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# Beneficial effects of 2,4-diacetylphloroglucinol-producing pseudomonads on the marine alga *Saccharina latissima*

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ABSTRACT: *Pseudomonas* strains were shown to be regularly associated with the brown macroalga *Saccharina latissima* from the Baltic Sea, studied over several years, and were identified as producers of the antimicrobially active compound 2,4-diacetylphloroglucinol. These findings support the assumption of a stable association between the *Pseudomonas* spp. strains and *S. latissima* in the Baltic Sea. The metabolite profile of the *Pseudomonas* spp. comprised monoacetylphloroglucinol, 2,4-diacetylphloroglucinol, pyoluteorin and several rhizoxins, which exhibited broad-spectrum antibiotic activities against Gram-positive and Gram-negative bacteria as well as against fungi. Because the antibiotic activities included the inhibition of the 2 algal pathogens *Pseudoalteromonas elyakovii* and *Algicola bacteriolytica*, we propose a beneficial effect of these marine pseudomonads on their host *S. latissima*.

KEY WORDS: *Pseudomonas protegens* · Alga–bacteria association · 2,4-diacetylphloroglucinol · Pyoluteorin · Rhizoxin · Brown macroalgae



## INTRODUCTION

Marine macroalgae continuously come into contact with microorganisms (Potin 2002), which eventually colonise the algal surfaces. As a result of numerous biological interactions, complex and changeable communities have evolved on macroalgal surfaces (Goecke et al. 2010). Given this and the fact that algae lack cell-based immune responses, it is reasonable to postulate the production of bioactive secondary metabolites as a fundamental defence mechanism against microbial attack (Engel et al. 2002, Thomas et al. 2008). It is well known that many substances within microbe–host interactions are produced by the microorganisms (Jensen & Fenical 1994, Schmidt 2005, König et al. 2006, Egan et al. 2008, Lane & Kubanek 2008). *Pseudomonas* species are well known producers of bioactive compounds (Bérdy 2005) with diverse chemical structures (Isnansetyo & Kamei 2009), as reflected by the ca. 900 compounds described in the Dictionary of Natural Products (Buckingham 2011).

Though marine *Pseudomonas* isolates have been obtained from various marine macroorganisms, such as tunicates, sponges, and turtle grass but also red algae (Isnansetyo et al. 2001, Isnansetyo & Kamei 2009), their secondary metabolites are not well studied (Isnansetyo & Kamei 2009). As an example, the lethal activity of marine *Pseudomonas stutzeri* against the red tide toxic phytoplankton *Chattonella antiqua* has been reported (Hayashida et al. 1991). Neither deleterious effects nor beneficial interactions with macroalgae have been reported for *Pseudomonas* species prior to the present study.

The bacterial communities of the brown macroalga *Saccharina latissima* (synonym *Laminaria sac-*

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*charina*) harbour a large number of antimicrobially active bacteria, including several *Pseudomonas* species (Wiese et al. 2009). Secondary metabolites are proposed to positively affect the survival of their producers (for review, see Goecke et al. 2010). Also, the host may benefit from inhibition of competing, degrading and potentially pathogenic surface-colonising microorganisms. The complex situation of microbe–macroorganism interaction is poorly understood, especially *in situ*. However, more and more evidence has recently been provided by studies on the effect of microbes on their hosts (see Goecke et al. 2010). The present study contributes to the understanding of these interactions using the system of the marine alga *S. latissima* and its associated bacteria: we have identified several secondary metabolites produced by pseudomonads isolated from *S. latissima*. Their production is considered to be in favour of both the producing bacteria and their host, as described for other systems (see Goecke et al. 2010). Algae are afflicted by diseases, like many other living organisms. However, basic information concerning their pathogens is lacking (Gachon et al. 2010). Because 2,4-diacetyl-phloroglucinol (DAPG), monoacetylphloroglucinol (MAPG) and pyoluteorin (PLT) are active against *Pseudoalteromonas elyakovii* and *Algicola bacteriolytica*, which are hypothesized to cause disease of *Saccharina japonica* (Sawabe et al. 1998, 2000), we suppose that through production of these antibiotic compounds, the marine pseudomonads may be beneficial for the macroalgal host.

## MATERIALS AND METHODS

### Collection of algae

Live specimen of *Saccharina latissima* (phyllosphere) were collected in October 2002, May 2003 and January 2010 in the Kiel Fjord, Baltic Sea (Germany) at 6 m depth by SCUBA divers. Additionally, in 2010, 5 specimens each of the rhizosphere and *S. latissima* were taken from the same habitat.

### Isolation and identification of antimicrobially active *Pseudomonas* spp.

In 2002 and 2003, bacteria were isolated from *Saccharina latissima* according to Wiese et al. (2009). From a greater number of isolates, 13 *Pseudomonas* strains demonstrated antibiotic activity and were selected for investigation of secondary metabolite production. Three further isolates were chosen from the collection of *Pseudomonas* spp. strains obtained in 2010 by the following procedure: The algae were homogenised, and the suspensions of different dilution series as well as the rhizosphere suspension were plated on 3 different media: (1) Medium G, GSP agar according to Kielwein (1971), (2) Medium P prepared according to Kalinovskaya et al. (2004) and modified by supplementation with 18 g Tropic Marin sea salt (Dr. Biener) and 15 g Bacto agar in 1 l deionised water, pH 7.6, and (3) Medium C, a *Pseudomonas* selective agar base supplemented with *Pseudomonas* CFC selective supplement (Merck), pH 7.2. Incubation was carried out in the dark at 28°C for 5 d. The light regime of the sampling site was not considered as a parameter for setting the culture conditions. Pure cultures were obtained by subsequent plating steps on Bacto marine broth agar (MB) with 37.4 g $l^{-1}$ marine broth and 15 g $l^{-1}$ agar. The pure cultures were stored at −80°C using the Cryobank System (Mast Diagnostica), according to the manufacturer's instructions. '*Pseudomonas*-like colonies' (as determined by growth on selective media, colony form and microscopic analysis) were identified by 16S rRNA gene sequence analysis according to Thiel et al. (2007) using the Basic Local Alignment Search Tool with the European Molecular Biology Laboratory (EMBL) nucleotide database (Altschul et al. 1990) and the Ribosomal Database Project II (RDP-II) website (Cole et al. 2007).

### Strain identification and phylogenetic analysis

The Ribosomal Database Project database was used to select the next related type strains (http://rdp.cme.msu.edu; Cole et al. 2009). Sequence similarity values were determined with the 'bl2seq' tool of the National Center for Biotechnology Information database (http://blast.ncbi.nlm.nih.gov/; Tatusova & Madden 1999). The alignment of the 16S rRNA gene sequences of the strains LD45, LD46, LD47, LD49, LD51, LD52, LD53, LM59, LB062a, LD115, LD118, LD119, LD120, LB183, LB184 and LB185, including type strains of *Pseudomonas fluorescens* and *Azotobacter vinelandii*, was performed with Clustal X (Larkin et al. 2007) and refined manually. *A. vinelandii* was selected as an outgroup. All sequences were cut to the same length of 1401 base pairs. LD120, LB183 and LB184 were selected as representatives of the alga-associated isolates for the phylogenetic analysis. The phylogenetic trees were calcu-

lated by the neighbour-joining method (Saitou & Nei 1987) and maximum-likelihood method (Felsenstein 1981) using MEGA version 3.1 (Kumar et al. 2004) and PhyML Online (Guindon et al. 2005), respectively. A total of 1000 bootstrap replicates were applied for the neighbour-joining analysis and 500 bootstrap replicates for the maximum-likelihood analysis. The maximum-likelihood tree was calculated using the GTR model and an estimated proportion of invariable sites as well as the Gamma distribution parameter. NJplot was used to display the phylogenetic trees (http://pbil.univ-lyon1.fr/software/njplot.html; Perrière & Gouy 1996).

Additionally, to confirm the phylogenetic identification by 16S rRNA gene sequence analysis, strain LD120 was examined for its physiological characteristics and pigment production. For the physiological characterisation, the utilisation of 95 carbon sources by strain LD120 was examined with the GN2 MicroPlate system (BIOLOG) following the manufacturer's instructions in duplicates. Biolog GN2 plates contain 96 wells. Each well, except for a negative control (water), contains a unique carbon source and the metabolic indicator tetrazolium violet. A colour change of tetrazolium violet allows identification of metabolism based on the exchange of electrons generated during respiration. The results of strain LD120 were compared to the phenotypic characterisation chart according to Rico & Preston (2008).

Pigment production is a characteristic of fluorescent pseudomonads (Stanier et al. 1966). Strain LD120 was grown on Medium P for 3 d at 28°C in the dark and examined for the production of fluorescein using a UV lamp (366 nm).

### Substrate utilisation of *Pseudomonas* sp. strain LD120

To prove whether strain LD120 is able to digest algal compounds, it was grown on minimal medium containing 1.5% (w/v) Bacto agar for solidification and the following substrates (1% (w/v)): sodium alginate, cellulose, mannitol, glucose, freeze dried *Saccharina latissima* (powder), agarose, DL sodium malate, sodium succinate, sodium fumarate and L sodium glutamate. All substrates were dissolved in sterile sea water with salinity according to the sampling site. Inoculation was accomplished by streaking a 5 d old culture from an MB agar plate. Growth was monitored for 12 d in the dark at 28°C. As a control for growth, cultures were grown on MB agar.

### Selection of DAPG-producing *Pseudomonas* strains

According to their production of DAPG, 16 *Pseudomonas* strains were selected, all of which showed a colour change of MB agar plates indicating DAPG production as reported by Bangera & Thomashow (1996) and Keel et al. (1996). DAPG production in culture extracts was verified by high-performance liquid chromatography (HPLC) with diode-array detection and mass spectrometry (HPLC-DAD-MS) analysis (Bonsall et al. 1997).

### Cultivation and extraction of agar plates

Cultures of all strains were grown for 7 d at 28°C in the dark on MB agar medium until a dense bacterial lawn was obtained on the plates. The cultures, including the agar, were homogenised and extracted using 20 ml ethyl acetate. The organic phase was separated, dried *in vacuo* and subsequently dissolved in 200 µl methanol. These extracts were used for chemical analyses.

### Cultivation of *Pseudomonas* strain LD120

*Pseudomonas* strain LD120 was chosen for isolation of substances and was grown in 2 l Erlenmeyer flasks each containing 1 l of TM medium (5.0 g $l^{-1}$ papaic digest of soybean meal, 1.0 g $l^{-1}$ Bacto yeast extract, 30.0 g $l^{-1}$ Tropic Marin sea salt and 1 l deionised water at pH 7.7) for 48 h at 28°C under shaking conditions (120 rpm). Each culture was inoculated separately with a 2 $cm^2$ piece from a preculture grown on agar plates (20 d old, 25°C, dark). A total of 10 l of culture broth was extracted with the same volume of ethyl acetate. The extract was the source of compounds for chemical analyses and antimicrobial testing.

### Analytical HPLC analyses

Chemical analyses and dereplication of substances was done according to Schneemann et al. (2010b) by comparison of MS and UV data obtained by HPLC-UV-MS analyses. Reversed phase HPLC experiments were performed using a C18 column (Phenomenex Onyx Monolithic C18, 100 × 3.00 mm) applying an $H_2O$ (A)/acetonitrile ($C_2H_3N$) (B) gradient with 0.1% HCOOH added to both solvents (gradient 0 min 5% B, 4 min 60% B, 6 min 100% B; flow 2 ml $min^{-1}$) on a

VWR Hitachi Elite LaChrom system coupled to an ESI-ion trap detector (Esquire 4000, Bruker Daltonics). The retention times of MAPG, DAPG and PLT were 1.9 to 2.1 min, 3.5 to 3.7 min and 3.0 to 3.1 min, respectively. Rhizoxins eluted between 3.5 and 4.9 min.

### Preparative HPLC analyses

The extract was subjected to preparative HPLC (eluents: $H_2O$ [A], acetonitrile [B]; gradient 0 min 20% B, 18 min 100% B; flow 15 ml $min^{-1}$). MAPG, DAPG and PLT eluted at 5.7 to 5.9 min, 7.0 to 7.2 min and 8.6 to 8.8 min, respectively. Preparative HPLC was carried out using a Merck Hitachi system consisting of an L-7150 pump, an L-2200 autosampler and an L-2450 diode array detector with a Phenomenex Gemini-NX C18 column (5 µm, 110 Å, Axia packed; 100 × 21.20 mm).

### Antimicrobial assays

Antimicrobial assays were performed using 18 test strains (see Table 3). Among them were *Algicola bacteriolytica*, which is hypothesized to be the causative agent of red-spot disease in the brown alga *Saccharina japonica* (Sawabe et al. 1998), and *Pseudoalteromonas elyakovii*, an alginolytic marine bacterium isolated from a spot-diseased *S. japonica* (Sawabe et al. 2000). Both *Trichophyton* species (see Table 3) were from the Department of Dermatology, Allergology and Venerology, University Hospital Schleswig-Holstein, Kiel, Germany.

For cultivation of the test strains, the following media were used: (1) TSB3+ medium (3 g $l^{-1}$ tryptic soy broth, 5 g $l^{-1}$ NaCl), (2) TSB12+ medium (12 g $l^{-1}$ tryptic soy broth, 5 g $l^{-1}$ NaCl), (3) M1 medium (5 g $l^{-1}$ Bacto-peptone, 3 g $l^{-1}$ meat extract, pH 7.0), (4) modified M186 medium (1 g $l^{-1}$ yeast extract, 1 g $l^{-1}$ malt extract, 1.67 g $l^{-1}$ peptone from soybeans, 3.3 g $l^{-1}$ glucose), (5) PGY medium (5 g trypticase peptone, 5 g Bacto-peptone, 10 g yeast extract, 5 g beef extract [BD], 5 g glucose, 2 g $KH_2PO_4$, 1 ml Tween 80, 40 ml salt solution, 10 ml haemin solution, 0.2 ml vitamin $K_1$ solution, 0.5 g cysteine-HCl × $H_2O$, 950 ml deionised water, pH 7.2; salt solution: 0.25 g $CaCl_2 \times 2\ H_2O$, 0.5 g $MgSO_4 \times 7\ H_2O$, 1 g $K_2HPO_4$, 1 g $KH_2PO_4$, 10 g $NaHCO_3$, 2 g NaCl, 1000 ml deionised water; haemin solution: 50 mg haemin, 1 ml 1 N NaOH, add distilled water to 100 ml; vitamin $K_1$ solution: 0.1 ml vitamin $K_1$, 20 ml 95% ethanol), (6) MA medium (20 g malt extract, 1000 ml deionised water), (7) PM medium (150 g $l^{-1}$ pea, 5 g $l^{-1}$ glucose, pH 6.5), (8) MYA medium (10 g malt extract, 4 g yeast extract, 4 g glucose, pH 5.6) and (9) SA medium (10 g $l^{-1}$ Bacto-peptone, 20 g $l^{-1}$ glucose, pH 5.6).

To obtain spores of the fungal strains for the subsequent testing, the method according to F. Horter (pers. comm.) was used: Agar plates with 2 wk old cultures were covered with 10 ml of sterile 10 mM $Na_2HPO_4$ (pH 7.3). Using a Drigalski-spatula, the spores were scraped off of the plates. The spore suspension was poured through a gauze bandage and washed 2 times with 10 mM $Na_2HPO_4$ (pH 7.3).

*Pseudomonas* sp. LD120 metabolites were adjusted to 10 mM. For all tests, a 1:5 dilution (except for *P. infestans* with a 1:10 dilution) in phosphate-buffered saline (8 g $l^{-1}$ NaCl, 0.2 g $l^{-1}$ KCL, 1.44 g $l^{-1}$ $Na_2HPO_4$ and 0.24 g $l^{-1}$ $KH_2PO_4$, pH 7.4) was used.

For activity tests, 96 well microtiter plates were used. Aliquots of 10.5 µl of the metabolite dilution and 200 µl of an overnight culture of the test strain or spore suspension were used in triplicate. Non-inoculated medium, chloramphenicol (100 µM) or cycloheximide (100 µM), was used as the positive control; the test strains alone were used as negative controls. Bacterial growth and cell viability were estimated by measuring the optical density at 600 nm and using the resazurin/resorufin method as modified by Schneemann et al. (2010b). Specific conditions for the different test strains concerning the optical density of the inoculum, medium used, microtiter plate incubation temperature, aeration, light conditions, incubation time and incubation conditions are listed in Appendix 1. The assay against *Propionibacterium acnes* was performed according to Schneemann et al. (2010a). The inhibition of fungal strain growth was determined by measuring the optical density at 600 nm after 48 or 72 h.

## RESULTS

### Phylogenetic identification of *Pseudomonas* strains from *Saccharina latissima*

Among a larger number of bacteria isolated from the phyllosphere and rhizosphere of *Saccharina latissima* specimens from the Baltic Sea, 16 strains were affiliated to the genus *Pseudomonas* according to 16S rRNA gene sequences. These isolates originated from different sampling campaigns. Nine *Pseudo-*

*monas* strains were isolated from the phyllosphere of the alga sampled in November 2002: strains LD45 (AM913891), LD46 (AM913892), LD47 (AM913893), LD49 (AM913894), LD51 (AM913895), LD52 (AM913896) and LD53 (AM913897), LM 59 (AM913898) and LM63 (AM913900). Four strains were obtained from phyllosphere samples in May 2003: LB062a (FR686532), LD155 (AM913903), LD118 (AM913943) and LD120 (AM913905). Three further strains were isolated in January 2010: strain LB183 (FR675975) from the phyllosphere and strains LB184 (FR675976) and LB185 (FR675977) from the rhizosphere. The accession numbers of the 16S rRNA gene sequences in the EMBL database are given in parentheses.

A neighbour-joining tree was calculated to show the phylogenetic relationship of the strains (Fig. 1). The phylogenetic analysis using the maximum-likelihood method revealed the same results (data not shown). Two groups of strains were found. Most of the *Pseudomonas* strains associated with *Saccharina latissima* affiliated to *P. protegens* CHA0$^T$ with a sequence similarity of >99.5% (Fig. 1), which also includes some strains previously misclassified as *P. fluorescens* (such as strain Pf-5) (Ramette et al. 2011). We refer to these strains as the 'LD120-group' because their 16S rRNA gene sequences showed a similarity of 100% to each other. The second group with strains LB183 and LB184 affiliated to *P. brassicacearum* DBK11$^T$ (AF100321) and formed a separate cluster with similarity ranges from 97.5 to 97.8% to the 'LD120 group'.

## Metabolical and biochemical characterisation of strain LD120

Because differentiation of closely related *Pseudomonas* species cannot be accomplished clearly based on 16S rRNA gene sequences (Peix et al. 2009), further experiments were carried out. As is typical for fluorescent *Pseudomonas* strains, LD120 produced a fluorescent yellowish-green-coloured pigment that diffused into the agar and showed fluorescence under the UV light. According to the Biolog system, the substrate utilisation pattern of

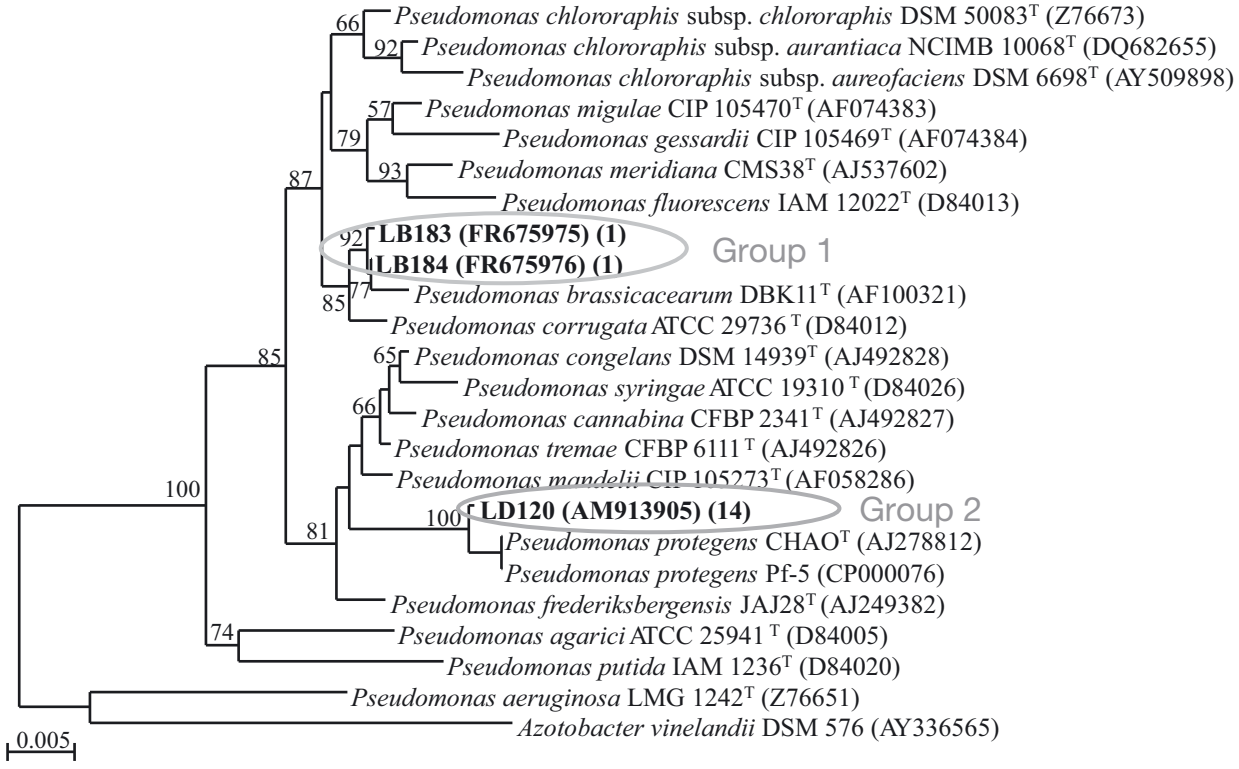


Fig. 1. Phylogenetic analysis of *Pseudomonas* strains associated with *Saccharina latissima* using the neighbour-joining method. Bootstrap values are given as percentages (only numbers above 50% are shown). The scale bar indicates the number of substitutions per nucleotide position. The strains isolated in the present study are highlighted in **bold**, and the number of isolated strains is shown in parentheses. Group 2, i.e. the 'LD120 group', includes sequences from the isolates LD45, LD46, LD47, LD49, LD51, LD52, LD53, LM59, LB062a, LD115, LD118, LD119, and LB185

*Pseudomonas* sp. LD120 was 89% similar to that of *P. protegens* Pf-5 as reported by Rico & Preston (2008). Values of 86 and 76% similarity to the substrate utilisation patterns of *P. putida* KT2440 and *P. syringae* pathovar tomato DC3000, respectively, were detected. Furthermore, strain LD120 produced the metabolites MAPG, DAPG, PLT, which are known products from *P. protegens* (Keel et al. 1996, Ramette et al. 2011). On the basis of its chemical and biochemical properties, strain LD120 was classified as a strain of *P. protegens*. According to the phylogenetic and chemical analyses, the strains from the LD120 group were classified as strains of *P. protegens* as well.

Growth of *Pseudomonas* sp. LD120 was not detected on sodium alginate, cellulose, freeze-dried *S. latissima* powder, agarose or agar, indicating that strain LD120 was not able to digest polymeric sugars originating from the alga. On Biolog GN2 microplates, the strain LD120 utilizes a wide spectrum of carbon sources, including a number of sugars (e.g. D-fructose, α-D-glucose, D-mannose, sucrose, D-gluconic acid) and amino acids (e.g. L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, γ-aminobutyric acid, D-alanine, L-alanine, L-leucine, L-ornithine, L-threonine, L-alanyl-glycine and glycyl-L-glutamic acid). Growth occurred on minimal media with sodium salts of DL-malate, succinate, fumarate, citric acid and α-ketoglutaric acid as substrates, with the strain showing a clear preference for dicarboxylic acids (citric acid cycle compounds) and complex media (marine broth) containing proteins.

### Secondary metabolites produced by *Pseudomonas* strains

Concerning the strains' secondary metabolite production, 4 different production patterns of MAPG, DAPG, PLT and the rhizoxins were obtained within the *Pseudomonas* strains, as shown in Table 1. While the majority of the strains produced at least 2 of these metabolites in several combinations, some produced only 1 of the compounds. Strain LD120 showed the broadest metabolite pattern with the production of all 4 compounds. Therefore, strain LD120 was chosen for further analyses.

### Chemical analysis of culture extracts

The secondary metabolite pattern of extracts of strain LD120 showed 15 substances (Table 2), 12 of which were identified, with MAPG, DAPG and PLT as major compounds and mainly various rhizoxin derivatives as minor compounds.

### Antimicrobial activities of the major secondary metabolites

A comprehensive panel of test strains was used to study the biological activities of the isolated compounds. The selection included bacteria from a variety of groups that are present on macroalgae in the Baltic Sea (Staufenberger et al. 2008, Wiese et al. 2009), algal and plant pathogens and medically relevant strains. This broad panel should give information to indicate a possible ecological function of the compounds and identify the potential for eventual biotechnological applications.

The metabolites isolated from strain LD120 exhibited various antimicrobial activities against different test organisms (Table 3). All metabolites (Table 1) were active against the 2 tested Gram-positive bacteria, but only PLT was also active against several Gram-negative bacteria. In addition to antibacterial activities, MAPG and DAPG exhibited activities against fungi, especially against the plant pathogens *Septoria tritici* and *Phytophthora infestans* as well as against the 2 dermatophytes *Trichophyton mentagrophytes* and *T. rubrum*. In addition, DAPG was weakly active against the yeast *Candida glabrata* and the phytopathogenic fungus *Botrytis cinerea*. PLT was active against the plant pathogens *Pseudomonas syringae*, *Erwinia amylovora*, *Ralstonia solanacearum* and *Xanthomonas campestris* as well as the algal pathogens *Algicola bacteriolytica* and *Pseudoalteromonas elyakovii,* whereas DAPG showed inhibition of *E. amylovora* and *A. bacteriolytica*.

Table 1. Patterns of secondary metabolites produced by *Pseudomonas* strains as revealed by HPLC-UV-MS analyses. MAPG: monoacetylphloroglucinol, DAPG: 2,4-diacetylphloroglucinol, PLT: pyoluteorin, +: production of metabolite was detected, –: production of metabolite was not detected

| Strain | MAPG | DAPG | PLT | Rhizoxins |
|---|---|---|---|---|
| LD120 | + | + | + | + |
| 13 strains[a] | – | + | + | – |
| LB184 | – | + | – | – |
| LB183 | + | + | – | – |

[a]Strains LB062a, LB185, LD46, LD47, LD49, LD51, LD52, LD53, LD115, LD118, LM59, LM63, LD45

Table 2. *Pseudomonas protegens.* Secondary metabolites identified in cultures of strain LD120. MS: mass spectrometry, UV: ultraviolet detection, $^{1}$H-NMR: proton nuclear magnetic resonance; m/z: mass-to-charge ratio; $[M+H]^+$: protonated molecular mass

| No. | Compound | Dereplication | m/z $[M+H]^+$ |
|---|---|---|---|
| 1 | Monoacetylphloroglucinol | MS, UV, $^{1}$H-NMR | 169 |
| 2 | 2,4-diacetylphloroglucinol | MS, UV, $^{1}$H-NMR | 211 |
| 3 | Pyoluteorin | MS, UV, $^{1}$H-NMR | 272 |
| 4 | Rhizoxin S1 | MS, UV | 614 |
| 5 | Rhizoxin S2 | MS, UV | 628 |
| 6 | Rhizoxin D1 | MS, UV | 580 |
| 7 | Rhizoxin D3 | MS, UV | 598 |
| 8 | Rhizoxin M1 | MS, UV | 628 |
| 9 | Rhizoxin Z1 | MS, UV | 628 |
| 10 | WF1360B (rhizoxin derivative) | MS, UV | 596 |
| 11 | WF1360D (rhizoxin derivative) | MS, UV | 596 |
| 12 | WF1360F (rhizoxin derivative) | MS, UV | 610 |
| 13 | Unknown compound 1 | MS, UV | 348 |
| 14 | Unknown compound 2 | MS, UV | 322 |
| 15 | Unknown compound 3 | MS, UV | 320 |

## DISCUSSION

### *Saccharina latissima* and pseudomonads

As revealed in a previous cultivation-based study, members of the genera *Streptomyces* and *Bacillus* together with pseudomonads, associated with the marine brown macroalga *Saccharina latissima*, represented high proportions of antimicrobially active bacteria (Wiese et al. 2009). Based on the repeated isolation of pseudomonads from the Baltic Sea site, we have demonstrated an enduring association of pseudomonads with *S. latissima*. Specific association of bacterial communities was found on different parts of *S. latissima* originating from the Baltic Sea and North Sea (Staufenberger et al. 2008). This likely stable association with the alga raises the question of whether the pseudomonads are of particular importance for *S. latissima*. As epibiotic bacteria are fast colonisers, highly adaptive and rapidly metabolise algal exudates (Goecke et al. 2010), pseudomonads could have an advantage compared to other bacteria due to their production of secondary metabolites, which are active against competitive microorganisms.

The ability of marine bacteria to produce secondary metabolites has been established. In addition to structural variety, bioactive compounds obtained from marine microorganisms are known for their broad range of biological effects, which include antimicrobial antiprotozoan, antiparasitic, and antitumour activities. Surface-associated microorganisms supposedly have developed specialised and stable adaptations specific to the microenvironment created by a particular host (Penesyan et al. 2010). Therefore, our research focused on antimicrobially active

Table 3. Antimicrobial activities of metabolites from *Pseudomonas protegens* strain LD120 tested at concentrations of 100 µM (50 µM for *Phytophtora infestans*). Activities of ≥80% inhibition are highlighted **in bold**. MAPG: monoacetylphloroglucinol, DAPG: 2,4-diacetylphloroglucinol, PLT: pyoluteorin

| Test strain | Description | Inhibition (%) | | |
|---|---|---|---|---|
| | | MAPG | DAPG | PLT |
| *Bacillus subtilis* (DSM 347) | Gram-positive | **100** | **100** | **100** |
| *Staphylococcus lentus* (DSM 6672) | Gram-positive | **97** | **98** | **100** |
| *Escherichia coli* (DSM 498) | Gram-negative | 31 | 39 | **93** |
| *Pseudomonas fluorescens* (NCIMB 10586) | Gram-negative | 0 | 0 | 37 |
| *Pseudomonas syringae* (DSM 50252) | Phytopathogenic bacterium | 0 | 0 | **97** |
| *Erwinia amylovora* (DSM 50901) | Phytopathogenic bacterium | 0 | 75 | **93** |
| *Ralstonia solanacearum* (DSM 9544) | Phytopathogenic bacterium | 0 | 0 | **100** |
| *Xanthomonas campestris* (DSM 2405) | Phytopathogenic bacterium | 0 | 46 | **99** |
| *Botrytis cinerea* (BASF) | Phytopathogenic fungus | 26 | 40 | 42 |
| *Phytophthora infestans* (BASF) | Phytopathogenic fungus | **86** | **86** | **89** |
| *Septoria tritici* (BASF) | Phytopathogenic fungus | **100** | **100** | 0 |
| *Algicola bacteriolytica* (CIP 105725) | Algal pathogen | 59 | **80** | **94** |
| *Pseudoalteromonas elyakovii* (CIP 105338) | Algal pathogen | 0 | 0 | **94** |
| *Pseudomonas aeruginosa* (DSM 50071) | Clinical pathogen | 0 | 0 | 0 |
| *Propionibacterium acnes* (DSM 1897) | Clinical pathogen | **95** | **97** | **97** |
| *Candida glabrata* (DSM 6425) | Yeast | 30 | 29 | 0 |
| *Trichophyton mentagrophytes* | Dermatophyte | **94** | **93** | 20 |
| *Trichophyton rubrum* | Dermatophyte | **98** | **98** | 34 |

*Pseudomonas* strains, which were isolated from *Saccharina latissima* and showed a reddish colour change on MB medium, indicating the production of the bioactive DAPG (Bangera & Thomashow 1996, Keel et al. 1996). The classification of these pseudomonads revealed the affiliation to 2 separate clusters within the genus *Pseudomonas* (Fig. 1). Most of the strains, including strain LD120, affiliated to *Pseudomonas protegens*. In addition, these strains produced at least 1 of the biologically active metabolites MAPG, PLT and rhizoxins. The most potent producer was *P. protegens* strain LD120, which was characterized in regard to utilization of substrate possibly originating from the alga and was chosen for detailed chemical analyses.

### Metabolic properties of strain LD120

The analysis of strain LD120 in respect of its capability to degrade selected carbon sources possibly deriving from the alga showed that growth did not occur on minimal media with powdered and freeze-dried *Saccharina latissima* or on minimal media supplemented with alginate, an important algal polysaccharide comprising 30 to 36% of algal dry weight (Obluchinskaya 2008). Mannitol, a main storage component of *S. latissima* that can be accumulated up to 15% of absolute algal dry weight (Obluchinskaya 2008), was not utilised as a growth substrate either. These findings indicate that macromolecular structures of living, healthy algae are not attacked by this bacterium (Kremer & Markham 1979), and strain LD120 is proposed to be non deleterious to the healthy algal host. However, macroalgae are known to release large amounts of organic carbon, e.g. amino acids and sugars, into the surrounding environment (providing nutrients for microorganisms) (Goecke et al. 2010), which are also released during fragmentation of kelp. These compounds presumably represent substrates for the *Pseudomonas* strains, a hypothesis that is supported by the wide spectrum of substrates used from the BIOLOG test system, including a number of organic acids (e.g. tricarboxylic acid cycle compounds), sugars and amino acids.

### Bioactivity of metabolites produced by *Pseudomonas* strains

The metabolites DAPG, MAPG, PLT and rhizoxins, which were produced by the *Pseudomonas* strains associated with the marine alga *Saccharina latissima*, have also been discussed as products of *Pseudomonas* isolates from terrestrial plants (Tatusova & Madden 1999, Brodhagen et al. 2004, Weller et al. 2007, Loper et al. 2008). Fluorescent *Pseudomonas* species have been studied for decades with regard to their plant growth-promoting effects through the effective suppression of soilborne plant diseases (Nowak-Thompson et al. 1994, Weller 2007). The modes of action playing a role in disease suppression by these bacteria include siderophore-mediated competition for iron, production of lytic enzymes, induced systemic resistance and antibiosis (Bakker et al. 2007). Root-colonising, plant-beneficial pseudomonads release a remarkable diversity of metabolites with antibiotic activity, such as the polyketidic compounds DAPG and PLT (Brodhagen et al. 2004, Dubuis et al. 2007). We demonstrated that these metabolites are produced by *Pseudomonas* strains associated with *S. latissima*. DAPG, MAPG, PLT and rhizoxins are thus proposed to be chemical mediators of antibacterial, antifungal, cytotoxic and antiprotozoal effects for possible protection of the alga, as shown for terrestrial pseudomonad-host plant associations (Iwasaki et al. 1984, Kiyoto et al. 1986, de Souza et al. 2003, Isnansetyo et al. 2003, Jousset et al. 2006). Both DAPG and PLT produced by the *Pseudomonas* strains in the present study are able to suppress the algal pathogens *Algicola bacteriolytica* and *Pseudoalteromonas elyakovii*.

Previously, Armstrong et al. (2001) reported that epibionts in the marine environment play a protective role by releasing compounds into the surrounding seawater and thereby acting against other microorganisms, potentially both competitors and pathogens. Other reports indicated that epiphytic bacteria, which produce bioactive substances, enhanced the fitness of their algal host (Rao et al. 2007). We suggest that the metabolites DAPG, MAPG, PLT and rhizoxins act against competitors and degrading or other deleterious microorganisms, like fungal and bacterial pathogens of *Saccharina latissima.* The rhizoxins, highly antibiotic and antimitotic compounds are known products of the fungus-associated *Burkholderia rhizoxinica* as well as of *Pseudomonas protegens* Pf-5 (Partida-Martinez & Hertweck 2005, Scherlach et al. 2006, Loper et al. 2008). Rhizoxin and its derivatives were reported to significantly inhibit growth of the plant pathogenic fungus *Fusarium oxysporum*. However, rhizoxin S2 is also the causal agent of rice seedling blight, and therefore, the ecological role of the rhizoxins may well be considered to be context-dependent (Brendel et al. 2007).

In our bioassay panel, DAPG, MAPG and PLT demonstrated a broad range of bioactivities. Noticeably, they showed strong biological activities against phytopathogenic bacteria and fungi and also against specific algal pathogens. In particular, PLT showed good activity against the 2 algal pathogens *Algicola bacteriolytica* and *Pseudoalteromonas elyakovii*. *A. bacteriolytica* is a marine bacterium that might be the causative agent of red spot disease of *Saccharina japonica* (synonym *Laminaria japonica*), leading to a decrease in the seed supply of the alga (Sawabe et al. 1998). *P. elyakovii* is an alginolytic marine bacterium and was isolated from spot-wounded fronds of *S. japonica*. Both bacteria may induce severe damage to *Saccharina* crops (Sawabe et al. 2000) owing to complete thallus degradation of kombu within 1 wk (Sawabe et al. 1992). Thus, concerted action of these 3 compounds (DAPG, MAPG and PLT) could promote protective properties against these pathogenic microorganisms. Inhibitory activities against other epiphytic bacteria are of great importance in microhabitats on the algal surface, where competition for an attachment site is frequent. The competition for space between epibiotic bacteria based on bioactive compounds may provide an antifouling protection to the algal basibiont.

## CONCLUSION

We demonstrated that members of *Pseudomonas species* regularly occur on the surface of *Saccharina latissima* in the Baltic Sea. The secondary metabolites acetylphloroglucinols (MAPG and DAPG) and PLT produced by these bacteria exhibited antimicrobial activities against algal pathogens and a broad range of bacteria and fungi. Based on these findings, we propose a beneficial effect of these *Pseudomonas* strains on *S. latissima* and their possible activity in preventing fouling processes. Further studies are needed to clarify the localisation and seasonal occurrence of *Pseudomonas* cells on *S. latissima* and also identify the antimicrobial compounds *in situ* to demonstrate their ecological role.

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**Appendix 1.** Conditions applied for the growth inhibition assays. $OD_{600}$: optical density at 600 nm

| Test strain | Cell density of inoculum | Medium | Temp. (°C) | Aerobe/ anaerobe | Light/ dark | Time (h) | Shaking/ standing |
|---|---|---|---|---|---|---|---|
| **Gram-positive bacteria** | | | | | | | |
| *Bacillus subtilis* | $OD_{600}$ = 0.02 | TSB3+ | 28 | Aerobe | Dark | 14–16 | 600 rpm |
| *Staphylococcus lentus* | $OD_{600}$ = 0.02 | TSB3+ | 28 | Aerobe | Dark | 14–16 | 600 rpm |
| **Gram-negative bacteria** | | | | | | | |
| *Escherichia coli* | $OD_{600}$ = 0.03 | TSB12+ | 28 | Aerobe | Dark | 14–16 | 600 rpm |
| *Pseudomonas fluorescens* | $OD_{600}$ = 0.03 | TSB12+ | 28 | Aerobe | Dark | 14–16 | 600 rpm |
| **Yeast** | | | | | | | |
| *Candida glabrata* | $OD_{600}$ = 0.05 | M186 | 28 | Aerobe | Dark | 14–16 | 600 rpm |
| **Clinically relevant bacteria** | | | | | | | |
| *Pseudomonas aeruginosa* | $OD_{600}$ = 0.03 | TSB12+ | 37 | Aerobe | Dark | 14–16 | 600 rpm |
| *Propionibacterium acnes* | $OD_{600}$ = 0.03 | PGY | 37 | Anaerobe | Dark | 48 | Standing |
| **Clinically relevant fungi** | | | | | | | |
| *Trichophyton mentagrophytes* | $5 \times 10^4$ spores $ml^{-1}$ | SA | 28 | Aerobe | Dark | 72 | 120 rpm |
| *Trichophyton rubrum* | $5 \times 10^4$ spores $ml^{-1}$ | SA | 28 | Aerobe | Dark | 72 | 120 rpm |
| **Phytopathogenic bacteria** | | | | | | | |
| *Pseudomonas syringae* | $OD_{600}$ = 0.03 | TSB12+ | 28 | Aerobe | Dark | 14–16 | 600 rpm |
| *Xanthomonas campestris* | $OD_{600}$ = 0.03 | TSB12+ | 28 | Aerobe | Dark | 14–16 | 600 rpm |
| *Erwinia amylovora* | $OD_{600}$ = 0.03 | TSB12+ | 28 | Aerobe | Dark | 14–16 | 600 rpm |
| *Ralstonia solanacearum* | $OD_{600}$ = 0.03 | M1 | 28 | Aerobe | Dark | 14–16 | 600 rpm |
| **Phytopathogenic fungi** | | | | | | | |
| *Botrytis cinerea* | $5 \times 10^4$ spores $ml^{-1}$ | MA | 20 | Aerobe | Dark | 48 | Standing |
| *Phytophthora infestans* | $1 \times 10^4$ spores $ml^{-1}$ | PM | 20 | Aerobe | Dark | 72 | Standing |
| *Septoria tritici* | $OD_{600}$ = 0.05 | MYA | 20 | Aerobe | 16 h light:8 h dark | 48 | 600 rpm |
| **Algae pathogenic bacteria** | | | | | | | |
| *Pseudoalteromonas elyakovii* | $OD_{600}$ = 0.01 | MB | 28 | Aerobe | Dark | 14–16 | 600 rpm |
| *Algicola bacteriolytica* | $OD_{600}$ = 0.03 | MB | 28 | Aerobe | Dark | 14–16 | 600 rpm |

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