). EI-MS: *m*/*z* 169 (1) $[M–55]^+$, 153 (1), 135 (1), 126 (2), 111 (16), 96 (10), 81 (11), 67 (12), 55 (100), 41 (23).

9-Methylundec-1-en-3-yl acrylate (**52b**). Yield: 61% (111 mg, 0.47 mmol). TLC (pentane/Et_2O 10:1): R_f 0.50. GC: *I* 1552. ^{1}H-NMR ($CDCl_3$): 0.85–0.89 (*m*, 6 H); 1.06–1.16 (*m*, 2 H); 1.21–1.35 (*m*, 8 H); 1.45 (*non*, *J* = 6.6, 1 H); 1.57–1.71 (*m*, 2 H); 5.17 (*dt*, *J* = 1.2, 10.5, 1 H); 5.25 (*dt*, *J* = 1.3, 17.2, 1 H); 5.31 (*q*, *J* = 6.3, 1 H); 5.81 (*ddd*, *J* = 6.4, 10.5, 17.2, 1 H);

5.81 (*dd*, *J* = 1.6, 10.4, 1 H); 6.13 (*dd*, *J* = 10.4, 17.3, 1 H); 6.41 (*dd*, *J* = 1.5, 17.3, 1 H). ^{13}C-NMR ($CDCl_3$): 11.4 (Me); 19.2 (Me); 25.1 (CH_2); 26.9 (CH_2); 29.5 (CH_2); 29.7 (CH_2); 34.2 (CH_2); 34.4 (CH); 36.5 (CH_2); 75.0 (CH); 116.6 (CH_2); 128.8 (CH); 130.5 (CH_2); 136.5 (CH); 165.6 (C). EI-MS: *m*/*z* 181 (1) $[M-57]^+$, 137 (3), 126 (2), 111 (15), 96 (9), 81 (11), 67 (12), 55 (100), 41 (21).

Preparation of α,β-*unsaturated* γ-*lactones*. A mixture of the acrylate **52a,b** (1 eq.) and benzylidene-[1,3-bis-(2,4,6-trimethylphenyl)-2-imidazolidinylidene]-dichloro-(tricyclohexylphosphane)ruthenium (Grubbs catalyst, 2. generation, 2 mol-%) in dry toluene (0.05 mol l^{-1} acrylate concentration) was heated to reflux over night. The solvent was removed under reduced pressure. The crude product was purified by CC (silica gel; pentane/Et_2O 3:1) to give the *α,β*-unsaturated *γ*-lactones **35** and **36** as colorless liquids.

10-Methylundec-2-en-4-olide (**35**). Yield: 83% (660 mg, 3.37 mmol). TLC (pentane/Et_2O 3:1): R_f 0.15. GC: *I* 1638. ^{1}H-NMR ($CDCl_3$): 0.86 (*d*, *J* = 6.7, 6 H); 1.12–1.18 (*m*, 2 H); 1.23–1.36 (*m*, 4 H); 1.38–1.49 (*m*, 2 H); 1.52 (*non*, *J* = 6.7, 1 H); 1.61–1.71 (*m*, 1 H); 1.73–1.82 (*m*, 1 H); 5.03–5.06 (*m*, 1 H); 6.08–6.11 (*m*, 1 H); 7.49 (*dd*, *J* = 1.3, 5.7, 1 H). ^{13}C-NMR ($CDCl_3$): 22.4 (2 Me); 24.8 (CH_2); 27.0 (CH_2); 27.7 (CH); 29.4 (CH_2); 33.0 (CH_2); 38.6 (CH_2); 83.3 (CH); 121.2 (CH); 156.4 (CH); 173.0 (C). EI-MS: *m*/*z* 196 (6) $[M]^+$, 181 (2), 163 (5), 153 (7), 136 (32), 122 (20), 111 (17), 97 (84), 83 (54), 69 (30), 55 (100), 41 (95).

10-Methyldodec-2-en-4-olide (**36**). Yield: 80% (78 mg, 0.37 mmol). TLC (pentane/Et_2O 3:1): R_f 0.15. GC: *I* 1758. ^{1}H-NMR ($CDCl_3$): 0.84 (*d*, *J* = 6.3, 3 H); 0.85 (*t*, *J* = 7.3, 3 H); 1.07–1.17 (*m*, 2 H); 1.24–1.37 (*m*, 7 H); 1.39–1.50 (*m*, 2 H); 1.62–1.70 (*m*, 1 H); 1.71–1.81 (*m*, 1 H); 5.04 (*ddt*, *J* = 1.7, 5.6, 7.3, 1 H); 6.11 (*dd*, *J* = 2.0, 5.7, 1 H); 7.46 (*dd*, *J* = 1.5, 5.7, 1 H). ^{13}C-NMR ($CDCl_3$): 11.3 (Me); 19.1 (Me); 25.0 (CH_2); 26.8 (CH_2); 29.4 (CH_2); 29.6 (CH_2); 33.2 (CH_2); 34.3 (CH); 36.4 (CH_2); 83.4 (CH); 121.5 (CH); 156.3 (CH); 173.1 (C). EI-MS: *m*/*z* 210 (5) $[M]^+$, 181 (10), 163 (17), 150 (21), 135 (22), 122 (24), 109 (20), 97 (89), 83 (61), 69 (28), 55 (100), 41 (90).

Preparation of 2-alkylfurans. A solution of BuLi in hexane (1.6 mol l^{-1}, 1.1 eq.) was diluted with dry THF (0.25 mol l^{-1} BuLi concentration) and cooled to 0°C. Furan (3 eq.) was added dropwise. The reaction mixture was stirred for 24 h at room temperature. A solution of the alkyl bromide **54a,b** (1 eq.) in dry THF (1 mol l^{-1}) was added dropwise. The reaction mixture was stirred for 24 h at room temperature and then quenched by the addition of 2N HCl. The aqueous layer was separated and extracted three times with Et_2O. The combined organic layers were dried ($MgSO_4$) and concentrated. Purification by CC (pentane/Et_2O 20:1) gave the 2-alkylfurans **55a,b** as colorless liquids.

2-(6-Methylheptyl)furan (**55a**). Yield: 87% (1.27 g, 7.06 mmol). TLC (pentane/Et_2O 20:1): R_f 1.00. GC: *I* 1265. ^{1}H-NMR ($CDCl_3$): 0.86 (*d*, *J* = 6.6, 6 H); 1.14–1.21 (*m*, 2 H); 1.23–1.35 (*m*, 4 H); 1.52 (*non*, *J* = 6.6, 1 H); 1.60–1.67 (*m*, 2 H); 2.61 (*t*, *J* = 7.6, 2 H); 5.96 (*dd*, *J* = 0.8, 3.1, 1 H); 6.26 (*dd*, *J* = 1.9, 3.1, 1 H); 7.28 (*dd*, *J* = 0.8, 1.8, 1 H). ^{13}C-NMR ($CDCl_3$): 22.6 (2 Me); 27.1 (CH_2); 28.0 (CH); 28.0 (CH_2); 28.1 (CH_2); 29.5 (CH_2); 38.9 (CH_2); 104.5 (CH); 110.0 (CH); 140.6 (CH); 156.6 (C). EI-MS: *m*/*z* 180 (12) $[M]^+$, 165 (2), 137 (5), 123 (9), 109 (4), 95 (31), 81 (100), 67 (10), 53 (33), 41 (37).

2-(6-Methyloctyl)furan (**55b**). Yield: 99% (2.85 g, 14.7 mmol). TLC (pentane/Et_2O 20:1): R_f 1.00. GC: *I* 1370. ^{1}H-NMR ($CDCl_3$): 0.83–0.87 (*m*, 6 H); 1.07–1.19 (*m*, 2 H); 1.23–1.44 (*m*, 7 H); 1.60–1.67 (*m*, 2 H); 2.61 (*t*, *J* = 7.6, 2 H); 5.96 (*dd*, *J* = 0.8, 3.2, 1 H); 6.27 (*dd*, *J* = 1.9, 3.1, 1 H); 7.29 (*dd*, *J* = 0.8, 1.8, 1 H). ^{13}C-NMR ($CDCl_3$): 11.4 (Me); 19.2 (Me); 26.8 (CH_2); 28.0 (CH_2); 28.1 (CH_2); 29.5 (CH_2); 29.6 (CH_2); 34.4 (CH); 36.5 (CH_2); 104.5 (CH); 110.0 (CH); 140.6 (CH); 156.6 (C). EI-MS: *m*/*z* 194 (8) $[M]^+$, 165 (4), 137 (5), 123 (7), 109 (4), 95 (30), 81 (100), 67 (10), 53 (39), 41 (48).

Preparation of β,γ-*unsaturated* γ-*lactones*. The method of Pelter and Rowlands [25] was used. Flask A was charged with the 2-alkylfuran **55a,b** (1 eq.) and dry Et_2O (0.25 mol l^{-1}). The solution was cooled to 0°C. A solution of BuLi in hexane (1 eq., 1.6 mol l^{-1}) was added dropwise. The reaction mixture was stirred for 3 h to give solution A. Flask B was charged with

$B(OMe)_3$ (100 eq.) and dry Et_2O (v/v = 1/2). The mixture was cooled to 0°C. A solution of BCl_3 in hexane (0.33 eq., 1 mol l^{-1}) was added dropwise. The reaction mixture was stirred for 3 h at room temperature. The resulting solution was cooled to 0°C and the cooled (0°C) solution A was transferred to flask B. The reaction mixture was stirred for 1 h at room temperature to give solution B. Flask C was charged with K_2CO_3 (10 eq.) and Et_2O (1 mol l^{-1}). Then *m*-CPBA (70%, 2 eq.) was added. The reaction mixture was stirred for 30 min at room temperature. Solution B was transferred to flask C. The reaction mixture was stirred for 10 min and then quenched by the addition of H_2O. The aqueous layer was separated and extracted three times with Et_2O. The combined organic layers were dried ($MgSO_4$) and concentrated. The pure β,γ-unsaturated γ-lactones **37** and **38** were obtained as colorless liquids by CC (silica gel; pentane/Et_2O 10:1).

10-Methylundec-3-en-4-olide (**37**). Yield: 61% (384 mg, 1.96 mmol). TLC (pentane/Et_2O 10:1): R_f 0.25. GC: *I* 1550. ^{1}H-NMR ($CDCl_3$): 0.87 (*d*, *J* = 6.6, 6 H); 1.13–1.19 (*m*, 2 H); 1.26–1.36 (*m*, 4 H); 1.52 (*non*, *J* = 6.6, 1 H); 1.54–1.59 (*m*, 2 H); 2.26–2.31 (*m*, 2 H); 3.16–3.19 (*m*, 2 H); 5.10–5.12 (*m*, 1 H). ^{13}C-NMR ($CDCl_3$): 22.6 (2 Me); 25.7 (CH_2); 27.0 (CH_2); 27.9 (CH); 28.2 (CH_2); 29.2 (CH_2); 33.9 (CH_2); 38.8 (CH_2); 98.1 (CH); 157.3 (C); 177.0 (C). EI-MS: *m*/*z* 196 (2) $[M]^+$, 181 (1), 153 (10), 140 (15), 122 (15), 111 (51), 98 (57), 83 (18), 70 (30), 55 (100), 41 (74).

10-Methyldodec-3-en-4-olide (**38**). Yield: 63% (580 mg, 2.76 mmol). TLC (pentane/Et_2O 10:1): R_f 0.26. GC: *I* 1663. ^{1}H-NMR ($CDCl_3$): 0.84 (*d*, *J* = 6.4, 3 H); 0.86 (*t*, *J* = 7.2, 3 H); 1.07–1.18 (*m*, 2 H); 1.21–1.38 (*m*, 7 H); 1.50–1.62 (*m*, 2 H); 2.26–2.31 (*m*, 2 H); 3.16–3.18 (*m*, 2 H); 5.10–5.11 (*m*, 1 H). ^{13}C-NMR ($CDCl_3$): 11.7 (Me); 19.5 (Me); 26.0 (CH_2); 27.0 (CH_2); 28.6 (CH_2); 29.6 (CH_2); 29.8 (CH_2); 34.2 (CH_2); 34.6 (CH); 36.7 (CH_2); 98.4 (CH); 157.7 (C); 177.3 (C). EI-MS: *m*/*z* 181 (3) $[M–29]^+$, 153 (4), 140 (7), 122 (7), 111 (26), 98 (31), 95 (9), 83 (16), 70 (14), 41 (76), 55 (100).

Acknowledgements

J. S. D. thanks the Fond der Chemischen Industrie and the BMBF for a stipend. We thank Renate Gahl-Janssen for excellent technical assistence.

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VI

Reclassification of *Roseobacter gallaeciensis* Ruiz-Ponte *et al.* 1998 as *Phaeobacter gallaeciensis* gen. nov., comb. nov., and description of *Phaeobacter inhibens* sp. nov., antibiotic-producing members of the *Roseobacter* clade

Submitted to
International Journal of Systematic and Evolutionary Microbiology

Reclassification of *Roseobacter gallaeciensis* Ruiz-Ponte *et al.* 1998 as *Phaeobacter gallaeciensis* gen. nov., comb. nov., and description of *Phaeobacter inhibens* sp. nov., antibiotic-producing members of the *Roseobacter* clade.

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Keywords: Antagonistic activity, *Phaeobacter*, *Roseobacter*, tropodithietic acid, Wadden Sea

Running title: Description of antibiotic-producing *Phaeobacter* spp.
Subject category: *Proteobacteria*
Format of description: Full paper

The GenBank accession number for the 16S rRNA gene sequence of *Phaeobacter inhibens* $T5^T$ is AY177712.

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SUMMARY

A heterotrophic, antibiotic-producing bacterium, strain T5^T, was isolated from the German Wadden Sea, located in the southern region of the North Sea. Sequence analysis of the 16S rRNA gene of this strain demonstrated close affiliation with *Roseobacter gallaeciensis* BS107^T (99% similarity), although genotypic (DNA-DNA similarity, DNA G + C content) and phenotypic characterization revealed that strain T5^T represents a new species. The organism is strictly aerobic, Gram-negative, rod-shaped, motile, and forms brown pigmented colonies. The strain produces the antibiotic tropodithietic acid throughout exponential phase, which inhibits the growth of bacteria affiliated with different taxa, as well as marine algae. Strain T5^T requires sodium ions and utilizes a wide range of substrates, including oligosaccharides, sugar alcohols, organic acids and amino acids. The DNA G + C content was 55·7 mol %. Comparative 16S rRNA gene sequence analysis revealed that strain T5^T and *Roseobacter gallaeciensis* BS107^T affiliate with *Leisingera methylohalidivorans* as their closest described relative within the *Roseobacter* clade (T5^T = 97% similarity; BS107 ^{T} = 97·1% similarity), of the alpha subclass of the *Proteobacteria*. Comparison of T5^T and *Roseobacter gallaeciensis* BS107^T with *Roseobacter denitrificans* and *Roseobacter litoralis* showed striking differences with respect to phylogenetic affiliation, pigmentation, presence of bacteriochlorophyll *a* and antibiotic production. On the basis of our results we propose reclassification of *Roseobacter gallaeciensis* BS107^T as the type species of a new genus, *Phaeobacter gallaeciensis* BS107^T (= CIP 105210^T = DSM 12440^T) and describe strain T5^T (= LMG 22475^T = DSM 16374^T) as a new species of this genus, *Phaeobacter inhibens*.

INTRODUCTION

The so-called *Roseobacter* clade (Giovannoni & Rappe, 2000) within the class α-*Proteobacteria* includes 16S rRNA gene sequences of cultivated and uncultivated organisms found in marine habitats all over the world. Characterization of isolates demonstrated that organisms in this group have very diverse metabolisms, including production of secondary metabolites (Gram, et al., 2002, Lafay, et al., 1995). Some species of this cluster are even capable of producing antibiotic compounds (Ruiz-Ponte, et al., 1999); Brinkhoff *et al*., 2004; (Wagner-Döbler, et al., 2004). The eponymous genus of the *Roseobacter* clade is currently comprised of three species: *Roseobacter gallaeciensis* (Ruiz-Pont *et al.* 1998), *Roseobacter denitrificans* and the type species, *Roseobacter litoralis* (Shiba, 1991). Recently it was demonstrated that an isolate from the German Wadden Sea, strain T5^T, as well as *Roseobacter gallaeciensis* BS107^T are able to produce a new tropolone derivative, tropodithietic acid, which exhibits strong antibiotic properties against marine bacteria of various taxa and marine algae (Brinkhoff *et al*., 2004). Sequence similarity of the 16S rRNA gene and physiological characteristics of strain T5^T revealed close affiliation with *Roseobacter gallaeciensis* BS107^T, however, phenetic comparison of these two bacteria with *Roseobacter denitrificans* and *Roseobacter litoralis* showed striking differences regarding genetic similarity, pigmentation, presence of bacteriochlorophyll (Bchl) *a* and production of antibiotics. Although in many cases there is reason to assign two phylogenetically closely related bacteria to the same genus, this species should not be greatly different with respect for their physiology of taxonomic purposes. On these grounds the inclusion of non-phototrohic strains into the existing genera Roseobacter, criticized in different reviews (e.g. Yurkov & Beatty, 1998; Rathgeber, et al., 2004), over emphasize the significance of 16S rRNA analysis. On the basis of our results we propose the reclassification of *Roseobacter gallaeciensis* BS107^T as the type species of a new genus, *Phaeobacter gallaeciensis* BS107^T, and describe strain T5^T as a new species of this genus, *Phaeobacter inhibens*.

METHODS

Source of bacteria. Strain T5^T was isolated from a 10^0 dilution of a most-probable-number series with Marine Broth 2216 (MB 2216, Difco, Becton Dickinson, USA) as medium, inoculated with surface water from a tidal mud flat (53°42´20´´N, 07°43´11´´E) of the

German Wadden Sea (Brinkhoff, et al., 2004). *Roseobacter gallaeciensis* BS107^T (= CIP105210^T = DSM 12440^T) was obtained from the Collection de l'Institut Pasteur (Paris, France), *Roseobacter denitrificans* DSM 7001^T and *Leisingera methylohalidivorans* DSM 14336^T were obtained from the were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and cultured as recommended by the supplier.

Growth experiments. Strain T5^T was subcultured in MB 2216 or in artificial seawater (ASW) consisting of: 372 mM NaCl; 49·1 mM $MgCl_2$; 25·5 mM Na_2SO_4; 9·3 mM $CaCl_2$; 8·5 mM KCl; 3·2 mM $NaHCO_3$; 672 µM KBr; 360 µM H_3BO_4; 213 µM $SrCl_2$; 57 µM NaF; 32 µM Na_2SiO_3; 20 µM $(NH_4)NO_3$; 0·4 mM Fe-citrate; 6 mM NH_4Cl and 0·263 mM K_2HPO_4 (denoted as artificial seawater, ASW). ASW medium was supplemented with different carbon sources as described below. Growth was determined by an increase in optical density at 650 nm (photometer model DU 520, Beckmann, Germany). Unless stated otherwise, assays were done using cells grown in MB 2216.

Temperature range for growth was determined between 4 and 45 °C in intervals of 5 °C, while the optimum was defined in 2 -°C increments between 25 and 35 °C. After plotting the growth curve for each temperature the maximum growth rate (μ_{max}) was calculated. The pH range (4 - 10·5) was tested in increments of 0·5 adjusted with sterile NaOH and HCl solutions (1 M each). The optimal pH value was determined by growth experiments in a temperature- and pH-controlled, 1·5-l batch fermenter system (FairMenTec, Germany), with filter-sterilized medium. Oxygen concentration and pH value were monitored by oxygen and pH electrodes (Mettler Toledo, Germany), respectively. In determining optimal pH, the pH was adjusted to 6·0, 7·0, 7·5, 8·0 and 8·5 by automatic titration with NaOH (1 M) or H_2SO_4 (1 M). Incubation of each experiment was performed at 30 °C. After plotting the growth curve, μ_{max} was calculated for each pH value.

Experiments for optimal salinity were carried out in 250-ml shake flasks at 180 rpm, 25 °C and a culture volume of 50 ml. Composition of the medium was (g l^{-1}): Trypton (5), yeast extract (2·5), K_2HPO_4 (0·05) and $SrCl_2$ (0·034) and a trace element stock solution (1 ml l^{-1}). The trace element stock solution consisted of (g l^{-1}): KBr (80), H_3BO_3 (22), Na_2SiO_3 (4), NaF (2·4) and $(NH_4)NO_3$ (1·6). For determining the optimal salinity the following concentrations were adjusted: 0, 12·5, 25, 37·5, 50, 65 g l^{-1}, using a concentrated stock solution of 106 g l^{-1}

[in g l^{-1}: NaCl (72), KCl (2·1), $MgCl_2$ (15·5), $CaCl_2$ (3·4), Na_2SO_4 (12), $NaHCO_3$ (0·9)]. Since higher concentrations of some components in the mineral salt stock solution did not easily dissolve, the range of osmotolerance was studied with NaCl only. To determine the salinity range in which strain $T5^T$ was able to grow, a concentrated NaCl solution was added to a minimal medium consisting of glucose (26 mM), NH_4Cl (6 mM), $MgCl_2$ (2,5 mM), K_2HPO_4 (0·263 mM) and 1 ml l^{-1} trace element solution SL10 (Tschech & Pfennig, 1984), to adjust final concentrations of 0·01, 0·05, 0·1, 0·25, 0·37, 0·5, 0·7, 1, 1·5 and 2 M NaCl. Requirement of Na^+ was studied in ASW supplemented with glucose (26 mM), where Na^+ was exchanged with K^+.

Cytochrome oxidase and catalase reaction assays were carried out according to the methods described by Smibert & Krieg (Smibert & Krieg, 1994). Exoenzyme activities (Hydrolyses of gelatin, starch and tween 80) were analyzed with MB 2216 solidified with 4% (w/v) gelatin or 1·5% (w/v) agarose and supplemented with 0·2% (w/v) starch and 1% (v/v) tween 80, respectively, as described previously (Smibert & Krieg, 1994). Reduction of nitrate was tested by the method of Smibert & Krieg (Smibert & Krieg, 1994) in ASW supplemented with glucose (26 mM) and a mixture of lactate (20 mM) and glucose (5 mM). The experiments were analysed after 10 weeks of incubation at 20 °C in a 12-h daylight rhythm with *Roseobacter denitrificans* used as a positive control. Production of Bchl *a* was determined by spectrophotometric analysis. Cells grown at 25 °C for 24 h either in the dark or with a natural daylight rhythm were collected by centrifugation of 2 ml culture broth (7000 rpm/ 5 min) and resuspended in a drop of medium. Pigments were extracted in 1·5 ml of an ice cold acetone-methanol solution (7:2 v/v) by incubation in the dark for 12 h. Relative absorption was determined with a spectrophotometer (DU 520, Beckmann, Germany) in the range of 400-900 nm. *Roseobacter denitrificans* was used as a positive control.

To determine the substrate spectrum of strain $T5^T$ 30 different carbon sources were tested in testtubes. ASW, supplemented with the vitamins nicotinic acid, thiamine, pantothenate, pyridoxalhydrochloride, cyanocobalamine, riboflavin and biotin (0·05 mg l^{-1} each), was used. Growth experiments with ASW + glucose (26 mM) and with and without vitamins showed no growth limitations without the vitamins, but a slightly enhanced biomass production if all vitamins were supplied. The following carbon sources were added to the medium to a final concentration of 1 g l^{-1}: D(+)-xylose, D(+)-glucose, D(+)-mannose, D(+)-galactose, D(-)-fructose, L(+)-arabinose, D(-)-ribose, L(+)-rhamnose, L(-)-fucose, D(-)-mannitol, D(-)-

sorbitol, D-glucosamine, sucrose, maltose, cellobiose, trehalose, lactose, sodium formate, sodium acetate, sodium pyruvate, sodium malate, sodium citrate, disodium succinate, sodium lactate, sodium propionate, starch, inulin, xylan, glycerol and tween 80. The polysaccharides starch, inulin and xylan were dissolved at 80 °C, only xylan was not completely dissolved and the saturated solution was used. All media were adjusted to pH 7·6 and filter-sterilized (Satorius, pore size 0·2 µm). Three replicates of 7 ml medium were inoculated with 15 µl cells (washed twice with ASW) from an exponentially growing culture of strain T5^T incubated with MB2216. The medium without any carbon source was used as a control. Growth was determined by measuring the optical density at 650 nm after incubation at 20 °C and 100 rpm shaking for 14 days. These experiments were repeated for *L. methylohalidovorans* with 11 carbon sources as listed in Table 1.

Utilization of L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamine, L-glutamic acid, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-trytophan, L-tyrosine, and L-valine (final concentration 1 mM) by strain T5^T was tested in MB 2216 without organic carbon sources at 20 °C for one week. Growth was inspected daily and determined by an increase in optical density at 550 nm.

To determine differences in sole carbon usage of strain T5^T, *Roseobacter gallaeciensis* and the methylotrophic *L. methylohalidivorans*, growth of these organisms on select, methylated substrates (all except butyrate) was tested in ASW, supplemented with the vitamin solution described above. Butyrate (1 mM), betaine (1 mM), methionine (10 mM), dimethylsulphate (DMS, 0·05 mM), methyl iodide (0·13 mM) and methyl chloride (0·37 mM) were added to the medium in test tubes or in sealed 50-ml serum flasks with an air-liquid ratio of 2:1 to reduce oxygen limitation. Comparison of growth of the organisms with MB 2216 in sealed flasks or cotton-stoppered Erlenmeyer flasks did not reveal growth limitations during 5 days of incubation. Methyl iodide was added to sealed culture vessels as an aqueous solution and methyl chloride was added as a gas. Growth was inspected daily by measuring optical density at 650 nm over a one-week incubation period.

Antibiotic susceptibility of strain T5^T and *Roseobacter gallaeciensis* was tested as described (Brinkhoff *et al.* 2004).

Microscopic analyses. Colony morphology was studied with a Leitz Aristoplan Microscope. Motility of strain T5^T was observed by light microscopy of an exponentially growing culture, presence of flagella was examined with staining procedures described by Ryu (Ryu, 1937). Number and type of flagella were examined by transmission electron microscopy (TEM), using cells from early exponential phase grown in ASW and supplied with 15mM glucose. For TEM observations cells were negatively stained with 1% (w/v) phosphotungstic acid or watched unstained as described before (Chavez, et al., 2004) to preserve the native shape and, after air drying, the copper grids (200 mesh, Plano, Germany) were examined with a model EM 902A transmission electron microscope (Zeiss, germany)

Antimicrobial activity. Tests for antibiotic production and detection of antimicrobial activity of *L. methylohalidivorans, Roseobacter litoralis* and *Roseobacter denitrificans* followed the method described by Brinkhoff *et al*. (Brinkhoff, et al., 2004).

Phylogenetic analysis. PCR amplification and sequencing of the nearly complete 16S rRNA gene of strain T5^T were performed according to the methods described by Brinkhoff & Muyzer (Brinkhoff & Muyzer, 1997). Phylogenetic analysis was performed with the ARB software package [http://www.mikro.biologie.tu-muenchen.de (Strunk, 1998)]. For tree calculation only sequences with more than 1300 bp were considered. A phylogenetic tree was constructed using neighbour-joining and maximum-likelihood analyses. Alignment positions at which less than 50% of sequences of the entire set of data had the same residues were excluded from the calculations to prevent uncertain alignments.

DNA base composition. The G + C content of cells of strain T5^T was determined by extracting genomic DNA according to Cashion *et al*. (Cashion, et al., 1977) and subsequent determination of deoxyribonucleosides by HPLC (Mesbah, et al., 1989); (Tamaoka & Komagata, 1984).

DNA-DNA hybridization. DNA-DNA hybridization analysis was performed using the renaturation method of Deley *et al.* (Deley, et al., 1970) with the modifications of Huss *et al.* (Huss, et al., 1983) and Escara & Hutton (Escara & Hutton, 1980). Renaturation rates were computed with the TRANSFER.BAS program by Jahnke (Jahnke, 1992).

RESULTS AND DISCUSSION

Morphology

Colonies of strain T5^T on agar are smooth and convex, with regular edges that become brownish with media containing ferric-citrate (e.g. MB 2216) after 24 h incubation at 20 °C. Older colonies become dark brown. Single cells are ovoid rods, 1·4-1·9 μm by 0·6-0·8 μm (Fig. 1). Strain T5^T was shown to have a Gram-negative cell wall structure by cell lyses after the addition of 3% (w/v) KOH (Gregersen, 1978). Spore formation was absent. Cells are motile by means of polar flagella (the exact number could not be identified).

Physiological characteristics

The results of the physiological tests demonstrated that strain T5^T is a marine, strictly aerobic, and Na^+-requiring bacterium, with growth occurring between 0·01 to below 1·5 M NaCl and optimally at 0·51 to 0·68 M NaCl. Strain T5^T is capable of growth in a pH range of 6·0-9·5, with an optimum at 7·5. Calculated growth rates were maximal between 27–29 °C with μ_{max} = 0·43 under optimal conditions during growth in a fermenter system. Above 36 °C no growth was observed.

The following carbon sources supported cell growth of strain T5^T: D(+)-xylose, D(+)-glucose, D(+)-mannose, D(+)-galactose, D(-)-fructose, D(-)-ribose, D(-)-mannitol, D(-)-sorbitol, D(-)-glucosamin, sucrose, maltose, cellobiose, trehalose, sodium acetate, sodium pyruvate, sodium malate, sodium citrate, disodium succinate, sodium lactate, sodium propionate, sodium butyrate, xylan and glycerol. In addition, strain T5^T showed growth with all amino acids tested (see method section). Further carbon sources that were tested but not utilized are listed in Methods.

Growth experiments with L(+)-arabinose, L(+)-rhamnose, L(-)-fucose, D(-)-sorbitol, D-glucosamine, cellobiose, trehalose, sodium butyrate, disodium succinate, tween 80, leucine DMS, methyl iodide, methyl chloride and betaine confirmed earlier results for *L. methylohalidivorans*, indicating that this organism appears to be an obligate methylotroph, capable of growth on a limited number of substrates (Schaefer, et al., 2002). *L. methylohalidivorans* utilized all tested methylated substrates, however, strain T5^T and *Roseobacter gallaeciensis* were not able to grow with DMS, methyl iodide or methyl chloride. In contrast to strain T5^T, *Roseobacter gallaeciensis* did not grow with betaine.

Strain T5^T and *Roseobacter gallaeciensis* are capable of producing the antibiotic tropodithietic acid (Brinkhoff *et al.*, 2004), which inhibits growth of other bacteria affiliated to different taxa and marine algae. For strain T5^T it was shown that the antibiotic is produced during the exponential growth phase (Brinkhoff *et al.*, 2004). For *Ruegeria algicola, Ruegeria gelatinovorans, L. methylohalidivorans, Roseobacter litoralis* and *Roseobacter denitrificans* no inhibitory effects could be observed (Brinkhoff, et al., 2004); this study)." Cells of strain T5^T and *Roseobacter gallaeciensis* are susceptible to penicillin G, streptomycin sulfate and chloramphenicol. The results of the phenotypic characterization are summarized in Table 1.

Phylogenetic interference.

The DNA G + C content of strain T5^T was determined to be 55·7 mol %. This value is similar to *Roseobacter gallaeciensis* (57·6 – 58 mol %) and *Roseobacter litoralis* (56·3 - 58·1 mol), but differs to a greater extent from the DNA base composition of *L. methylohalidivorans* (60·5 mol %) and *Roseobacter denitrificans* (59·6 mol %). Since 10 difference was proposed as a clear differentiating criterion for species at the genus level (Stackebrandt & Liesack, 1993), these results demonstrate no significant distinctive features.

Phylogenetic analysis of the 16S rRNA gene sequences of *Roseobacter gallaeciensis*, *Roseobacter denitrificans* and *Roseobacter litoralis* indicated that *Roseobacter gallaeciensis* does not affiliate with the other *Roseobacter* species but with *L. methylohalidivorans* as its closest described relative, regardless of which treeing method was used (Fig. 2). Other features also discriminate *Roseobacter gallaeciensis* from *Roseobacter denitrificans* and *Roseobacter litoralis*. *Roseobacter* spp. were originally defined as pink pigmented, aerobic phototrophic bacteria containing Bchl *a* (Shiba, 1991), however, *Roseobacter gallaeciensis* is brown pigmented, non phototrophic, and does not contain Bchl *a*. A former member of the genus *Roseobacter*, *Roseobacter algicola* ATCC 51440^T (Lafay, et al., 1995), isolated from a culture of the toxin-producing dinoflaggelate *Prorocentrum lima*, does not produce Bchl *a* and has since been reclassified as *Ruegeria algicola* (Uchino, et al., 1998). *Roseobacter gallaeciensis* was affiliated to the genus *Roseobacter*, since the 16S rRNA gene sequence of the type strain BS107^T clustered phylogenetically nearest to *Ruegeria algicola*, at a time when the numbers of bacteria and available sequences of the *Roseobacter* clade were significantly lower. The findings mentioned above, as well as further physiological differences listed in Table 1, support the reclassification of *Roseobacter gallaeciensis* into a novel genus.

DNA-DNA hybridization studies revealed low levels of DNA relatedness between *Roseobacter gallaeciensis* and *Roseobacter denitrificans, Roseobacter litoralis, L. methylohalidivorans* as well as *Ruegeria aligola*, which were all below 5% DNA-DNA similarity (Table 1), confirming that *Roseobacter gallaeciensis* does not belong to the genus *Roseobacter*. The hybridization results, differences in pigmentation, antibiotic production, exo-enzyme activities and utilization of a great variety of substrates, though not of methyl compounds, indicate that *Roseobacter gallaeciensis* also does not belong to the genus *Leisingera*, despite a 16S rRNA gene similarity of 97·1%. Thus we conclude that *Roseobacter gallaeciensis* represents a member of a new genus.

The 16S rRNA gene sequences of *Roseobacter gallaeciensis* and strain T5^T differ only at 13 positions (99% similarity). DNA-DNA hybridization analysis, however, revealed a DNA relatedness of 16·3 – 19·6% between *Roseobacter gallaeciensis* and strain T5^T. According to Wayne *et al*. (Wayne, et al., 1987) the phylogenetic definition of a species generally includes organisms with more than 70% DNA-DNA relatedness. Thus, *Roseobacter gallaeciensis* and strain T5^T are clearly distinguishable from each other. These results, as well as differences listed in Table 1, indicate that *Roseobacter gallaeciensis* and strain T5^T are two distinct species of a new genus, *Phaeobacter* gen. nov., for which we propose the names *Phaeobacter gallaeciensis* comb. nov. and *Phaeobacter inhibens* sp. nov. respectively

Description of *Phaeobacter* gen. nov.

Phaeobacter (Phae.o.bac´ter. Gr. adj. *phaeos*, dark, brown; N. L. masc. n. *bacter*, rod; N.L. masc. n. *Phaeobacter*, a brown rod)

Cells are Gram-negative, ovoid rods that multiply by binary fission. Tendency to aggregate in liquid MB 2216 medium. On marine agar 2216 colonies become brownish to dark brown and a diffusible brownish pigment is produced. Salinity optimum range from 0·2 to 0·68 M NaCl. pH optimum is between 7·0 - 7·5. Cells are motile by means of polar flagella. Photosynthetic growth does not occur, Bchl *a* is absent. Metabolism is chemoheterotrophic and obligatly aerobic. Not able to reduce nitrate. No growth was observed with methyl iodide, methyl chloride or DMS. Oxidase and catalase positive, amylase, gelatinase and tweenase negative. The type species is *Phaeobacter gallaeciensis*.

Description of *Phaeobacter gallaeciensis* comb. nov.

Phaeobacter gallaeciensis (L. n. Gallaecia, Galicia, North-West region of Spain; N.L. masc. adj. *gallaeciensis*, pertaining to Galicia)

(*Roseobacter gallaeciensis* (Ruiz-Ponte, et al., 1998). The description is the same as that for *Roseobacter gallaeciensis*, except that the species is sensitive to penicillin G, cells produce the antibiotic tropodithietic acid and growth also occurs with butyrate and methionine as sole carbon sources. No growth with betaine (*Roseobacter gallaeciensis* strain BS107^T)

Description of *Phaeobacter inhibens* sp. nov.

Phaeobacter inhibens (in.hi´bens. L. part. adj. *inhibens* inhibiting).

Cells are Gram-negative, ovoid rods (1·4-1·9 μm x 0·6-0·8 μm) and motile by means of polar flagella. Colonies on agar are smooth and convex, with regular edges that become brownish/ocher on ferric-citrate-containing media after 24-h incubation at 20 °C, and dark brown after incubation for 48 h, with diameter up to 0·8 mm. Tendency to aggregate in liquid MB 2216 medium. Cells grow at temperatures ranging from 4 - 36 °C, with an optimum between 27 - 29 °C, and pH ranging from 6·0 - 9·5 (optimum, 7·5). Cells grow in the presence of 0·01 and with concentrations below 1·5 M Na^+, the optimal salinity is between 0·51 - 0·68 M, no growth was observed without Na^+.

Oxidase and catalase positive, amylase, gelatinase and tweenase negative. Photosynthetic growth does not occur, Bchl *a* is absent. Not able to reduce nitrate. The following substrates are utilized: D(+)-xylose, D(+)-glucose, D(+)-mannose, D(+)-galactose, D(-)-fructose, D(-)-ribose, D(-)-mannitol, D(-)-sorbitol, D(-)-glucosamine, sucrose, maltose, cellobiose, trehalose, sodium acetate, sodium pyruvate, sodium malate, sodium citrate, disodium succinate, sodium lactate, sodium propionate, sodium butyrate, xylan, glycerol, betaine, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamine, L-glutamic acid, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-trytophan, L-tyrosine, and L-valine. Substrates that did not support growth are: L(+)-arabinose, L(+)-rhamnose, L(-)-fucose, lactose, sodium formate, starch, inulin, tween 80, DMS, methyl iodide, and methyl chloride. No vitamin requirements were observed. Cells produce the antibiotic tropodithietic acid during the exponential growth phase, and are susceptible to penicillin G, streptomycin sulfate and chloramphenicol.

The DNA G + C content is 55·7 mol %. Isolated from surface water of a tidal mud flat in the German Wadden Sea, a southern region of the North Sea. Type strain is T5^T (= DSM 16374^T = LMG 22475^T).

ACKNOWLEDGEMENTS

We thank Erko Stackebrandt for helpful discussions, Hans Trüper for help with the epithet and Renate Gahl-Janssen for technical assistance. This work was supported by grants from the Volkswagen Foundation within the Lower Saxonian priority program Marine Biotechnology.

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Table 1. Major properties that differentiate *Phaeobacter gallaeciensis* BS107^T and *Phaeobacter inhibens* T5^T from each other and from related species.

Species: **1**, *Ruegeria algicola* DSM 10251^T; **2**, *Leisingera methylohalidivorans* DSM 14336^T; **3**, *Phaeobacter inhibens* T5 ^{T}; **4**, *Phaeobacter gallaeciensis* BS107^T; **5**, *Roseobacter denitrificans* DSM 7001^T; **6**, *Roseobacter litoralis* DSM 6996^T ; + = positive result or growth, - = negative result or no growth, V = variable, ND = no data available.

Characteristic	**1** ¦	**2** ≠	**3**	**4** †	**5** ‡	**6** ‡
Colony colour	beige/pinkish	no pigment	brown	brown	pink	pink
Bchl *a*	-	- #	-	-	+	+
DNA G + C content (mol %)	60 †	60·5	55·7	57·6 - 58	59·6	56·3 - 58·1
DNA-DNA hybridization with *P. gallaeciensis* BS107^T	2- 4 % †	< 1 %	16·3 – 19·6 %	100 %	2 % †	3 % †
Growth at 4 °C	-	+ #	+	-	+	+
Growth at 37 °C	+	- #	-	+	-	-
Nitrate reduction	-	-	-	-	+ (light)	-
Enzyme activity tested:						
Amylase	(+)	+	-	-	-	-
Tweenase 80	-	- #	-	-	+	+
Gelatinase	+	- #	-	-	+	+
Substrate used:						
Arabinose	+ *	- #	-	-	+ *	V *
Rhamnose	- / + *	- #	-	-	+ *	+ *
Fucose	- / + *	- #	-	ND	+ *	+ *
Cellobiose	-	- #	+	+	- ¦	- *
Trehalose	+	- #	+	+	- ¦	ND
Glucosamine	-	- #	+	-	+ *	+ *
Acetate	-	-	+	+	+	+
Citrate	+	-	+	-	+	+
Succinate	-	- #	+	+	+ / - *	+ / - *
Butyrate	-	- #	+	+ #	-	-
Tween 80	- *	- #	-	- #	+ *	+ *
Glycerol	-	-	+	+	- *	- *
Sorbitol	- / + *	- #	+	+	+ *	+ *
Serine	ND	-	+	-	ND	ND
Leucine	- *	- #	+	+	- *	- *
Methyl iodid	ND	+	-	- #	ND	ND
Methyl chlorid	ND	+	-	- #	ND	ND
DMS	ND	+	-	- #	ND	ND
Betaine	ND	+	+	-	ND	ND
Vitamin requirement:						
Thiamine	+	-	-	+	+	+
Nicotinic acid	-	-	-	-	+	+
Biotin	+	-	-	-	+	+

¦ data from (Lafay, et al., 1995)
† data from (Ruiz-Ponte, et al., 1998)
‡ data from (Shiba, 1991)
≠ data from (Schaefer, et al., 2002)
* data from (Labrenz, et al., 1999)
# this study

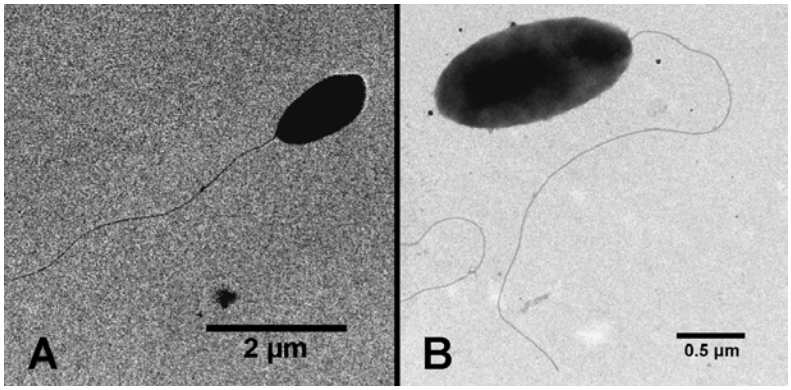

Fig. 1. Electron micrographs of *Phaeobacter inhibens* T5^T. TEM of unstained (A) and negatively stained cells (B) showing the typical rod-shaped cell morphology with a single polar flagellum.

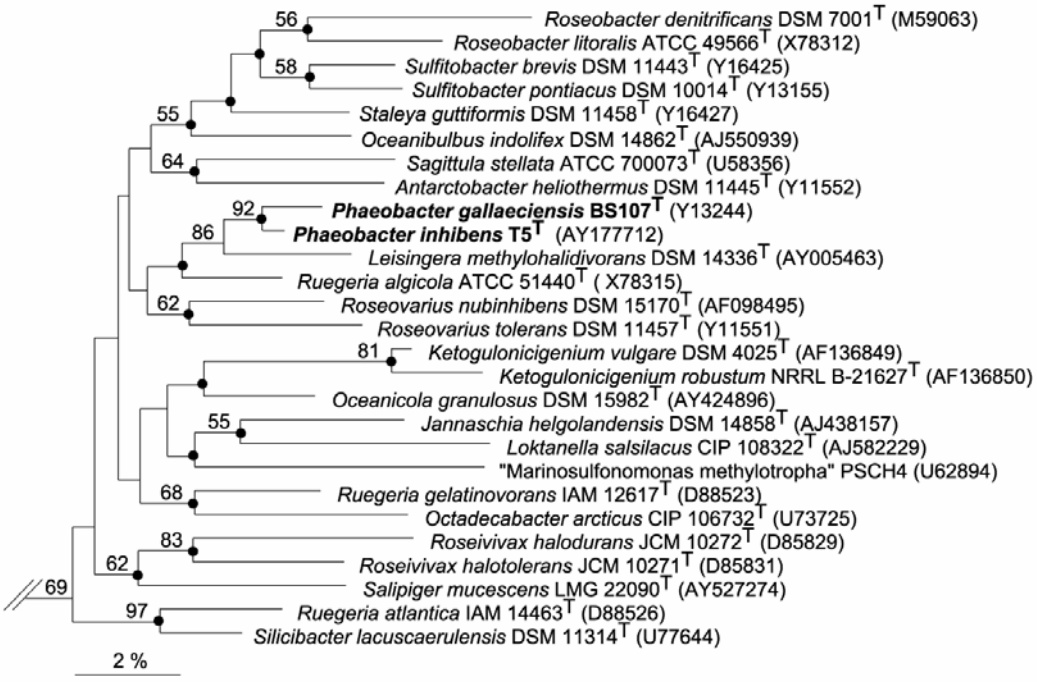

Fig. 2. Neighbour-joining tree showing the phylogenetic relationship between *Phaeobacter inhibens* T5^T, *Phaeobacter gallaeciensis* BS107^T and representatives of the *Rhodobacteraceae* within the class α-*Proteobacteria* based on 16S rRNA gene sequence similarity. Only boostrap values greater than 50% (derived from 2000 replicates) are shown. Filled circles at each node indicate nodes recovered reproducibly with maximum likelihood. Selected members of the class γ-*Proteobacteria* were used as an outgroup to define the root of the tree. The GenBank sequence accession numbers are given in parenthesis. Scale bar indicates 0·02 substitutions per nucleotide position.

VII

Schlussbetrachtung und Ausblick

Schlussbetrachtung und Ausblick

Im Rahmen des niedersächsischen Forschungsschwerpunktes „Marine Biotechnologie“ sollten marine Naturstoffe aus neu isolierten Bakterien gefunden und charakterisiert werden. Schon dessen interdisziplinäre Ausrichtung mit Biotechnologen, Chemikern und Biologen verdeutlicht die Notwendigkeit der Zusammenarbeit, um sich diesem Forschungsziel zu nähern. Ausgangspunkt der gemeinsamen Untersuchung waren erfolgreiche Kultivierungsversuche aus der Nordsee und dem deutschen Wattenmeer, bei denen über 350 verschiedene Bakterienstämme isoliert werden konnten (Brinkhoff, et al., 2004; Stevens, 2004; Grossart, et al., 2004). Viele dieser Isolate stellen nach Vergleich der 16S rRNA Gensequenzen mit den Sequenzen der GenBank Datenbank neue Arten und sogar Gattungen dar. Im Schwerpunkt parallel arbeitende Gruppen konnten über 400 Stämme aus der Nordsee bei Helgoland isolieren (Wagner-Döbler, et al., 2003). Aus der Vielzahl der neuen Isolate wurden im Rahmen der Promotion von Langfang Liang (Liang, 2003) 38 Bakterienstämme einem chemischen Screening unterzogen, und lediglich bei sechs Bakterien konnten die Untersuchungen so intensiviert werden, dass eine Reihe bisher unbekannter Sekundärmetabolite isoliert und charakterisiert werden konnte. Von diesen vier Stämmen wurde einzig für Stamm T5 ein Verfahren zum „scale up“ der Sekundärmetabolit-Produktion eingehend untersucht (Heidorn, 2003). Dies bedeutet, dass trotz einiger Erfolge bei der Entdeckung neuer Sekundärmetabolite die Untersuchungsrate des chemisch biologischen Screenings deutlich langsamer ist, als die Entdeckungsrate neuer Bakterienstämme. Die Beschleunigung des chemischen Screenings wird somit zum entscheidenden Geschwindigkeitsfaktor bei der Suche nach neuen Metaboliten. An dieser Stelle konnten die Ergebnisse der eigenen Arbeiten (siehe Kapitel II und III) sinnvolle Unterstützung leisten und eine Vorauswahl der Stämme ermöglichen. Hierbei wurden in den biologischen Hemmtests eine bioaktive Wirkung gegen Bakterien bei rund 16% aller untersuchten Stämme entdeckt. Dieser Anteil liegt auf dem Niveau von Ergebnissen anderer Arbeitsgruppen im Verbundprojekt. So konnten Wagner-Döbler et al. (2002) in 35 aus 188 Isolaten (19%) eine antibiotische Wirkung gegen Bakterien feststellen. Die Tatsache, dass im Hinblick auf neue Produkte des Sekundärstoffwechsels die *Roseobacter*-Gruppe als eine potente Gruppe anzusehen ist, wurde ebenfalls durch parallel durchgeführte Studien an einzelnen Vertretern dieser Gruppe, sowie der Genomsequenzierung von *Silicibacter pomeroyi* DSS-3 bestätigt (Liang, 2003, Schröder, 2001; Moran, et al., 2004; Wagner-Döbler, et al., 2004). Somit könnte in Zukunft eine intensivere Erforschung der *Roseobacter*-Gruppe die eher

unsystematische Auswahl der Nordsee-Isolate ablösen. Einzig der späte Beginn des PCR-basierten Screenings auf Gene des Sekundärstoffwechsel innerhalb des zweiten Antragzeitraumes, begrenzte die Ausnutzung der Erkenntnisse auf den Stamm GWS-BW-H260 (Liang, 2003) (siehe unten). Insgesamt erwies sich die interdisziplinäre Zusammenarbeit innerhalb der Forschungsschwerpunktes bei der Entdeckung neuer Sekundärmetabolite als förderlich und sinnvoll. Dies zeigt z.B.sich in der Untersuchung des *Streptomyces* Stammes GWS-BW-H5, der bezüglich des PCR-basierten Screenings und der Inhibitionstests unauffällig blieb (siehe Kapitel I). Da frühere Untersuchungen die Gattung *Streptomyces* jedoch als besonders reich an Naturstoffen beschrieben, bot es sich an, das Screening auf weniger gut erforschte Verbindungen auszuweiten. Gemeinsam mit Jeroen Dickschaat und Stephan Schulz wurden in diesem Stamm bisher unbekannte Lacton-Verbindungen entdeckt und charakterisiert. Diese Entdeckung bestätigt Vermutungen, nach denen das Sekundärstoffpotential in dieser Gattung noch nicht ausgeschöpft ist (Watve, et al., 2001). Zusammenfassend war das Projekt von der erfolgreichen Entdeckung neuer Gene und Produkte des Sekundärstoffwechsels gekennzeichnet und bietet die Struktur auch für zukünftige Fragestellungen, die nur interdisziplinär gelöst werden können (siehe Ausblick).

Einsatz spezifischer Primer als Nachweissystem für Sekundärstoffgene

Frühere Studien, die mit Hilfe der PCR Gene des Sekundärstoffwechsels nachweisen konnten, beschränkten sich meist auf enge phylogenetische Mikroorganismengruppen (Christiansen, et al., 2001; Decker, et al., 1996; Neilan, et al., 1999; Sosio, et al., 2000; Hyun, et al., 2000; Moffitt & Neilan, 2003). Der erfolgreiche Einsatz in dieser Arbeit von Primern zur Detektion von Genen des nicht-ribosomalen Polypeptid-Stoffwechsels und der Polyketid Biosynthese in verschiedenen phylogenetischen Bakteriengruppen beweist die Breite der Anwendungsmöglichkeiten dieser Methode. Besonders hervorzuheben ist die Tatsache, dass mit den in früheren Arbeiten publizierten Primern (Rajendran, 1999) keine PCR-Produkte mit Isolaten aus dem Wattenmeer amplifiziert werden konnten. Dies ist mit hoher Wahrscheinlichkeit auf die stark limitierte Anzahl der für das Design benutzten Sequenzen aus gram-positiven Bakterien zurückzuführen. Erst die Berücksichtigung von Gensequenzen der in marinen Habitaten dominierenden *Proteobacteria* ermöglichte die Entwicklung funktionierender Primer.

Gleichzeitig macht dieses Beispiel aber auch auf die Limitierungen der Methode aufmerksam. Letztendlich können nur Genfragmente entdeckt werden, deren Sequenzen homolog sind zu

bereits bekannten Genen. Die Entdeckung von ungewöhnlichen Gen-Anordnungen im Cluster (Funa, et al., 1999; Moore & Hopke, 2001; Shen, 2003) zeigt die Bedeutung, die alternative Methoden wie die Shotgun-Klonierung leisten, da diese Cluster mit einem PCR-basierten Verfahren übersehen worden wären (Ueda, et al., 1995).

Ein weiterer entscheidender Nachteil ist, dass man eine enzymatische Aktivität und das Genprodukt nicht nachweisen kann, somit bleibt das PCR-basierte Screening eine Methode, die als Ergänzung und Erleichterung von chemischen Screening-Verfahren anzusehen ist und erst die Zusammenführung der verschiedenen Methoden schöpft das komplette Potential dieser Untersuchung aus. Ein konkretes Beispiel hierfür ist die Zusammenarbeit mit der Arbeitsgruppe Zeeck (Universität-Göttingen), wo der Nachweis eines NRPS-Gens im Stamm GWS-BW-H260 durch die Isolierung eines bioaktiven Peptides bestätigt wurde (Liang, 2003). Da dieser Stamm in den vorangegangenen Inhibitions-Test unauffällig war, und auch die durch Kultivierung in MB2216 gewonnenen Kulturüberstände allein keine Bioaktivität aufwiesen, wäre dieser Stamm als Produzent neuer Sekundärstoffe nicht zu erkennen gewesen. Während unter Standard-Kultivierungsbedingungen nur sehr geringe Mengen der bioaktiven Substanz produziert wurden, konnte durch die Verwendung eines definierten Mediums mit D-Mannitol und Sojamehl als C-Quelle die Produktion so gesteigert werden, dass eine Isolierung des Produktes ermöglicht wurde.

Für andere Gene des Sekundärstoffwechsels, wie die dNTP Glucose Dehydratase ist das Primer-basierte Screening nur in geringem Maße tauglich. Obwohl schon in früheren Studien die grundsätzliche Durchführbarkeit demonstriert wurde (Decker, et al., 1996; Hyun, et al., 2000), führte ein Sreening an eigenen Isolaten zu PCR-Produkten in mehr als 53% aller untersuchten Stämme (Daten nicht gezeigt). Zwar konnte nachgewiesen werden, dass dNTP Glucose Dehydratase Gene bei der Biosynthese von Sekundärmetaboliten mit Polyketid-Strukturen eine wesentliche Rolle spielen (Chen, et al., 2000b; Amann, et al., 2001; Wang, et al., 2002), woraus sich eine kombinierte Untersuchungsstrategie nach beiden Enzym-Genen anbot. Weitere Untersuchungen zeigten jedoch, dass die dNTP Glucose Dehydratase ebenso im Primärstoffwechsel eine Rolle spielen könnten (Madduri, et al., 2001). Das Enzym besitzt besondere Bedeutung bei der Zellwand-Synthese, auch in Bakterien die bereits als Sekundärstoffproduzenten charakterisiert wurden. Dies könnte eine Erklärung für die Vielzahl an positiven PCR-Ergebnissen sein, erschwert aber die Interpretation der Befunde, sodass von weiteren Untersuchungen in dieser Richtung abgesehen wurde.

Ein wesentlicher Vorteil der molekularbiologischen Verfahren ist der höhere Probendurchsatz im Vergleich zu den chemischen Screening Methoden. Die unter Standardbedingungen oft

unproduktiven Bakterienstämme müssen häufig durch gezielte Veränderung der Medienzusammensetzung und Fermentationsprozesse zur Sekundärstoffsynthese angeregt werden (Marwick, et al., 1999; Bode, et al., 2002). Diese, auch als OSMAC (one strain many compounds)- Ansatz, bezeichnete Methode eröffnet Möglichkeiten, das volle metabolische Potential der Bakterien auszuschöpfen, ist aber zeitaufwendig, da die Variationsmöglichkeiten extrem vielfältig sind. Natürlich sind diese erhöhten Probendurchsatzraten nicht zu vergleichen mit dem industriellen „high-throughput screening“ (über 100000 Proben im Jahr), wobei meistens hoch selektive Testsysteme zum Einsatz kommen. Die hohe Probenanzahl wird vornehmlich durch die äußerst geringe Detektionsrate von 1:10000 diktiert (Wagner-Döbler, et al., 2002). Bei einer Auswahl von weniger als 1000 Stämmen, wie sie z.B. im gemeinsamen Verbundprojekt „marine Biotechnologie“ isoliert wurden, wären die Erfolgsaussichten vermutlich enttäuschend niedrig. So bleiben das PCR-basierte und das chemische Screening mit ihrer geringeren Selektivität und dem niedrigeren Budget-Einsatz nach wie vor attraktive Alternativen für manuelle Untersuchungen an kleinen Kultursammlungen.

Phylogenie und Physiologie der *Roseobacter*-Isolate

Auffällig ist die Diskrepanz zwischen phylogenetischer Ähnlichkeit (ca. 4-5% Unterschied der 16S rRNA Gen-Sequenz) und phenotypischer Heterogenität innerhalb der *Roseobacter*-Gruppe (Vergleich Kapitel VI). Dieser Befund ist unter Bakterien nicht singulär. So gibt es mehrere bekannte Beispiele von Stämmen, welche zwar kaum einen Unterschied in der Gensequenz der 16S rRNA aufweisen, aber dennoch verschiedene ökologische Nischen besetzen und aufgrund dieser als unterschiedliche Arten beschrieben wurden, z.B. *Bacillus anthracis* und *Bacillus cereus* (Ash, et al., 1991) oder *Neisseria meningitis* und *Neisseria gonorrhoeae* (Guibourdenche, et al., 1986). Auch die eigenen Untersuchungen zur Begründung einer neuen Gattung *Phaeobacter* beschäftigten sich mit dieser Problematik. Durch detaillierte Charakterisierung von *R. gallaeciensis* und „*Phaeobacter inhibens*“ und dem Vergleich mit phylogenetisch verwandten Spezies, darunter die phototrophen Vertreter *R. denitrificans* und *R. litoralis*, konnte gezeigt werden, dass das hohe Maß an Übereinstimmung des 16S rRNA Genes nicht mit der gesamtgenetischen und damit auch der physiologischen Ähnlichkeit korrespondierte. Dieser Unterschied kann von den spezifischen Eigenarten der 16S rRNA als phylogenetischer Marker herrühren und die geringe Evolutionsrate mit überwiegend neutralen Substitutionen widerspiegeln (Palys, et al., 1997). Dies bedeutet, dass die Abgrenzung aufgrund von DNA/ DNA Hybridisierung oder 16S

rRNA-Gen Analyse für diese Gruppe weniger aussagekräftig ist, eine Tendenz, die mit der steigenden Anzahl an Isolaten innerhalb einer phylogenetischen Gruppe einhergeht. Während die Analyse der 16S rRNA Gensequenzen bei der Bestimmung möglicher evolutionärer Beziehungen zwischen Organismen sinnvoll ist, bedeutet Taxonomie die Eingruppierung aufgrund von typischen Merkmalen, wie sie allen Vertretern innerhalb eines Taxons eigen sind. Die Ähnlichkeit von 16S rRNA Genen kann daher nur ein weiteres Kriterium, nicht aber das ausschlaggebende Argument sein (Palys, et al., 1997).

Ungeachtet dieser Kritik an der reinen 16 S rRNA-Gen Analyse, bleibt zu klären wie die hohe physiologische Diversität innerhalb der *Roseobacter*-Gruppe entstanden ist. Pradella et al. (2004) fanden in Vertretern der *Roseobacter*-Gruppe bis zu 8 Plasmide. Lineare Plasmide sind weitverbreitet und enthalten Gene für eine Reihe von ökologisch relevanten Stoffwechselmechanismen, wie Abbau von Xenobiotika (Shimizu, et al., 2001), Schwermetall-Resistenz (Ravel, et al., 1998), Antibiotika-Produktion (Netolitzky, et al., 1995) oder Photosynthese (Pradella, et al., 2004) . Jüngste Versuche mit den in dieser Arbeit vorgestellten *Roseobacter*-Isolaten aus dem Wattenmeer wiesen ebenfalls eine Vielzahl an Plasmiden auf (Tim Engelhardt, unveröffentlicht). Etwa 80% der Plasmide besaßen eine lineare Form. Obwohl die Gene auf diesen extrachromosomalen Elementen noch nicht näher untersucht werden konnten, lässt schon allein die Größe der Plasmide von teilweise über 400 kbp komplexe Gencluster vermuten. In der Gesamtheit der Bakterien aus der *Roseobacter*-Gruppe lässt sich eine hoch komplexe und sehr verschiedenartige Zusammensetzung der Plasmide in den einzelnen Stämmen erkennen. Dieses zeigt den starken evolutionären Druck, dem die einzelnen Genome der verschiedenen Organismen unterliegen und der sich in den Genomstrukturen widerspiegelt, wenn man davon ausgeht, dass die Plasmide einen Teil zur hohen physiologischen Vielfalt der *Roseobacter*-Gruppe beitragen.

Diese phenotypische Heterogenität erlaubt es den Vertretern der *Roseobacter*-Gruppe verschiedene ökologische Nischen zu besetzen und könnte auch der Grund sein, warum sich Organismen der *Roseobacter*-Gruppe weit verbreitet haben und z.T. hohen Abundanzen zufinden sind, wie durch verschiedenen Studien belegt wurde. So konnten mit Hilfe der Dot-Blot-Hybridisierung 28% der rDNA aus Proben der Südostküste der USA dem *Roseobacter*-Cluster zugeordnet werden (Gonzalez & Moran, 1997). Rappé und Mitarbeiter fanden, dass 21% der Klonsequenzen aus Proben von Cape Hatteras (USA) zu Sequenzen von *R. denitrificans* und *R. litoralis* ähnlich waren (Rappe, et al., 1997). Aber auch in der Nordsee konnte unter Einsatz spezifischer Sonden nachgewiesen werden, dass ca. 12% der

detektierbaren Bakterien dem *Roseobacter*-Cluster angehörten (Eilers, et al., 2001). Selje et al. (2004) konnten die weltweite Verbreitung eines unkultivierten *Roseobacter*-Cluster nachweisen, deren Vertreter zwischen 20 und 7 % der Gesamtzellzahl in der deutschen Bucht ausmachten.

Das Sekundärstoffpotential mariner Bakterien aus dem Wattenmeer und Überlegungen zur ökologischen Relevanz

Unter den möglichen Quellen für Sekundärmetabolite stellen Bakterien eine der ergiebigsten Ressourcen dar, wobei nur eine überraschend kleine Anzahl Taxa die große Mehrheit der entdeckten Verbindungen synthetisiert. So sind von den 53 bekannten Bakterien-Phyla nur fünf bekannt, die Anti-Infektionswirkstoffe produzieren (Keller & Zengler, 2004). Unter diesen fünf Phyla werden durch Bakterien der Ordnung *Actinomycetales* und insbesondere der Gattung *Streptomyces* die überwiegende Mehrheit an natürlichen Verbindungen synthetisiert. Mit der anfänglichen Beschränkung des PCR-basierten Screenings auf gram-positive Bakterien aus dem deutschen Wattenmeer innerhalb dieser Studie sollten zwei potentielle Ziele erreicht werden. Zum ersten waren zu diesem Zeitpunkt deutlich mehr PKS- und NRPS-Gensequenzen aus gram-positiven Bakterien, insbesondere Streptomyceten, bekannt, was die Wahrscheinlichkeit homologe Gensequenzen zu entdecken erhöhte. Zum anderen waren die *Actinobacteria* und *Firmicutes* terrestrischer Standorte als potente Sekundärstoffproduzenten bekannt, wodurch ein hoher Entdeckungsgrad an neuen Naturstoffen auch in marinen gram-positiven Mikroorganismen erwartet werden sollte. So zeigte sich in früheren Studien, dass marine gram-positive Isolate ein pharmazeutisch interessantes Sekundärstoffpotential besitzen (He, et al., 2001; Feling, et al., 2003; Jensen, et al., 2005) und mit über 100 beschriebenen Verbindungen eine ergiebige Quelle für neue Naturstoffe darstellen (Blunt, et al., 2004). Asolkar et al. (2004) entdeckten in *Janibacter limosus,* welcher aus der Nordsee bei Helgoland isoliert wurde, ebenfalls interessante neue Stoffe. Diese Ergebnisse aus dem vorherigen Abschnitt passen zu den eigenen Untersuchungen, die bei den gram-positiven Isolaten einen deutlich höheren Anteil an Stämmen mit antagonistischer Wirkung (21 %) als bei den alpha-*Proteobacteria* (8,3 %) fanden.

Für die Ergebnisse des PCR-basierten Screenings ergab ein direkter Vergleich zwischen gram-positiven Isolaten und Vertretern der *Roseobacter*-Gruppe allerdings ein anderes Bild für die Bakterienstämme aus dem deutschen Wattenmeer. Hierbei stellten sich die Bakterien der *Roseobacter*-Gruppe als deutlich potenteres phylogenetisches Cluster heraus. Dies steht im Widerspruch zu jüngst veröffentlichten Ergebnissen eines primerbasierten Screenings von

über 200 Referenz-Stämmen der meisten Familien in der Ordnung *Actinomycetales*. Ayus-Sacido und Genilloud (2005) fanden in 56,7 % und 79,5 % aller Stämme PCR-Produkte mit Primern passend für PKS I-, bzw. NRPS-Gene. Während sich die NRPS-Gene in fast allen Gruppen amplifizieren ließen, waren die PKS I Gene in weniger Gattungen konzentriert. Hierbei ist allerdings zu beachten, dass andere Primer eingesetzt wurden und die Spezifität der PCR-Produkte nur für zwei Organismen exemplarisch gezeigt wurde. Die beträchtlichen Unterschiede zu den Ergebnissen dieser Arbeit lassen sich vielleicht am leichtesten durch die Zusammensetzung der untersuchten Stämme erklären. So gehörten ca. 15 % aller gestesteten Stämme der Gattung *Streptomyces* an und insgesamt waren für 19 % schon PKS- oder NRPS-Produkte bekannt.

Dieses starke Potential zur Sekundärstoffbildung ist vermutlich einer der Gründe, warum Bakterien der Ordnung *Actinomycetales* in terrestrischen Habitaten so erfolgreich sind. Actinomyceten machen rund ein Drittel aller beschriebenen Bakterienarten aus (Goodfellow, et al., 1984; Goodfellow, 1989) und kommen in Böden zum Teil in hohen Abundanzen vor (McCaig, et al., 1999; Smalla, et al., 2001; Gremion, et al., 2003). Darüber hinaus spielt die Sekundärstoffproduktion, insbesondere bei Organismen der Gattung *Streptomyces*, häufig eine Rolle bei Symbiosen (Piel, 2004). So schützen Antibiotika-produzierende *Streptomyces* Arten den Nachwuchs von Grabwespen (Boomsma & Aanen, 2005) oder das Nest der Blattschneideameisen vor Parasiten (Currie, et al., 2003).

Auch *Roseobacter* wurden oft in Assoziation mit eukaryotischen Organismen gefunden. *R. dentrificans* und *R. litoralis* wurden von Makrophyten isoliert (Shiba, 1991). *R. algicola* stammt aus einer Dinoflagellatenkultur von *Prorocentrum lima* (Lafay, et al., 1995) und *R. gallaeciensis* von Larven der Muschel *Pecten maximus* (Ruiz-Ponte, et al., 1998)

Eine andere Studie bringt Vertreter des *Roseobacter*-Gruppe als Symbionten der Auster *Crassotrea virginica* mit dem Auftreten von „juvenile oyster desease“ in Verbindung (Boettcher, et al., 2000). Aus Symbiosen von Bakterien mit Schwämmen ist Produktion von antibiotisch wirkenden Verbindungen durch Mikroorganismen (z.B. als Abwehrstrategie) in marinen Habitaten gut untersucht (Faulkner, et al., 2000; Belarbi, et al., 2003). So ist es wahrscheinlich, dass aufgrund zahlreicher Wechselwirkungen mit anderen Organismen verschiedene Arten der *Roseobacter*-Gruppe ebenfalls pro- oder antibiotische Substanzen produzieren. Lafay et al. (Lafay, et al., 1995) vermuteten die Bildung des Toxins Okadasäure durch einen Stamm von *Ruegeria algicola* und Ruiz-Ponte et al. (Ruiz-Ponte, et al., 1999), sowie Hjelm et al. (Hjelm, et al., 2004) zeigten, dass Vertreter der *Roseobacter*-Gruppe eine

antagonistische Wirkung gegenüber verschiedenen γ-*Proteobacteria* besitzen. Über die antibiotisch wirksame Substanz Tropodithietsäure aus „*Phaeobacter inhibens*“ T5 (Brinkhoff, et al., 2004) wurde im Rahmen dieser Arbeit bereits berichtet. Die Ergebnissen unserer Studie untermauern auch die frühere Vermutung, dass weitere *Roseobacter*-Arten zur Synthese von Naturstoffen befähigt sind (Giovannoni & Rappe, 2000) und somit ein noch weitgehend unentdecktes Potential für die Biotechnologie darstellen. Eine der wichtigsten Fragen ist, warum verschiedene Vertreter der *Roseobacter*-Gruppe assoziiert mit eukaryotischen Organismen leben und /oder als Produzenten von antagonistisch wirkenden Verbindungen auftreten. Ein möglicher Erklärungsversuch setzt ein verändertes Konzept der chemischen Verteidigung vor marinen Mikroorganismen bei Eukaryonten voraus. Analog zu den für *Streptomyces* beschriebenen Symbiosen gibt es Beweise, dass symbiotische Proteobakterien ihre Wirte chemisch verteidigen können. So wurde in verschiedenen Experimenten eine erhöhte Resistenz von Eiern des Krebses *Palaemon macrodactylus* gegenüber einem pathogenen Pilz durch Besiedelung mit einem *Alteromonas* Stamm festgestellt (Gil-Turnes, et al., 1989). Eine Ähnliche Abwehrstrategie trat auch beim amerikanischen Hummer *Homarus americanus* auf, dessen Eier mit einem gram-negativen Bakterium kolonisiert waren (Gil-Turnes & Fenical, 1992). Im Gegensatz dazu fanden Grossart et al. (Grossart, et al., 2005) bei der Untersuchung der bakteriellen Gemeinschaft der Diatomeen *Thalassiosira rotula* und *Skeletonema costatum* viele *Roseobacter*-Arten nur in der Phycossphäre, nicht aber auf der Oberfläche wachsender Zellen. In einer anderen Arbeit wurde der Einfluss von angehefteten Bakterien auf die Besiedlung künstlicher Partikel mit verschieden Stämmen untersucht (Grossart, et al., 2003). Der Antibiotika produzierende Vertreter der *Roseobacter*-Gruppe, Stamm T5, verhinderte nicht die Besiedlung von Partikeln mit Bakterien, gegen die er in Inhibitionstests mit Agarplatten eine antagonistische Wirkung zeigte. In einem anschließenden Vergleichsexperiment mit einer Mutante von T5, die nicht mehr in Lage war, das entsprechenden Antibiotikum zu produzieren, wurden keine Unterschiede gegenüber dem Wildtyp bezüglich der Bakterienbesiedlung festgestellt. Allerdings war die Dauer dieser Experimente auf 160 Minuten begrenzt, möglicherweise zu kurz, um einen antagonistischen Effekt zu beobachten. Ob die oben beschriebene mutualistischen Symbiose, obwohl grundsätzlich möglich, auch von *Roseobacter*-Stämmen genutzt wird, muss angesichts dieser Experimente offen bleiben.

Ausblick

Im Zuge dieser Arbeit konnten wertvolle Erkenntnisse über die Möglichkeit der tatsächlichen und potentiellen Sekundärstoffproduktion mariner Bakteriengemeinschaften des deutschen Wattenmeeres gewonnen werden. Viele der von uns untersuchten Stämme zeigten in herkömmlichen chemischen oder biologischen Tests jedoch keine oder nur geringe Produktion der postulierten Verbindungen. Eine der großen Herausforderungen für die Zukunft bleibt es daher, die neuen Naturstoffe auch zu isolieren.

Mit ausgewählten Stämmen, bei denen Nachweise auf genetischer und zum Teil bereits auch auf chemischer Ebene für die Produktion vermutlich neuer Sekundärstoffe vorliegen, kann untersucht werden, unter welchen Bedingungen die nachgewiesenen Gene abgelesen werden und wie die Produktion dieser Substanzen durch unterschiedliche Kultivierungbedingungen optimiert werden kann..

Wie sich aus der Vielzahl der beschriebenen Verfahren (u.a. Bode, et al., 2002; Grond, et al., 2002; Demain, 1998; Nakata, et al., 1999: Chen, et al., 2000a: Sashihara, et al., 2001) erkennen lässt, gibt es keine allgemeine Vorgehensweise für die Induktion und Optimierung der Sekundärstoffproduktion von Bakterien. Der Nachweis einer erfolgreichen Induktion eines bestimmten Stoffwechselweges lässt sich heute aber zumindest sehr gezielt verfolgen, wenn man die beteiligten Gene und deren Sequenzen kennt. Dabei ist die *messenger* RNA (mRNA) als Produkt der Genexpression mit einer sehr hohen Umsatzrate der Schlüssel für diesen Nachweis, da man die Menge an mRNA als direktes Maß der Transkriptionsaktivität in der Zelle werten kann (z.B., Dawes, et al., 2003; Neretin, et al., 2003; Rokbi, et al., 2001; Savli, et al., 2003). Die *real-time* RT (*reverse transcriptase*)-PCR ist aufgrund der höheren Spezifität und des deutlich geringeren Zeitaufwands anderen Methoden wie *Northern* Hybridisierung, *Ribonuclease Protection Assay* und Kompetitiver Reverser Transkriptions PCR (RT-PCR) vorzuziehen (Ferré, 1998). Die zu erwartenden Ergebnisse könnten zur Lösung der schwierigsten Probleme des chemischen Screenings beitragen, nämlich die zu geringen Produktausbeuten und die redundante Isolierung bereits bekannter Naturstoffe.

Auf diese Weise ließen sich die postulierten Verbindungen gewinnen und anschließend chemisch charakterisieren. Mit den synthetisierten Verbindungen könnten gezielte Tests bezüglich ihrer biologischen Wirkung auf andere Organismen durchgeführt werden, wie dies ansatzweise für die Lactone aus Stamm GWS-BW-H5 bereits geschehen ist (Kapitel IV). Die Gewinnung der Produkte bildet somit die Grundlage für den nächsten wichtigen Schritt,

nämlich die ökologische Bedeutung der Naturstoffe eingehend zu untersuchen und zu verstehen.

Die in dieser Studie sequenzierten PKS-Gene der *Roseobacter*-Gruppe wiesen auch untereinander hohe Ähnlichkeiten auf und bilden ein phylogenetisches Cluster (Abb. 2 Kapitel III). Bisher ist spekulativ, ob dieser Befund die phylogenetische Ähnlichkeit der untersuchten Organismen widerspiegelt oder als Hinweis auf lateralen Gentransfer verstanden werden muss. Dieselben Isolate wiesen auch einen hohen Plasmidgehalt (durchschnittlich 6 pro Stamm) auf. Mit Hilfe der Gensequenzen aus diesen Isolaten könnten Sonden für eine Hybridisierung hergestellt werden, die Aufschluss darüber gäben ob die gesuchten Gene auf extrachromosomalen Elementen liegen. Dies würde unser Wissen über die Entwicklung oder auch Verbreitung der Gene für Sekundärstoffproduktion erweitern und Fragen nach der Homologie der entdeckten Gene beantworten helfen.

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Danksagung

Ganz herzlich möchte ich mich bei Meinhard Simon bedanken, der mir viel Vertrauen entgegen gebracht, meine manchmal schroffe Art ertragen, und der trotz widriger Finanzierungsmöglichkeiten immer wieder einen Weg für eine Fortsetzung dieser Arbeit geschaffen hat.
Außerdem danke ich Prof. Dr. Heribert Cypionka für die freundliche und unkomplizierte Übernahme des zweiten Gutachtens dieser Arbeit, und dass er Jacqueline Süß eingestellt hat.....

Ganz besonderer Dank gilt Thorsten Brinkhoff für fruchtbare Diskussionen und guten Ideen. Er hat mich die letzten Jahre mit seiner sprichwörtlichen Geduld ertragen und mir auf verschiedenste Weise geholfen. Ich erinnere mich gerne an gemeinsame Tagungen und den ein oder anderen nützlichen Tip fürs (Forscher-) Leben.

Im Rahmen der gemeinsam veröffentlichten Arbeiten gilt mein Dank Prof. Dr. Lone Gram, in deren Labor die AHL-Screenings durchgeführt wurden, für wertvolle Beiträge zur Diskussion. Ebenso gilt mein Dank Jeroen Dickschat und Prof. Dr. Stephan Schulz für die gute Zusammenarbeit und den netten Austausch während meines Besuches in Braunschweig.

Ohne den besonderen Einsatz von Renate Gahl-Janssen während der letzten Phase dieser Arbeit, wäre ich wohl nie fertig geworden, dafür mein herzlicher Dank.

Bedanken möchte ich mich auch bei allen anderen Kollegen meiner Arbeitsgruppe, für gute fachliche Zusammenarbeit, praktische Hilfe im Labor, für Fragen und Antworten jeder Art. Es gab manches soziale Auf und Ab zu überwinden, ich denke dies ist uns gemeinsam gelungen. Danke für gelungene Inselausflüge, Grillparties, philosophische Bürogespräche, feucht fröhliche Kohlfahrten, also für alles was das gemeinsame Arbeitsleben aufregend und lustig machte.

Ebenso bedanke ich mich bei den „Cypis“ meiner netten Nachbararbeitsgruppe. Ihr gabt mir ein Asyl in Zeiten der Not ☺ , wart bei Fragen immer für mich da. Was wäre ich ohne euren Chemikalien-Schrank!

Lebenslauf

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Erklärung:

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst und nur die angegebenen Quellen und Hilfsmittel verwendet habe.

Oldenburg, 14. Juli 2005