Characterisation of inhibitory substances produced by two *Pseudoalteromonas* species and the cyanobacterial strain Flo1

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Jan Erik Rau

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1. Gutachter: Prof. Dr. Ulrich Fischer
2. Gutachter: Dr. Olav Grundmann

1. Prüfer: Prof. Dr. Wolfgang Heyser
2. Prüfer: Prof. Dr. Karl-Heinz Blotevogel

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Für meine Familie

Table of contents

Abbreviations

BBD	Black band disease
BLAST	Basic local alignment search tool
bp	Base pairs
CBB	Coomassie brilliant blue
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EC	Enzyme commission (number)
FAD	Flavin adenine dinucleotide
FPLC	Fast protein liquid chromatography
kDa	Kilodalton
L-AAO	L-amino acid oxidase
LB	Lysogeny broth
MALDI-TOF/TOF	Matrix-assisted laser desorption ionization-time-of-flight/time-of-flight
MWCO	Molecular weight cut off
MRSA	Methicillin-resistant *Staphylococcus aureus*
MYG medium	Malt/yeast/glucose medium
PCR	Polymerase chain reaction
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
T	Time
TEMED	N,N,N',N'-Tetramethylethylenediamine
TSA	Tryptic soy agar
TSB	Tryptic soy broth

Abstract

The two *Pseudoalteromonas* species *P. aurantia* NCIMB 2052^T, *P. citrea* NCIMB 1889^T and the marine filamentous cyanobacterial strain Flo1 have been known for the production of inhibitory substances for many years, but the character and the mode of action of these substances remained uncovered. Hence, in the present study the inhibitory substances of *P. aurantia* and *P. citrea* were investigated with respect to their substantial and functional nature, their inhibitory potential, their stability against various treatments as well as the growth phases and incubation conditions when the substances are produced. In addition, an inhibitory substance produced by strain Flo1 was examined regarding its structure, function, and inhibitory potential. Furthermore, its taxonomic position amongst closely related cyanobacteria was investigated by re-constructing phylogenetic trees, showing that strain Flo1 is most closely related to the genus *Geitlerinema*.

For the inhibitory substances from *P. aurantia* and *P. citrea* it could be revealed that they strongly inhibit growth of methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis*, and of several other bacterial strains. *Escherichia coli* UM255, a catalase negative mutant, was the best inhibited organism. Growth of the yeast *Saccharomyces pastorianus* was only partially inhibited by the substances from *P. aurantia*, but not by those from *P. citrea*, whereas *Bacillus subtilis* was not inhibited at all. Nearly the same results could be obtained for an inhibitory substance from *Geitlerinema* strain Flo1.

The inhibitor production by *P. aurantia* and *P. citrea* could be detected under standard incubation conditions during the late exponential to stationary growth phase, respectively. When incubated at 10°C, *P. citrea* produced inhibitory substances, whereas *P. aurantia* did not. The enzymes catalase, peroxidase, and proteinase K were neutralising the antibacterial activity of the inhibitory substances from both species, whereas α-amylase and trypsin did not. Thermolability and resistance of the inhibitory substances of both Pseudoalteromonads to acid-alkaline treatment could be shown. Isoelectric points of the inhibitors from *P. aurantia* and *P. citrea* were determined, at pH 8.7 respectively 8.9 and 9.4.

It could be investigated that the inhibitory substances of both Pseudoalteromonads and of *Geitlerinema* strain Flo1 are amino acid oxidases. In this context it could be revealed that the amino acid oxidases from *P. aurantia* and *P. citrea* have relatively

broad substrate spectra, whereas the amino acid oxidase from strain Flo1 is restricted to L-lysine as only substrate. D-amino acids tested could not be used as substrates by the different amino acid oxidases under investigation, revealing that they are all L-amino acid oxidases (L-AAOs). The L-AAO from strain Flo1 only enzymatical converted L-lysine by its activity, concluding that it is a specific L-lysine oxidase.

A practicable and simple method for in-gel detection of L-AAOs after non-denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) based on the visualisation of occurring hydrogen peroxide production was successfully developed for the first time to determine their approximate molecular weights and number. Non-denaturing SDS-PAGE could be used due to the finding that the activity of the inhibitory substances had shown to be relatively resistant to SDS as well as ß-mercaptoethanol. Using the newly developed application, it could be determined that *P. aurantia* is most probably producing three L-AAOs (60-65, 225-230, and >260 kilodalton; kDa), whereas *P. citrea* is producing only two (165-170 and 225-230 kDa). The three inhibitory substances from *P. aurantia* may all be composed of one structural unit of 60-65 kDa in size. *Geitlerinema* strain Flo1 formed only one specific ~55 kDa L-AAO.

Zusammenfassung

Die beiden *Pseudoalteromonas*-Arten *P. aurantia* NCIMB 2052^T und *P. citrea* NCIMB 1889^T als auch der marine cyanobakterielle Stamm Flo1 sind seit vielen Jahren für die Produktion von Hemmstoffen bekannt, allerdings wurden diese Substanzen nie genau charakterisiert und auch deren Wirkmechanismus wurde nicht hinreichend untersucht. Aus diesen Gründen wurden die Hemmstoffe beider Pseudoalteromonaden in der vorliegenden Arbeit hinsichtlich ihrer substanziellen sowie funktionellen Natur, ihres inhibitorischen Potentials und ihrer Stabilität gegenüber verschiedenen Behandlungsweisen charakterisiert. Zusätzlich wurde untersucht, in welchen Wachstumsphasen und unter welchen Inkubationsbedingungen die Hemmstoffe produziert werden. Weiterhin wurde auch eine Charakterisierung eines Hemmstoffs von Stamm Flo1 bezüglich seiner Substanz und Funktion wie auch seines Hemmpotentials vorgenommen. Auch wurde die taxonomische Stellung von Stamm Flo1 unter nahe verwandten Cyanobakterien mittels der Erstellung von phylogenetischen Stammbäumen bestimmt, wodurch gezeigt wurde, dass die nächstverwandten Arten von Flo1 zur Gattung *Geitlerinema* gehören.

Es konnte festgestellt werden, dass die inhibitorischen Substanzen von *P. aurantia* und *P. citrea* das Wachstum eines Methicillin-resistenten *Staphylococcus aureus* (MRSA) sowie von *Staphylococcus epidermidis* und das von verschiedenen anderen bakteriellen Stämmen hemmen. *Escherichia coli* UM255, eine Katalase-negative Mutante, wurde am meisten an ihrem Wachstum gehindert, wohingegen das von der Hefe *Saccharomyces pastorianus* nur durch die Substanzen von *P. aurantia* inhibiert wurde, aber nicht durch die von *P. citrea*. Das Wachstum von *Bacillus subtilis* wurde in keinem der Fälle eingeschränkt. Annähernd die gleichen Ergebnisse konnten für den von Stamm Flo1 produzierten Hemmstoff erzielt werden, mit der Ausnahme, dass der Einfluss auf MRSA nicht getestet wurde.

Die Ergebnisse zeigten weiterhin, dass *P. aurantia* und *P. citrea* ihre Hemmstoffe während der späten exponentiellen bzw. der stationären Wachstumsphase produzierten. Bei einer Inkubationstemperatur von 10°C produzierte *P. citrea* Hemmstoffe, *P. aurantia* hingegen nicht. Die Enzyme Katalase, Peroxidase und Proteinase K hoben die antibakterielle Wirkung der inhibitorischen Substanzen beider Pseudoalteromonaden auf, wohingegen α-Amylase und Trypsin dies nicht

vermochten. Es konnte festgestellt werden, dass die Hemmstoffe beider Arten hitzeempfindlich und in einem weiten pH-Wert-Bereich noch aktiv sind. Weiterhin wurden die isoelektrischen Punkte der Hemmstoffe bei pH-Werten von 8.7 bzw. 8.9 und 9.4 bestimmt.

Es konnte außerdem gezeigt werden, dass die inhibitorischen Substanzen beider Pseudoalteromonaden, ebenso wie die von *Geitlerinema* Stamm Flo1 produzierte Substanz, Aminosäureoxidasen sind. Weitere Untersuchungen zeigten, dass die Aminosäureoxidasen von *P. aurantia* und *P. citrea* relativ breite Substratspektra aufweisen, die Aminosäureoxidase von Stamm Flo1 hingegen ein sehr spezifisches. Wenn D-Aminosäuren als Substrate angeboten wurden, konnten diese von keiner der untersuchten Aminosäureoxidasen genutzt werden, woraus geschlossen werden konnte, dass es sich bei allen Inhibitoren um L-Aminosäureoxidasen handelt. Die von Stamm Flo1 produzierte L-AAO konnte nur L-Lysin enzymatisch umwandeln, was für eine spezifische L-Lysinoxidase spricht.

Um die ungefähren Molekulargewichte und die jeweilige Anzahl an L-AAOs bestimmen zu können, wurde eine einfache sowie praktikable Methode zur Detektion dieser Enzyme nach nicht-denaturierender Natriumdodecylsulfat-Polyacrylamidgelelektrophorese (SDS-PAGE) entwickelt, basierend auf der Visualisierung aufkommender Wasserstoffperoxidproduktion. Dadurch konnten die L-AAOs direkt im Polyacrylamidgel detektiert werden. Die genannte Methode konnte verwendet werden, da die Hemmstoffe relativ resistent gegen SDS und ß-Mercaptoethanol sind. So konnte ermittelt werden, dass *P. aurantia* höchstwahrscheinlich drei L-AAOs mit Molekulargewichten von 60-65, 225-230 und >260 Kilodalton produziert, wohingegen *P. citrea* nur zwei von 165-170 und 225-230 kDa bildet. Es konnten Hinweise erzielt werden, dass die drei Hemmstoffe von *P. aurantia* aus nur einer strukturellen Grundeinheit von jeweils 60-65 kDa aufgebaut sind. Weiterhin konnte ermittelt werden, dass die von *Geitlerinema* Stamm Flo1 produzierte L-AAO ein Molekulargewicht von ~55 kDa aufweist.

1. Introduction

1.1 Antibiotics

The word “antibiotic" (from the ἀντί - anti, "against", and βίος - bios, "life") was originally defined by Selman A. Waksman as "inhibiting the growth or the metabolic activities of bacteria and other micro-organisms by a chemical substance of microbial origin" (Waksman, 1947). Nowadays, the term “antibiotic” generally describes any compound that kills or inhibits the growth of microorganisms, such as bacteria, fungi, and protozoa, while causing no (or less) harm to the host (Madigan et al., 2009).

Antibiotic substances which act against bacteria can be divided into 13 classes, whereof ten are dissipated from a natural product template: The molecular structures of the aminoglycosides (streptomycin), ß-lactams (penicillins, cephalosporins, carbapenems, monobactams), glycopeptides (vancomycin), macrolides (erythromycin), phenylpropanoids (chloramphenicol), pleuromutilins (tiamulin and valnemulin), polyketides (tetracycline), streptogramins (pristinamycin), and, most recently, the glycylcyclines (tigecycline), lipopeptides (daptomycin) as well as another pleuromutilin (retapamulin) (Holzmann et al., 1984; Stipkovits et al., 2005; Yan et al., 2006; Hughes and Fenical, 2010). The other three classes, the quinolones (ciprofloxacin), oxazolidinones (linezolid), and sulfonamides, have no prototype in nature (Hughes and Fenical, 2010). In addition, a type of enzymes, the L-amino amino acid oxidases (L-AAOs), for example, have antibacterial properties also (see chapter 1.3, pages 22-24).

Antibiotics are produced by a diverse group of living beings, namely algae, animals (e.g. amphibians, arthropods, molluscs, reptiles, and tunicates), lichens, microorganisms (bacteria, fungi), and plants (Gräfe, 1992) and are known to be intermediates (Bu'Lock, 1961; Haslam, 1995), waste or end products of the metabolism (Vining et al., 1990; Demain and Fang, 2000) as well as other substances with antibiotic characteristics (Vallon et al., 1993; Chen et al., 2010b). The application of antibiotic substances in medical therapy as a standard practice was a milestone in the struggle against several threatening diseases, such as

endocarditis, gonorrhea, meningitis, pneumonia, septicaemia as well as tuberculosis (Noone, 1978; Grüneberg et al., 1984; Katz et al., 1992). Most common fields of applications for antibiotics are the human and veterinary medicine as well as the agriculture (Gräfe, 1992). Over two thirds of the clinically used antibiotics have been discovered from natural sources or are semi-synthetic derivatives of natural substances (Newman and Cragg, 2007), most of them derived from organisms living in a terrestrial environment (Okami, 1982). Recently, marine environments were coming more and more into focus also, especially marine actinomycete bacteria seem to be a promising source for new antibiotics (Bull and Stach, 2007; Bredholt et al., 2008).

Antibiotics are not only highly praised health-bringing agents, which humans can use for their purposes, they are also problematic. Thus, due to the extensive use and misuse of antibiotics in human and veterinary medicine as well as in agriculture, an increasing number of resistance mechanisms used by pathogenic bacteria against these substances can be noticed (Tait-Kamradt et al., 2000; Tenover, 2006; Kumarasamy et al., 2010; Vashishtha, 2010). Unfortunately, this is not directly followed by the discovery and development of new and effective antibiotic substances (Nathan, 2004). Thereby, it should be kept in mind that the time and costs in developing a new antibiotic, from discovery to clinical usage, averages 10-15 years respectively $800 million (Bragonzi, 2010). Since the early 1960s, just four new classes of antibiotics have found their way into use, so the approximately $30 billion global market for these substances - each year around 500 metric tons are produced (Madigan et al., 2009) - is still dominated by antibiotic classes discovered half a century ago (Fischbach and Walsh, 2009; see also page 12, Figure 1.1 A and B).

Staphylococcus aureus and, in particular, the methicillin-resistant *S. aureus* (MRSA) is a species, with a high pathogenic potential (Naimi et al., 2003; Fridkin et al., 2005). The organism is a Gram-positive spherical bacterium, frequently occurring as cell clusters, which was first identified as a nosocomial pathogen in the 1960s (Barrett et al., 1968). *S. aureus* is able to expeditiously mutate to gain resistance against several antibacterial agents (Schaaff et al., 2002) and, thus, is strongly resistant to commercially available antibiotics: Various investigations have identified not only

methicillin-resistant, but also vancomycin-resistant (Smith et al., 1999; Tenover et al., 2004) and teicoplanin-resistant *S. aureus* strains (Mainardi et al., 1995).

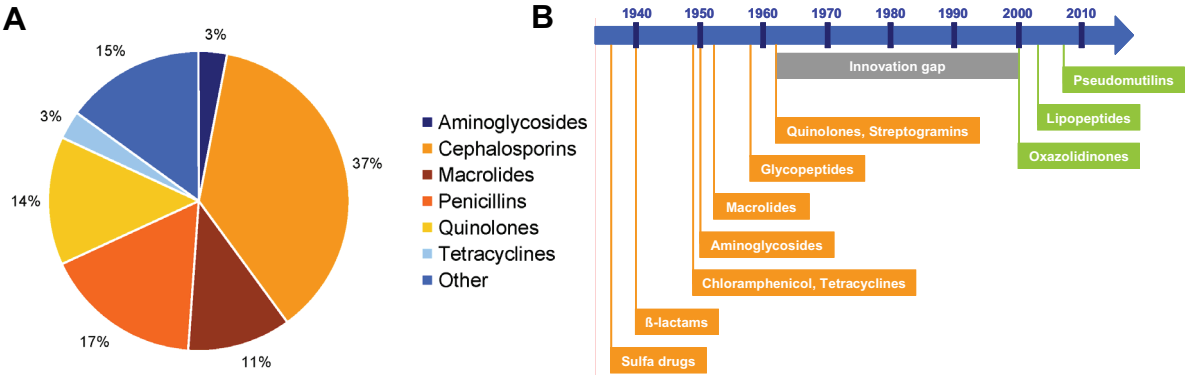

Fig. 1.1 A: Distribution of the annual global antibiotic production on the different agent classes (modified after Madigan et al., 2009). **B:** Time scale showing the distribution of the introduction of important antimicrobial chemotherapeutic agents with an innovation gap between 1962 and 2000 (modified after Fischbach and Walsh, 2009).

MRSA strains are known to be one of the main lethal causes among hospital-acquired infectious diseases (Fridkin et al., 2005). Over the last decades, MRSA infections have become endemic in hospitals all over the world (Diekema et al., 2001), affecting, with rare exception, only patients with established risk factors (Brumfitt and Hamilton-Miller, 1989). These risk factors include dialysis, indwelling percutaneous medical devices and catheters, recent hospitalization or surgery as well as residence in a longterm care facility (Lowy, 1998). More recently, however, cases of MRSA have been described among healthy community-dwelling individuals without established risk factors for MRSA acquisition (Gorak et al., 1999; Baggett et al., 2003; Naimi et al., 2003).

To account for all these facts, antibiotic-resistant *S. aureus* strains and other clinical relevant pathogenic microorganisms are becoming more and more a threatening global health care problem and, thus, effective therapeutic agents against these organisms are needed urgently.

1.2 Marine antibiotic-producing microorganisms

As already mentioned in chapter 1.1, marine microorganisms and their potential to produce antibiotics are coming increasingly into the focus of scientific research worldwide. But these organisms are not only a promising source for antibiotic substances, also other microbe-derived bioactive substances with various properties were already found and countless more are still waiting to be discovered.
In the following two chapters (1.2.1 and 1.2.2, pages 19-22), the genera *Pseudoalteromonas* and *Geitlerinema* are introduced in general and, in particular, their abilities to produce bioactive substances including antibiotics are addressed. In addition, aspects of the ecological significance of both genera in their natural environments are reviewed.

1.2.1 Genus *Pseudoalteromonas*

The genus *Pseudoalteromonas* (Gr. adj. *pseudês*, false; N.L. fem. n. *Alteromonas*, the genus *Alteromonas*; N.L. fem. n. *Pseudoalteromonas*, false *Alteromonas*) was first described by Gauthier and coworkers (1995). Taxonomically this genus is to be placed within the family *Pseudoalteromonadaceae*, within the order *Alteromonadales*, in the class of the *Gammaproteobacteria*. Originally, many members of the mentioned genus were associated with the genus *Alteromonas* (Baumann et al., 1972; Novick and Tyler, 1985), but, based on phylogenetic analysis, the latter genus was taxonomically re-organised by Gauthier and coworkers (1995). The genus *Pseudoalteromonas* currently comprises 36 validly published species. A phylogenetic tree showing the taxonomic relationships of the Pseudoalteromonads is given in Figure 1.2 (page 14).
Members of the genus *Pseudoalteromonas* can be found in various habitats of marine environments, often in association with marine eukaryotes such as algae, ascidians, mussels, sponges, and tunicates. Detailed overviews about the habitats of these organisms are given by Mikhailov and coworkers (2006) and by Bowman (2007).

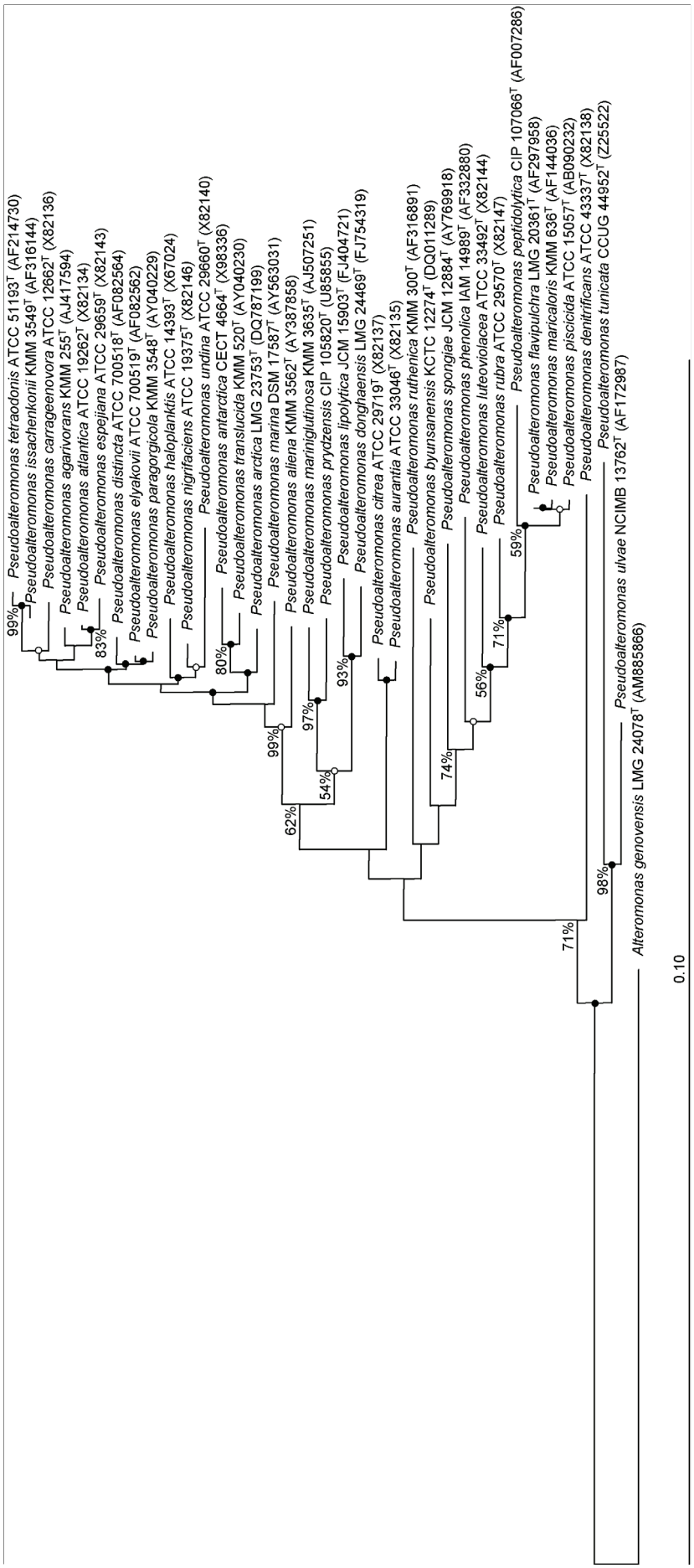

Fig. 1.2 Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences of all validly published *Pseudoalteromonas* species. Bootstrap values (>50%) based on 1,000 resamplings are shown. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-parsimony and maximum-likelihood algorithms. Open circles indicate that the corresponding nodes were also recovered with either the maximum-parsimony or the maximum-likelihood algorithm (see also chapter 6, pages 154-155, Figure S1 and S2). The sequence of *Alteromonas genovensis* LMG 24078^T (GenBank accession no. AM885866) was used as an outgroup. Bar, 0.1 substitutions per nucleotide position.

Pseudoalteromonas strains are strictly aerobic, Gram-negative, non-sporeforming, slightly curved or straight, rod-shaped bacteria of 0.2-1.5 µm × 1.8-4.0 µm in size with a chemoheterotrophic metabolism (Mikhailov et al., 2006). Strains of most species are motile by a single unsheathed polar flagellum, but also unsheathed lateral flagella and/or sheathed polar flagella are reported for some species (Novick and Tyler, 1985; Enger et al., 1987; Holmström et al., 1998; Ivanova et al., 2000).
All Pseudoalteromonads require Na^+ ions for growth (Mikhailov et al., 2006), therefore, they could be regarded as obligately marine microorganisms. All species can grow at 20-25°C, most are able to grow at 4°C (Mikhailov et al., 2006). The substrate D-glucose is utilised by all of them (Mikhailov et al., 2006), except by *P. distincta* (Romanenko et al., 1995).

The major part of the *Pseudoalteromonas* species is characterised by high hydrolytic activities and produces a variety of extracellular enzymes, such as alginases, amylases, caseinases, chitinases, DNAses, gelatinases, lecithinases, and lipases (Mikhailov et al., 2006). *P. atlantica*, *P. carrageenovora*, and some strains of *P. citrea* can decompose agar and/or carrageenan (Akagawa-Matsushita et al., 1992; Ivanova et al., 1998).

Various *Pseudoalteromonas* species are secreting bioactive compounds of low and high molecular weight with algicidal, anti-fouling, antimicrobial as well as various pharmaceutically-relevant activities (Bowman, 2007) and, therefore, these organisms and their compounds are in the research focus of a large group of scientists from various countries, increasingly. The bioactive substances from *Pseudoalteromonas* species were so far classified as L-AAOs (Gómez et al., 2008; Mai-Prochnow et al., 2008; Chen et al., 2010b; see also chapter 1.3, pages 22-24), cyclic peptides, polyanionic exopolymers, substituted phenolic and pyrolle-containing alkaloids, toxic proteins, and also several bromine-substituted agents (Bowman, 2007).
P. phenolica, for example, is producing the low molecular weight brominated biphenyl compound MC21-A (3,3',5,5'-tetrabromo-2,2'-diphenyldiol) with antibacterial activity against different MRSA strains (Isnansetyo and Kamei, 2003a,b; see also page 16, Figure 1.3 A). Egan and coworkers (2002) found that *P. tunicata* is producing at least four target-specific compounds with anti-fungal activities and at least two pigments, a yellow and a purple one. The yellow pigment itself also has

anti-fungal activity (Egan et al., 2002) and was identified as a tambjamine-like alkaloid, designated YP1 (Franks et al., 2005; see also Figure 1.3 B). Tambjamines are bioactive substances containing 4-methoxypyrrole, found in association with marine invertebrates, and are known to have antimicrobial, anti-proliferation, anti-tumour, ichthyodeterrent, and immunosuppressive activities (Lindquist and Fenical, 1991). They are likely acting as natural defensive substances against predators (Lindquist and Fenical, 1991). It seems that not the invertebrates themselves are the source of these substances, but bacteria, which are colonizing the surfaces of the higher organisms (König et al., 2006), like in the case of *P. tunicata* and its tambjamine-like alkaloid YP1.

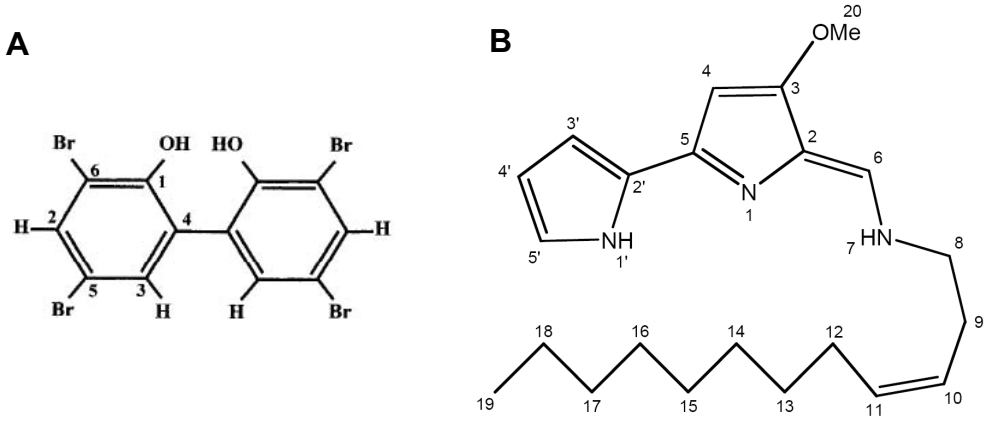

Fig. 1.3 A: Chemical structure of the brominated biphenyl compound MC21-A (Isnansetyo and Kamei, 2003b; figure used with the kind permission of the American Society for Microbiology). **B:** Chemical structure of the tambjamine-like alkaloid YP1 (Franks et al., 2005; figure is free of copyright due to publication in the open access journal Molecules).

As an example for the production of a high molecular weight compound, the *Pseudoalteromonas* species strain X153 was reported to secrete an unstable tetrameric protein of 280 kDa that also showed to have inhibitory activity against a broad spectrum of marine bacteria (Longeon et al., 2004). The same authors proposed strain X153 as an effective probiont, which is protecting commercial shellfish species against pathogenic *Vibrio* species.

Pseudoalteromonas species are in various ways ecologically significant. They are known for influencing settlement (Holmström et al., 1996; Holmström et al., 2002; Dobretsov et al., 2007), germination (Egan et al., 2000, 2001) as well as metamorphosis (Leitz and Wagner, 1993; Negri et al., 2001) of various algae and

invertebrates (see also chapter 4, pages 130-132, Table 4.1). In addition, they seem to be involved in a predatory-like manner within the microbial loop (Lovejoy et al., 1998; Skerratt et al. 2002). The latter mentioned author found swarming cells of *Pseudoalteromonas* strain ACEM 1 attracting to lysed cells of the dinoflagellate *Gymnodinium catenatum* providing the idea that the production of an unidentified algicidal substance is used with the function to supply nutrients for the bacterial species (see also page 18, Figure 1.4 A-D). According to the same author, the anti-algal activity is at its maximum during the peak of the *G. catenatum* bloom, possibly triggered by a quorum sensing mechanism, and probably thus contribute to the disappearance of the bloom.

Furthermore, members of the genus *Pseudoalteromonas* may also be hosted by marine fauna and flora for defence reasons (Lindquist and Fenical, 1991; Holmström et al., 1996; Holmström et al., 2002; Dobretsov et al., 2006; Dobretsov et al., 2007). As another important ecological feature, Pseudoalteromonads have been reported to influence biofilm formation in several marine habitats (Grossart et al., 2003; Mai-Prochnow et al., 2004, 2006; Burmølle et al., 2006; Saravanan et al., 2006; Bayles, 2007; Huang et al., 2007; Rao et al., 2010). In this respect, species such as *P. aurantia* (Gauthier and Breittmayer, 1979; Holmström et al., 2002) and *P. citrea* (Gauthier, 1977; Holmström et al., 2002; Patel et al., 2003; Kalinovskaya et al., 2004; Urvantseva et al., 2006) are of particular interest as the bioactive compounds formed by these species are preventing biofilm residents becoming overwhelmed by other colonising, potentially biofouling issue causing species (Grossart et al., 2003; Franks et al., 2006). In contrast to that, some members of the genus *Pseudoalteromonas* are highly effective in biofilm formation (Saravanan et al., 2006; Huang et al., 2007; Iijima et al., 2009) and accordingly may possibly cause biofouling. Thus, especially the algal polysaccharide degrading species *P. agarivorans*, *P. atlantica*, *P. carrageenovora*, and *P. citrea* (Akagawa-Matsushita et al., 1992; Romanenko et al., 2003a; Urvantseva et al., 2006) as well as the settlement of invertebrate larvae inducing species *P. denitrificans*, *P. luteoviolacea*, and *P. spongiae* (Hugget et al., 2006; Huang et al., 2007) could be problematic in this regard.

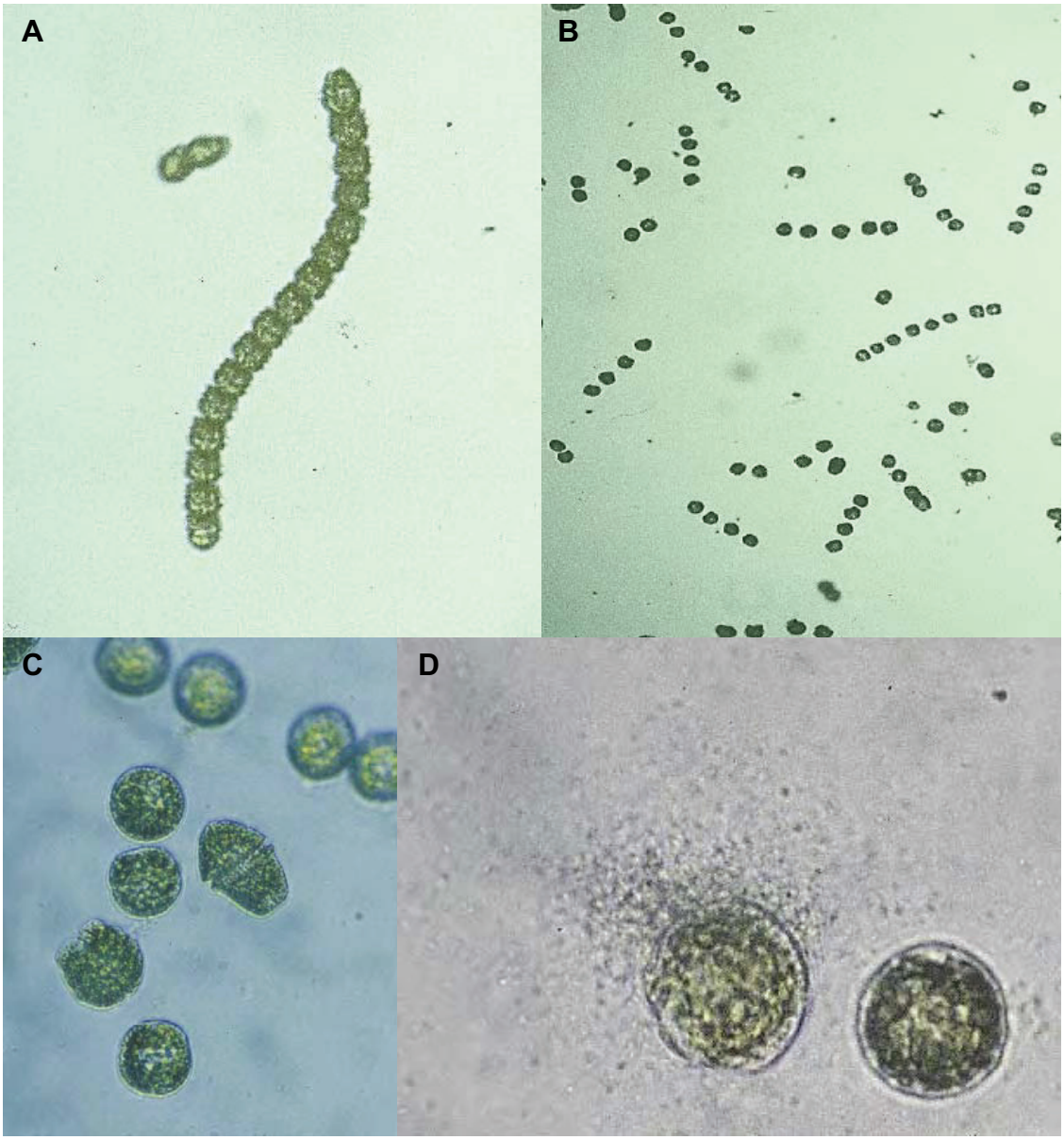

Fig. 1.4 Images showing a time series of vegetative *Gymnodinium catenatum* cells after addition of algicidal culture supernatant from *Pseudoalteromonas* species strain ACEM 1. **A:** Typical cell chain of *G. catenatum* (T = 0-10 min). **B:** Cell chains disaggregate into single cells (T = 20 min). **C:** Cells get rounded (T = 45 min). **D:** Cell lysis and drain off (T = 3 h); the addition of strain ACEM 1 cells is also shown in this part of the figure to demonstrate swarming of these bacteria to a lysed cell of *G. catenatum*. T, time. Data and figures taken from Skerratt and coworkers (2002) and Bowman (2007); the figures are free of copyright due to publication in the open access journal Marine Drugs.

Due to the fact that the hitherto studies included only a relatively small subset of *Pseudoalteromonas* strains in comparison to the known diversity of the genus, it can be reckoned that various more elucidating findings of novel bioactive compounds derived from members of this genus are very likely to be realised in the future.

1.2.2 Genus *Geitlerinema*

The genus *Geitlerinema* (named in honour of Prof. Dr. Lothar Geitler) was first described by Anagnostidis (1989). Taxonomically this genus is to be placed within the family *Pseudanabaenaceae* (subfamily *Pseudanabaenoideae*), within the order *Oscillatoriales*, in the class of the *Cyanophyceae*. The genus *Geitlerinema* currently comprises 33 species, but none of them has been validly published under the Bacteriological Code of Nomenclature. Their descriptions are mainly based on morphological features, e.g. the cell nature and ultrastructure. According to the website of the Pasteur Culture Collection of Cyanobacteria (PCC), only two cyanobacteria have been validly published following the rules of the mentioned code, namely *Prochlorothrix hollandica* strain PCC 9006^T (Burger-Wiersma et al., 1989) and *Prochlorococcus marinus* subsp. *pastoris* strain PCC 9511^T (Rippka et al., 2000).
Phylogenetic trees, showing the taxonomic relationships of *Geitlerinema* species with good quality 16S rRNA gene sequences of >1200 bp in length available, can be found in chapter 3 (Manuscript III, pages 113-114, Figure 3) and in chapter 6 (pages 158-159, Figure S4 and S5).

Members of the genus *Geitlerinema* are ubiquitous and frequently occur in several kinds of environments, such as freshwater as well as marine habitats (Anagnostidis, 1989; Romo et al., 1993; Margheri et al., 2003; Komárek and Anagnostidis, 2005). Overviews about the *Geitlerinema* habitats are given by Anagnostidis (1989) as well as Komárek and Anagnostidis (2005).

As described by Anagnostidis (1989), *Geitlerinema* species are filamentous, unsheated, non-heterocystous, mostly bright blue-green cyanobacteria with cylindrical, straight trichomes of 1-4 µm width, which are motile by gliding (see also page 20, Figure 1.5 A-C). Detailed surveys of the morphological and of some physiological features of this genus are given by the same author and also by Komárek and Anagnostidis (2005).

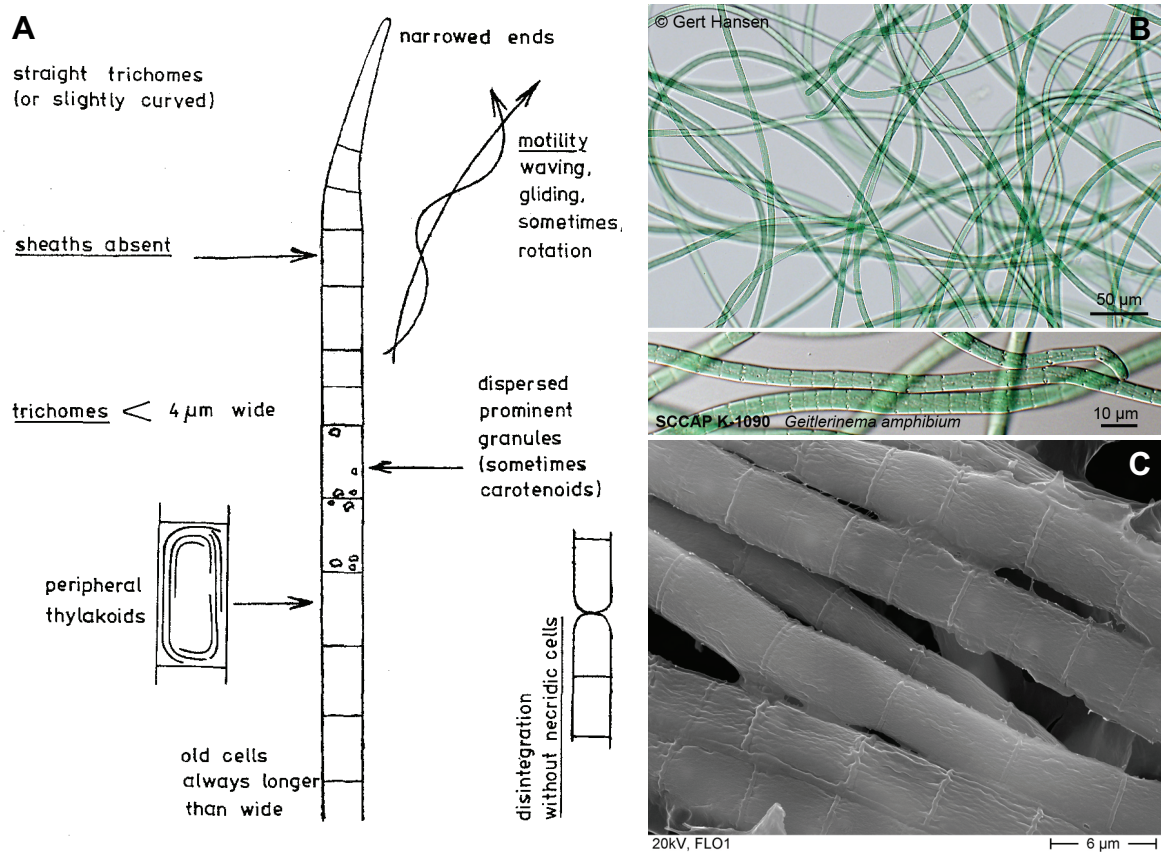

Fig. 1.5 A: Main generic characteristics of *Geitlerinema* (obligatory features are underlined). Figure was taken from Anagnostidis (1989), with the kind permission of the Springer-Verlag. **B:** Photographs of *Geitlerinema amphibium* strain K-1090, deposited in the Scandinavian Culture Collection of Algae and Protozoa (SCCAP) at the University of Copenhagen. They were taken from the website of the SCCAP, with the kind permission of Dr. Gert Hansen (University of Copenhagen, Denmark). **C:** Scanning electron microscopy picture of *Geitlerinema* strain Flo1, kindly provided by Dipl. Biol. Jan Schrübbers (University of Bremen, Germany). The picture was taken by Dr. Herbert Juling (Amtliche Materialprüfungsanstalt, Bremen, Germany).

Several *Geitlerinema* species are known for the production of bioactive compounds. Heyduck-Söller and Fischer (2000) found the culture supernatant of *Geitlerinema* strain Flo1 (in the mentioned study named *Oscillatoria limnetica* strain Flo1) to contain unidentified antibiotic compounds, which had growth inhibitory effects against several Gram-negative and Gram-positive bacteria as well as against the yeast *Saccharomyces cerevisiae*. Caicedo and coworkers (2010) reported that two different Amberlite-extracts of culture supernatant from strain Flo1 have antibacterial and antifungal activities, but the authors also did not identify the bioactive compound(s). Valdor and Aboal (2007) investigated *Geitlerinema splendidum* due to its bioactive potential. They revealed that the mentioned organism produces different

microcystins, whose secretion is postulated as the reason for growth inhibition and morphological as well as ultrastructural alterations of bacteria, cyanobacteria, and of a microalgae species (ultrastructural alterations were not observed for bacteria). Furthermore, *Geitlerinema* cf. sp. is producing a bioactive compound named mitsoamide, which is a linear peptide and has strongly cytotoxic activity against the human lung cancer cell line H-460 (IC50 460 nM) (Andrianasolo et al., 2007).

Apart from the fact that *Geitlerinema* species as phototrophic organisms are producing O_2, they have another important ecological role. Members of this genus are significant microbial mat-formers, especially their contribution concerning the black band disease (BBD) of corals seems to be relevant. The BBD is a pathogenic, sulfide-rich microbial mat, between 1 mm and several cm wide and up to 1 mm thick, which is dominated by different filamentous cyanobacteria species that infect corals on a global scale (Myers et al., 2007). The microbial mat migrates across the coral colonies in the shape of a band at rates averaging 3 mm per day and is able to kill whole colonies in a few months by separating apparently healthy tissue from freshly exposed coral skeleton (Rützler et al., 1983). In general, the BBD is comprised of a complex community of microbes consisting of various heterotrophic bacteria, of sulfide-oxidizing and sulfate-reducing bacteria as well as of non-heterocystous filamentous cyanobacteria (Gantar et al., 2009). Beyond the latter mentioned organisms, the presence of *Geitlerinema* species within this econiche was confirmed (Cooney et al., 2002; Myers et al., 2007; Richardson et al., 2007). Myers and coworkers (2007) found different BBD *Geitlerinema* and *Leptolyngbya* strains to prefer low light conditions and to carry out a sulfide-resistant oxygenic photosynthesis, which is relatively uncommon among cyanobacteria, thus most of them follow a strategy of sulfide-sensitive oxygenic photosynthesis, which involves an inhibition of photosynthesis even when low amounts of sulfide are present and, thereby, are considered to be obligate oxygenic phototrophs (Cohen et al., 1986). The advantage of carrying out a sulfide-resistant oxygenic photosynthesis is that it allows the BBD cyanobacteria to continue photosynthesizing despite that they are present below the oxygen/sulfide interface in the microbial mat (Myers et al., 2007). The ability of BBD cyanobacteria, including *Geitlerinema* species, to produce toxic compounds like microcystins (Richardson et al., 2007; Gantar et al., 2009; Stanić et al., 2011), may be of an additional ecological importance. These toxins were

assumed to be one of the mechanisms assisting to BBD-associated coral tissue lysis and death (Gantar et al., 2009; Stanić et al., 2011).

Such as the *Pseudoalteromonas* species, also the members of the genus *Geitlerinema*, due to their production of biotechnologically and therapeutically relevant bioactive compounds, are promising research subjects and thereby valuable prospective discoveries are likely to be made.

1.3 L-amino acid oxidases (L-AAOs)

L-AAOs (L-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.2.) are flavoenzymes, typically with a dimeric structure, first described by Zeller and Maritz (1944). Flavin adenine dinucleotide (FAD) is the strongly but non-covalently bound cofactor of these enzymes (Pawelek et al., 2000). L-AAOs are catalysing the stereospecific oxidative deamination of L-amino acid substrates to the respective α-keto acids (Du and Clemetson, 2002). The detailed chemical equation of the mentioned reaction is shown below:

$$\text{R-CHNH}_2\text{-COOH (L-amino acid)} + O_2 + H_2O \xrightarrow{\text{L-AAO}} \text{R-CO-COOH (α-keto acid)} + NH_3 + H_2O_2$$

The release of hydrogen peroxide during this reaction is the reason for the antimicrobial activity of L-AAOs (Stiles et al., 1991) and for their contribution to the toxicity of snake venoms (Samel et al., 2008). Indeed, the best studied enzymes of this kind were derived from those venoms (Li et al., 1994; Souza et al., 1999; MacHeroux et al., 2001; Lu et al., 2002; Stábeli et al., 2007; Samel et al., 2008). L-AAOs can be separated in groups with a broader spectrum (Gómez et al., 2008) and in those which have a more strict substrate specificity (Lucas-Elío et al., 2006). Sources, substrate specificities, and the molecular weights of different L-AAOs are summarised in Table 1.1 (page 24).

As can be seen in a part of Table 1.1, Gómez and coworkers (2008), Mai-Prochnow and coworkers (2008) as well as Chen and coworkers (2010b) recently found that the bioactive compounds of different *P. luteoviolacea* strains, *P. tunicata* strain D2 respectively *P. flavipulchra* strain C2 are L-AAOs. Promisingly, the L-AAO from

P. flavipulchra was able to inhibit growth of several MRSA strains and other clinical relevant bacteria.
Also different cyanobacteria are known for their production of L-AAOs or the presence of genes encoding for L-AAOs (Gau et al., 2007), but until now not including any species from the genus *Geitlerinema*.

L-AAOs are found to have different functions. As mentioned above, they are contributing to the toxicity of snake venoms (Samel et al., 2008). Furthermore, Palenik and Morel (1990a,b) found several marine phytoplankton species to contain cell-surface located L-AAOs for the utilization of L-amino acids as a nitrogen source. The green microalga *Chlamydomonas reinhardtii* produces L-AAOs, which were also found to supply ammonia from L-amino acids, but only when no primary nitrogen source is available (Muñoz-Blanco et al., 1990; Piedras et al., 1992; Vallon et al., 1993; see also page 24, Table 1.1). Bockholt and coworkers (1996) insertionally inactivated the *aoxA* gene encoding for the L-AAO of the cyanobacterium *Synechococcus elongatus* strain PCC 7942 with the effect that the organism could no longer grow with L-arginine as the sole N-source. The authors concluded that only the L-AAO enables strain PCC 7942 to utilize extracellularly added L-arginine as an N-source. In addition, *Proteus rettgeri* was found to have a membrane-bound L-AAO which probably interacts with the respiratory electron transport chain (Duerre and Chakrabarty, 1975). Apart from that, it has been reported that the red macroalga *Chondrus crispus* contains an L-AAO located in the apoplast which has a function in pathogen defence (Weinberger et al., 2005). Nagaoka and coworkers (2009) have shown that mice milk is containing an L-AAO which protects the mammary glands against bacterial infections due to the generation of hydrogen peroxide from L-amino acids present in the milk. Additionally, an L-AAO produced by *Pseudoalteromonas tunicata* was reported to play a crucial role during biofilm formation and dispersal (Mai-Prochnow et al., 2004, 2008; see also chapter 4.1, pages 122-123). Furthermore, Boulland and coworkers (2007) have shown that an L-AAO produced by human dendritic cells inhibits T-lymphocyte proliferation and, thus, has an immunomodulatory function.

Tab. 1.1 Sources, substrate specificities, and molecular weights (kDa) of different L-AAOs.

Source	Substrate specificity	Molecular weight (kDa)	Reference
Bacteria			
Marinomonas mediterranea ATCC 700492	high (only L-Lys)	~140-170	Lucas-Elío et al., 2005, 2006
Pseudoalteromonas flavipulchra C2	low	60	Chen et al., 2010b
Pseudoalteromonas luteoviolacea CPMOR-1 and CPMOR-2	low	110 (only determined for strain CPMOR-2)	Gómez et al., 2008
Pseudoalteromonas tunicata D2	high (only L-Lys)	190	James et al., 1996; Mai-Prochnow et al., 2008
Rheinheimera sp. GR5	high (only L-Lys)	71	Chen et al., 2010a
Streptococcus oligofermentans AS 1.3089	low	43	Tong et al., 2008
Cyanobacteria			
Synechococcus elongatus PCC 6301	high (only basic amino acids)	54	Gau et al., 2007
Fungi			
Aspergillus nidulans MH1	low	unknown	Davis et al., 2005
Trichoderma viride Y244-2	low	116	Kusakabe et al., 1980
Algae			
Chlamydomonas reinhardtii CW15 (green microalga)	very low (only L-Cys not)	~470, ~900-1000 and ~1200-1300	Piedras et al., 1992; Vallon et al., 1993
Gymnogongrus flabelliformis (red macroalga)	low	unknown	Fujisawa et al., 1982
Gastropoda			
Aplysia californica (sea hare)	high (only basic amino acids)	60	Yang et al., 2005
Insecta			
Venoms from several insect species	only L-Leu was tested	unknown	Ahn et al., 2000
Vertebrata			
Ink from *Platichthys stellatus* (flounder)	unknown	52	Kasai et al., 2010
Milk from *Mus musculus* (house mouse)	low	113	Sun et al., 2002
Dendritic cells from *Homo sapiens*	high (only L-Phe and L-Trp)	62	Boulland et al., 2007
Venom from *Bothrops moojeni* (fer-de-lance)	low	131	Stábeli et al., 2007
Venom from *Naja naja oxiana* (central asian cobra)	moderate	110	Samel et al., 2008

In the last 10-15 years, L-AAOs came more and more into scientific focus due to their diverse effects on blood components (platelets), on cells (including cancer cells), and on various microorganisms (Samel et al, 2008). The production of D-amino acids by enzymatic resolution of racemic amino acid solutions is the main commercial application for L-AAOs (Parikh et al., 1958; Takahashi et al., 1997). Furthermore, these enzymes were frequently used in biochemical studies for the identification of optical isomers of amino acids, preparation of α-keto acids, and production of thyroxine from 3,5-diiodotyrosine (Iwanaga and Suzuki, 1979).

1.4 Aims of the thesis

The *Pseudoalteromonas* species *P. aurantia* NCIMB 2052^T (Gauthier, 1977) and *P. citrea* NCIMB 1889^T (Gauthier and Breittmayer, 1979) as well as the *Geitlerinema* strain Flo1 (Heyduck-Söller and Fischer, 2000; Caicedo et al., 2010) are known for their production of inhibitory substances, but the substantial nature and the mechanism of inhibition of these substances are still remaining unknown.

Hence, regarding both *Pseudoalteromonas* species, the aims of the present study were

i) to elucidate the antibacterial potential of the inhibitory substances derived from *P. aurantia* and *P. citrea* against several bacteria including MRSA and other clinical relevant strains,
ii) to characterise these substances with respect to their functional nature by investigating the mechanism of inhibition as well as possible substrate spectra,
iii) to develop a new method for in-gel detection of these substances during non-denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine their approximate molecular weights and number, and
iv) to investigate and compare different growth phases and parameters at which they are produced.

Geitlerinema strain Flo1 was investigated

i) to elucidate the functional nature of one of the inhibitory substances produced by this strain Flo1 by studying its mechanism of inhibition as well as its possible substrate spectrum,
ii) to determine its approximate molecular weight by in-gel detection using the above mentioned newly developed method, and
iii) to analyse the phylogenetic position of strain Flo1 amongst closely related cyanobacteria.

2. Material and Methods

2.1 Culture media

2.1.1 Lysogeny broth (LB)

The LB was prepared according to Bertani (1951). The medium contained (l^{-1} distilled water) 10.0 g tryptone, 5.0 g yeast extract, and 10.0 g NaCl (pH 7.0). Solid LB contained (l^{-1} distilled water) 15.0 agar, additionally.

2.1.2 Malt/yeast/glucose medium (MYG)

The MYG medium contained (l^{-1} distilled water) 10.0 g malt extract, 4.0 g yeast extract, 20.0 g D-glucose, and 1.0 g $(NH_4)_2HPO_4$ (pH 6.5). Solid MYG contained (l^{-1} distilled water) 15.0 agar, additionally.

2.1.3 Tryptic soy broth (TSB)

The TSB contained (l^{-1} distilled water) 17.0 g tryptone, 3.0 g soytone, 5.0 g NaCl, 2.5 g D-glucose, and 2.5 g K_2HPO_4 (pH 7.3). Solid TSB contained (l^{-1} distilled water) 15.0 agar, additionally.

2.2 Microbial strains

The heterotrophic bacteria *Bacillus subtilis* ATCC 6051^T, *Cupriavidus metallidurans* DSM 2839^T, *Escherichia coli* K12, *Pseudoalteromonas aurantia* NCIMB 2052^T, *Pseudoalteromonas citrea* NCIMB 1889^T, *Pseudomonas fluorescens* ATCC 13525^T, and the yeast *Saccharomyces pastorianus* DSM 6580^{NT} were obtained from the "Deutsche Sammlung von Mikroorganismen und Zellkulturen" (DSMZ; Braunschweig, Germany).

The cyanobacterium *Geitlerinema* strain Flo1 was originally isolated by Dr. Jörg Rethmeier (University of Bremen, Germany) from a mangrove wood in Florida near Key Biscane and cultivated in the culture collection of the Department of Marine Microbiology at the University of Bremen (Germany). *Geitlerinema* PCC 7105 was

obtained from the Pasteur Culture Collection (Paris, France). Both strains were axenic, as evaluated by light microscopical studies. The cultivation of these strains was performed by Dipl. Biol. Jan Schrübbers (University of Bremen, Germany).
The bacterial strain *Escherichia coli* UM255 (catalase negative mutant; Mulvey et al., 1988) was kindly provided by Prof. Dr. Peter C. Loewen (University of Manitoba, Winnipeg, Canada).
Clinical isolates of the S2 classified heterotrophic bacteria *Enterococcus faecalis*, MRSA, and *Staphylococcus epidermidis* were provided by courtesy of the Molzym GmbH (Bremen, Germany). All experiments regarding these S2 strains were performed in the laboratory of the mentioned company.

2.3 16S rRNA gene sequence determination

The identities of the *Pseudoalteromonas* and *Geitlerinema* strains were verified respectively evaluated by 16S rRNA gene sequence determination. Therefore, extraction of the genomic DNA and PCR were carried out as described by Neilan (1995). The two primers 8F (5´-AGAGTTTGATCCTGGC-3´) and 1494R (5´-GTACGGCTACTACGAC-3´) were used for amplification. PCR products were purified with the Qiagen QIAquick^{TM} PCR product purification kit according to the manufacturer's instructions, and DNA sequencing was performed by GATC Biotech (Konstanz, Germany), using 517F (5´-CCAGCAGCCGCGGTAA-3´) as an additional primer together with the two other ones mentioned. The sequences obtained from amplification were merged together with Chromas Pro version 1.34, resulting in nearly full-length 16S rRNA gene sequences. Finally, the identities of both sequences were determined by using the basic local alignment search tool (BLAST).
In the case of both cyanobacterial strains, the whole procedure was performed by Dipl. Biol. Jan Schrübbers (University of Bremen, Germany).

2.4 Phylogenetic analyses

Phylogenetic analyses of 16S rRNA gene sequences were performed using the ARB software package (versions 5.1 and 5.2; Ludwig et al., 2004) and the corresponding SILVA SSURef 104 database (release October 2010; Pruesse et al., 2007). This dataset contains only good quality sequences of >1200 bp in length. The sequences

were aligned automatically using the PT server implemented in the ARB software package and the alignment was checked manually. Phylogenetic trees were reconstructed according to the neighbour-joining (Saitou and Nei, 1987), maximum-parsimony (Fitch, 1971), and maximum-likelihood (Felsenstein, 1981) methods. The stability of the groupings was estimated by bootstrap analysis (Felsenstein, 1985) of the neighbour-joining and maximum-parsimony methods based on 1,000 replications.

2.5 Cross-flow filtration

The sterile filtrated (0.2 µm cellulose acetate filter; Schleicher & Schuell) bacterial culture supernatants of *P. aurantia*, *P. citrea*, and of *Geitlerinema* strain Flo1 were concentrated via cross-flow filtration, which was realised by using a pump-system (pump: Masterflex L/S Economy Drive; pump head: Masterflex L/S Easy Load 7518-00) in combination with a cross-flow filtration module (Vivaflow 50; molecular weight cut off, MWCO: 10 kDa; polyethersulfone; Sartorius). The concentrated solutions were stored at -20°C until usage.

2.6 PAGE

PAGE was performed at 150 V using a vertical gel electrophoresis chamber (omniPAGE mini-System E-VS10-SYS; Biocom). Protocols for the preparation of the polyacrylamide gels with 40% acrylamide and 2% bis-acrylamide solutions according to Laemmli (1970) are given in Table 2.1 (page 29). For native PAGE the same protocols were used with the modification that all solutions were prepared without SDS.

Tab. 2.1 Composition of the stacking and resolving gels according to Laemmli (1970).

Solution	**Acrylamide concentration**		
	Stacking gel	**Resolving gel**	
	4%	**6%**	**10%**
40% Acrylamid	240 µl	730 µl	1.213 ml
2% Bis-acrylamid	130 µl	402 µl	667 µl
4x 0.5 M Tris-HCl, pH 6.8, 0.4% SDS	630 µl	-	-
4x 1,5 M Tris-HCl, pH 8.8, 0.4% SDS	-	1.25 ml	1.25 ml
Distilled water	1.485 ml	2.563 ml	1.8425 ml
N,N,N',N'-Tetramethylethylenediamine (TEMED)	5 µl	5 µl	5 µl
10% Ammonium persulfate	25 µl	50 µl	50 µl
Total volume	2.5 ml	5 ml	5 ml

More detailed information regarding the used materials and methods are given in the respective manuscripts (see chapter 3: Manuscript I, pages 37-42; Manuscript II, pages 66-70; Manuscript III, pages 93-98; Manuscript IV, pages 117-118).

3. Manuscripts

Explanation to my own contribution to each manuscript.

Manuscript I: Properties of bioactive substances produced by *Pseudoalteromonas aurantia* and *Pseudoalteromonas citrea* inhibiting growth of methicillin-resistant *Staphylococcus aureus*

Rau JE, Havemeyer S, and Fischer U (2011) Properties of bioactive substances produced by *Pseudoalteromonas aurantia* and *Pseudoalteromonas citrea* inhibiting growth of methicillin-resistant *Staphylococcus aureus*. Intended for publication in "Enzyme and Microbial Technology".

This manuscript is describing the initial characterisation of the inhibitory substances produced by two different *Pseudoalteromonas* strains.
The experimental setup was designed by me and I carried out all the laboratory work, except the experiments concerning the pH and temperature stability of the inhibitory substances which were performed by Sandra Havemeyer. Data analyses and writing were done by me, with support and input from all co-authors.

Manuscript II: Inhibitory substances from *Pseudoalteromonas aurantia* and *Pseudoalteromonas citrea* are L-amino acid oxidases with broad substrate spectra

Rau JE, Grundmann O, and Fischer U (2011) Inhibitory substances from *Pseudoalteromonas aurantia* and *Pseudoalteromonas citrea* are L-amino acid oxidases with broad substrate spectra. Intended for publication in "Enzyme and Microbial Technology".

Results concerning the further characterisation of the inhibitory substances produced by two different *Pseudoalteromonas* strains are revealed in this manuscript.
I developed the experimental setup and performed out all the laboratory work. Data analyses and writing were done by me, with support and input from all co-authors.

Manuscript III: L-lysine oxidase: an inhibitory substance produced by the marine filamentous cyanobacterium *Geitlerinema* strain Flo1

Schrübbers JP, Rau JE, and Fischer U (2011) L-lysine oxidase: an inhibitory substance produced by the marine filamentous cyanobacterium *Geitlerinema* strain Flo1. Submitted to "Zeitschrift für Naturforschung C" on June 7, 2011.

This manuscript is describing the characterisation of an inhibitory substance produced by *Geitlerinema* strain Flo1 and its phylogenetic positioning within the genus *Geitlerinema* and closely related cyanobacteria.
For the following parts of the manuscript I have designed the experimental setup, carried out the laboratory work, and analysed the data: SDS-PAGE, in-gel antibacterial assays, and investigation of the substrate spectrum of the inhibitory substance using a spectrophotometric assay. In addition, I re-constructed the phylogenetic trees. The manuscript was written by Jan Schrübbers and me, with support and input from the third author.

Manuscript IV: In-gel detection of L-amino acid oxidases based on the visualisation of hydrogen peroxide production

Rau JE, Fischer U (2011) In-gel detection of L-amino acid oxidases based on the visualisation of hydrogen peroxide production. J Microbiol Methods 85:228-229

This manuscript is dealing with the description of an easy and practicable technique for in-gel detection of L-amino acid oxidases after non-heating SDS-PAGE based on the visualisation of occurring hydrogen peroxide production.
I had the idea for the experimental setup and carried out the practical work. Data analyses and writing were performed by me, with support and input from the second author.

All manuscripts are formatted according to the rules of the respective journals.

Other publications (not part of the thesis)

Rau JE, Blotevogel KH, and Fischer U (2011) *Algoriphagus aquaeductus* sp. nov., isolated from a freshwater pipe. International Journal of Systematic and Evolutionary Microbiology, in press, doi: 10.1099/ijs.0.030809-0.

Bock M and Rau JE (2011) Growth and carotenoid synthesis of different *Algoriphagus* species in dependence of oxygen saturation and phosphate concentration. Submitted to “Zeitschrift für Naturforschung C” on April 1, 2011.

Poster communications

Rau JE, Blotevogel KH, and Fischer U (2007) *Hongiella aquaeductus* - a new aggregate-forming bacterium - isolated from a freshwater pipe. Biospektrum Sonderausgabe, Tagungsband zur VAAM-Jahrestagung 2007, p. 151.

Rau JE, Fischer U (2009) Characterisation of Inhibitory Substances Produced by *Pseudoalteromonas citrea* DSM 8771^T and *Pseudoalteromonas aurantia* DSM 6057^T. Biospektrum Sonderausgabe, Tagungsband zur VAAM-Jahrestagung 2009, p. 97.

Rau JE, Fischer U (2010) Development of a photometric test system for detecting inhibitory substances from *Pseudoalteromonas citrea* DSM 8771^T and *Pseudoalteromonas aurantia* DSM 6057^T. Biospektrum Sonderausgabe, Abstractband zur 3. Gemeinsamen Tagung von DGHM und VAAM, p. 28.

Manuscript I

3.1 Properties of bioactive substances produced by *Pseudoalteromonas aurantia* and *Pseudoalteromonas citrea* inhibiting growth of methicillin-resistant *Staphylococcus aureus*

Properties of bioactive substances produced by *Pseudoalteromonas aurantia* and *Pseudoalteromonas citrea* inhibiting growth of methicillin-resistant *Staphylococcus aureus*

Jan Erik Rau [1], Sandra Havemeyer [1,2], and Ulrich Fischer [1]

[1] Zentrum für Umweltforschung und nachhaltige Technologien, Fachbereich Biologie/Chemie, Abteilung Marine Mikrobiologie, Universität Bremen, Leobener Straße, D-28359 Bremen, Germany

[2] Max-Planck-Institut für Marine Mikrobiologie, Abteilung Ökophysiologie, Celsiusstrasse 1, D-28359 Bremen, Germany

Corresponding author:

Jan Erik Rau

Tel.: +49- (0) 421-218-63409

Fax: +49- (0) 421-218-7222

Email: jerau@uni-bremen.de

Abstract

In the present study, the inhibitory substances produced by *Pseudoalteromonas aurantia* NCIMB 2052^T and *Pseudoalteromonas citrea* NCIMB 1889^T have been investigated. The results reveal that these substances are able to strongly inhibit the growth of methicillin-resistant *Staphylococcus aureus* (MRSA; clinical isolate, Molzym GmbH, Bremen) and *Staphylococcus epidermidis* (clinical isolate, Molzym GmbH, Bremen) as well as several other microbial strains. The inhibitors are produced in the late exponential to stationary growth phase. Catalase, peroxidase, and proteinase K are neutralising the antibacterial activity of the inhibitory substances, whereas α-amylase and trypsin not. Hence, their substantial nature is very likely to be proteinogenous and hydrogen peroxide might be involved in the mechanism of antibacterial action. Isoelectric points of the inhibitory substances are 8.7 (*P. aurantia*) respectively 8.9 and 9.4 (*P. citrea*). Thus, most probably *P. aurantia* is producing only one inhibitory substance, whereas *P. citrea* is producing two. Thermolability and resistance of the inhibitory substances to acid-alkaline treatment is proven. This is one of the first reports on marine bacteria from the genus *Pseudoalteromonas* with high antibacterial potential against MRSA.

Keywords: Agar diffusion test, antibacterial activity, antibiogram, methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudoalteromonas aurantia*, *Pseudoalteromonas citrea*

1. Introduction

Antibiotics are defined as any substances produced by microorganisms, which are antagonistic upon growth of other microorganisms in high dilution [1]. Antibiotics may be intermediates, end or waste products of the metabolism as well as other compounds, which have antibiotic properties [2]. The use of antibiotics was groundbreaking in the fight against several diseases, such as endocarditis, gonorrhea, meningitis, pneumonia, septicaemia, and tuberculosis [3-5]. However, pathogenic microbial organisms that have become resistant to antibiotic therapy are an increasing problem for the public health [6]. The fact that microbes are highly stressable and have processed various ways to resist antibiotics is one part of the problem [7,8]. The increasing use and misuse of existing antibiotics in human and veterinary medicine and in agriculture is another one [9]. A species, which is highly problematic in the current time, is the methicillin-resistant *Staphylococcus aureus* (MRSA) [10,11]. As bacteria are continuously overcoming the medical tools for the fight against them, there is a constant need concerning the search for new antibiotics that affect the target [12-14].

An overview about the history, physiological features, and ecological significance of the genus *Pseudoalteromonas* was recently given by Bowman [15]. The author reviewed that the genus *Pseudoalteromonas* contains numerous marine species synthesizing biologically active substances which act upon a variety of target organisms. According to Bowman [15], this seems to be a unique characteristic for this genus and may greatly benefit *Pseudoalteromonas* cells in their competition for nutrients and colonisation of habitats. Furthermore, the author pointed out that many members of this genus are producing bioactive substances of low and high

Abbreviations:
Artificial sea water (ASW); methicillin-resistant *Staphylococcus aureus* (MRSA)

molecular weight with antimicrobial, anti-fouling, algicidal and various pharmaceutically-relevant activities.

Pseudoalteromonas aurantia NCIMB 2052^T and *Pseudoalteromonas citrea* NCIMB 1889^T are marine bacteria, which are also known to produce polyanionic antibiotics [16,17]. Their substantial nature as well as the mechanism of inhibition are still remaining unknown.

The objectives of this paper are i) to elucidate the antibacterial potential of the inhibitory substances derived from *P. aurantia* and *P. citrea* upon several bacteria including MRSA and other clinical relevant strains, ii) to partially characterise these substances and their mechanism of action as well as iii) to investigate and compare different growth phases and parameters at which they are produced.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

Pseudoalteromonas aurantia NCIMB 2052^T and *Pseudoalteromonas citrea* NCIMB 1889^T were cultivated using artificial sea water (ASW), modified after Kester et al. [18]. The medium contained (l^{-1} distilled water) 5.0 g Casamino acids, 1.0 g yeast extract, 1.0 g glucose, 17.54 g NaCl, 18.49 g $MgSO_4 \cdot 7H_2O$, 1.12 g KCl, 2.21 g $CaCl_2 \cdot 2H_2O$, 150.0 mg KBr, 50.0 mg Fe(III) citrate, 10.0 mg KH_2PO_4 (pH 7.5). Solid ASW contained (l^{-1} distilled water) 15.0 agar, additionally. The culture medium was adjusted to pH 7.6. In dependence of the assay to be performed and if not otherwise stated, cultivation was routinely performed at 26°C (*P. aurantia*) respectively 21°C (*P. citrea*) for 1-4 d on either solid or in liquid ASW by shaking at 150 rpm. In the

latter case, an OD_{600} of 0.1 was used to inoculate the bacterial cultures. Cultivation of the target organisms was routinely performed at the optimal growth temperature of the particular strains as described for the Pseudoalteromonads (further details see Table 1). Microorganisms were maintained at -80°C in appropriate medium with 30% (v/v) glycerol.

2.2 Preparation of concentrated supernatants

P. aurantia and *P. citrea* were grown in 1 l ASW for 24 h in 2 l Erlenmeyer flasks as described. The bacterial culture supernatants were collected by centrifugation at 10,000 rpm and 4°C for 60 min and sterile filtered. 50-fold concentration of the sterile supernatants was performed using pressure filtration with a cut off value of 10 kDa. Storage was done by freezing at -20°C.

2.3 Antibiograms

The antibacterial potential of concentrated bacterial culture supernatants from *P. aurantia* and *P. citrea* was investigated using agar diffusion tests, based on the method described by Bauer et al. [19] with the following modifications. Circular wells with a diameter of 6 mm were cut into the surface of an agar plate (type of medium depending on target organism; see Table 1). Approximately 15 ml agar medium were used per plate. Cultures of test strains (60 µl; OD_{600} = 0.1) were plated onto the agar surface using a Drigalski spatula. 50 µl of concentrated bacterial culture supernatant respectively of the control were added into the wells and zones of inhibition were determined after 24 h of incubation at the optimal growth temperature of the

123 particular target organism. As a control, 0.03% H_2O_2 solution was used. Mean
124 diameters of inhibition zones were quantified by subtracting the diameter of the well
125 (6 mm). Tests were performed in quadruplicate.
126 Antibacterial activities of the inhibitory substances produced by *P. aurantia* and
127 *P. citrea* were checked with the following organisms: *Bacillus subtilis* ATCC 6051^T,
128 *Cupriavidus metallidurans* DSM 2839^T, *Enterococcus faecalis* (clinical isolate,
129 Molzym GmbH, Bremen), *Escherichia coli* K12, catalase negative mutant
130 *Escherichia coli* UM255 [20], *Pseudoalteromonas aurantia* NCIMB 2052^T,
131 *Pseudoalteromonas citrea* NCIMB 1889^T, *Pseudomonas fluorescens* ATCC 13525^T,
132 methicillin-resistant *Staphylococcus aureus* (MRSA; clinical isolate, Molzym GmbH,
133 Bremen), *Staphylococcus epidermidis* (clinical isolate, Molzym GmbH, Bremen), and
134 the yeast *Saccharomyces pastorianus* DSM 6580^{NT}.
135
136 2.4 Growth and secretion of inhibitory substances under standard conditions
137
138 Production of the inhibitory substances during growth of *P. aurantia* and *P. citrea*
139 was determined under standard incubation conditions. The organisms were grown in
140 10 ml of ASW in 100 ml-Erlenmeyer flasks as described. Growth was followed by
141 measuring the turbidity of the cultures spectrophotometrically at 600 nm against
142 ASW. In addition, 150 µl per test culture were sampled, centrifuged at 13,000 rpm
143 and 4°C for 15 min, and frozen at -20°C until usage for the detection of antibacterial
144 activity by agar diffusion tests. OD measurements and sampling were done every 2 h
145 over a period of 36 h. The agar diffusion tests were performed as mentioned using
146 the catalase negative mutant *Escherichia coli* UM255 as target organism, except that

147 the strain was not plated onto the solid agar but the LB agar medium was inoculated
148 with an OD_{600} of 0.001 before pouring (plates were stored at most for 7 d).
149
150 2.5 Growth and secretion of inhibitory substances at low and high incubation
151 temperatures
152
153 Production of inhibitory substances during growth of *P. aurantia* and *P. citrea* was
154 determined under low in comparison to high temperature conditions. The incubation
155 parameters were 26°C (*P. aurantia*) respectively 21°C (*P. citrea*) and 10°C (for both);
156 without shaking. The organisms were grown in 10 ml of ASW in 100 ml-Erlenmeyer
157 flasks. Inoculation of test cultures, measurement of growth, sampling, and agar
158 diffusion tests were performed as described, except that OD measurements and
159 sampling were done every 24 h over a period of 16 d.
160
161 2.6 Effect of selected enzymes upon antibacterial activity
162
163 The effect of selected enzymes upon activities of inhibitory substances produced by
164 *P. aurantia* and *P. citrea* was investigated using agar diffusion tests, with the
165 following modifications. Additional circular wells from the same size were cut
166 neighbouring to the already existing ones (approximately 5 mm space between the
167 corresponding wells), in which the concentrated bacterial culture supernatants were
168 filled. Aqueous enzyme solutions were added into the additional wells (final volume
169 50 µl). The following enzymes were tested: 0.25 mg α-amylase from human saliva
170 (AppliChem, 100 units/mg), 5 µl catalase from *Aspergillus niger* (AppliChem,
171 5300 units/ml), 0.2 mg peroxidase from horseradish (Sigma, 116 units/mg), 0.8 mg

proteinase K from *Tritirachium album* (AppliChem, 30 units/mg), and 0.025 mg trypsin from porcine pancreas (Sigma, 1120 units/mg). Proteinase K and trypsin were activated with 5 mM $CaCl_2$. Enzyme solutions as well as distilled water without concentrated bacterial culture supernatant next to them were used as additional controls. Appearing inhibition of antibacterial activity was documented by the eclipse of the inhibition zones. Tests were performed in quadruplicate.

2.7 Isoelectric precipitation

Isoelectric precipitation was carried out to elucidate possible isoeletric points of the inhibitory substances produced by *P. aurantia* and *P. citrea*. Both organisms were cultivated and supernatants were collected as described, except that the supernatants were not concentrated and not frozen before usage. The isoelectric precipitation was performed according to Chen et al. [21]. Agar diffusion tests were performed as described using the catalase negative mutant *Escherichia coli* UM255 as target organism.

2.8 Temperature and pH stability of inhibitory substances

Temperature and pH stability of the inhibitory substances produced by both *Pseudoalteromonas* strains were investigated as described by Chen et al. [22], with the following modifications. The sources of inhibitory substances were bacterial culture supernatants. Cultivation and harvesting were performed as mentioned. The supernatants were incubated at 20, 50, 60, 70, and 80°C in a water bath over a period of 30 min. After this treatment, the samples were cooled in crushed ice and

stored until usage at 4°C. Determination of pH stability was evaluated in the range of pH 5-10 in intervals of one pH unit by adjusting the culture supernatants to the appropriate values. Conditions were maintained over 3 h before each sample was readjusted to its initial pH value of 7.6. Agar diffusion tests were carried out as described with *S. epidermidis* as target organism. Samples without treatment served as controls. Tests were performed in quadruplicate.

If not otherwise stated, all tests were performed in duplicates.

3. Results and Discussion

3.1 Antibacterial potential of inhibitory substances

The antibacterial effect of the inhibitory substances produced by *P. aurantia* and *P. citrea* was checked against 11 microbial strains, thereof 3 clinical isolates and 8 laboratory strains (Table 1). The overall inhibition patterns were nearly in total not significantly different for both inhibitor producing strains. Hence, it could be concluded that the substantial nature and also the concentration of the inhibitory substances in the samples of both Pseudoalteromonads might be quite similar. From all tested nosocomial strains, MRSA as well as *S. epidermidis* were highly susceptible to the inhibitory substances of both *Pseudoalteromonas* strains. This reveals that those inhibitors might possibly be used in future as new therapeutic antibiotic agents against both Staphylococci. Chen et al. [22] recently described that *P. flavipulchra* C2 also produces an inhibitory substance being an antibacterial protein, which inhibits growth of several clinical isolates, including 15 MRSA strains.

222 The nosocomial strain *E. faecalis* tested in the present study showed only weak
223 susceptibility. The highest inhibitions of 20 mm each were found when the catalase
224 negative mutant *Escherichia coli* UM255 was used as target organism. This
225 suggests that catalase probably plays an important role in the neutralisation of the
226 inhibitory effects. When *P. aurantia* NCIMB 2052^T was first described by Gauthier
227 and Breittmayer [17], they found a total lack of the antibacterial activity when the
228 organism was grown on blood-containing media. This phenomenon was explained
229 by the catalase activity of blood and was previously described for *P. citrea*
230 NCIMB 1889^T and *P. rubra* NCIMB 1890^T [16,23]. In the present study both
231 *Pseudoalteromonas* strains NCIMB 2052^T and NCIMB 1889^T were not only used as
232 source of inhibitory substances but additionally also as target organisms, revealing
233 that both *Pseudoalteromonas* strains exhibited autoinhibition. Holmström et al. [24]
234 investigated the antibacterial activity expressed by 10 different *Pseudoalteromonas*
235 species, including *P. citrea* NCIMB 1889^T. The authors also found that *P. citrea* does
236 inhibit its own growth, but that *P. rubra* NCIMB 1890^T and *P. tunicata* exhibited only
237 weak autoinhibitions. The other Pseudoalteromonads tested by Holmström et al. [24]
238 did not show autoinhibition. The results of the present study revealed that *E. coli* K12
239 was nearly resistant to the antibacterial effect of the inhibitory substances, whereas
240 *B. subtilis* was not affected at all. Most probably, both strains are producing catalase
241 in a concentration which is able to nearly respectively completely neutralise the
242 antibacterial effects of the inhibitors. The yeast *S. pastorianus* exhibited moderate
243 and only partial inhibition when being in contact with the inhibitory substances
244 produced by *P. aurantia*, whereas those from *P. citrea* had no effect on the growth.
245
246 3.2 Secretion patterns for inhibitory substances

247

248 To elucidate in which growth phase the inhibitory substances were secreted into the
249 medium, *P. aurantia* and *P. citrea* were grown under standard incubation conditions
250 and growth as well as the presence of inhibitors were checked every 2 h (Fig. 1 A
251 and B).
252 As can be seen, antibacterial activity could be detected in the culture medium of
253 *P. aurantia* after 6 h in the late exponential growth phase for the first time. The strain
254 showed no real stationary phase by turning directly into the declining phase. Most
255 probably this is due to the presence of the inhibitory substance and the fact that
256 *P. aurantia* is showing the phenomenon of autoinhibition. The highest inhibitor
257 production could be detected after 12 h over a period of 6 h. Thereafter, the
258 antibacterial activity became weaker until till the end of the experiment but did not
259 disappear completely. It seems that there is a direct correlation between the course
260 of the declining phase and inhibitor production, for which the autoinhibition of
261 *P. aurantia* might be the driving force.
262 Similar behaviour was observed for *P. citrea* with the exception that the inhibitory
263 activity first could be detected at the beginning of the declining growth phase after
264 12 h and that the highest inhibitor production lasted only for 2 h.
265 These results clearly have shown that *P. aurantia* is already secreting its inhibitory
266 substance in the late exponential growth phase, whereas *P. citrea* starts secreting at
267 the beginning of the declining phase. This is in accordance with findings of Chen
268 et al. [22] obtained with *P. flavipulchra* strain C2 where the antibacterial protein
269 production started when strain C2 entered the stationary growth phase. McCarthy
270 et al. [25] reported similar results for *P. luteoviolacea* strain 9K-V10. This strain starts
271 to produce an antibacterial protein during late exponential to stationary growth

phase. The point in time when the synthesis of inhibitory substances starts is possibly triggered by quorum sensing mechanisms [15].

The production of inhibitory substances during growth of *P. aurantia* and *P. citrea* at low (10°C without shaking) and high incubation temperatures (26 respectively 21°C without shaking) is documented in Fig. 2 A and B.

As can be seen, growth of both *Pseudoalteromonas* strains started immediately and reached their maxima at their optimal incubation temperature already after 1 d before directly entering their declining growth phases which remained more or less constant after 6 d of incubation. Growth at 10°C exhibited short lag-phases, and maximum growth occurred after 4 and 9 d respectively. Regarding production of the inhibitory substances, differences have been observed for both strains. *P. aurantia* produced the inhibitor only at 26°C during the whole time of incubation but not at 10°C (Fig. 2 A). This is in contrast to findings with *P. citrea*. This organism produced the inhibitory substance at 21°C with decreasing activity only during the first 3 d of incubation, while at 10°C the production of inhibitor started in the middle of the exponential growth phase reaching maximum activity at its end and the beginning of the declining one (Fig. 2 B).

Gauthier and Breitmayer [17] reported that the production of antibiotic compounds by *P. aurantia* strain NCIMB 2052^T was constant and independent of incubation temperature. This is in contrast with our observations with the same strain, since no inhibitor production was found at 10°C and the production was lesser at 21 than at 26°C (data not shown). This different production behaviour might be due to the use of diverse growth media, artificial seawater in the present study, and marine broth (Difco) in that of Gauthier and Breitmayer [17]. Regarding the production of inhibitory

297 substances by *P. citrea* strain NCIMB 1889^T, Gauthier [16] reported that higher
298 levels were formed at low temperatures. This finding is only partially in accordance
299 with the results obtained in the present study with the same strain, since the activity
300 of inhibitors produced at 10 or 21°C did not differ significantly. On the other hand,
301 more inhibitory substances were formed when the strain was grown by shaking with
302 150 rpm at 21 rather than at 26°C (data not shown).
303
304 3.3 Characterisation of inhibitory substances
305
306 The effect of selected enzymes on the activity of inhibitory substances produced by
307 *P. aurantia* and *P. citrea* was examined using agar diffusion tests (Table 2).
308 As can be seen, the antibacterial activities of the inhibitory substances produced by
309 *P. aurantia* respectively *P. citrea* were neutralised in the presence of catalase,
310 peroxidase, and proteinase K but not by α-amylase or trypsin. Due to the facts that
311 the antibacterial activities are neutralised by catalase and peroxidase, it is obvious
312 that possibly hydrogen peroxide is involved in the mechanism of inhibition. The
313 neutralisation caused by proteinase K indicates that the produced inhibitory
314 substances might probably be rather of proteinogenous than of amylaceous nature,
315 since treatment with α-amylase had no neutralising effect. The fact that the
316 antibacterial activity is not neutralised by trypsin might be an indication that the
317 inhibitory substances have only a low content of arginine- and lysine-residues, or
318 that these amino acids are located in the central parts of the inhibitory proteins
319 without any access for an enzymatic attack by trypsin. Controls with enzyme
320 solutions alone or destilled water had no effects on the growth of the used target
321 organism.

322 The neutralising effect of catalase respectively the involvement of hydrogen peroxide
323 in the mechanism of inhibition was previously described not only for *P. aurantia* and
324 *P. citrea* but also for other Pseudoalteromonads [15-17,22,26). Chen et al. [22] as
325 well as Gómez et al. [26] reported that the inhibitory substances of *P. flavipulchra*
326 strain C2 respectively different *P. luteoviolacea* strains are L-amino acid oxidases
327 (L-AAOs). L-AAOs (L-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.2.) are
328 flavoenzymes catalysing the oxidative deamination of L-amino acids to the
329 respective α-keto acids with the release of ammonium and hydrogen peroxide [27]
330 which determines the antimicrobial property of L-AAOs [28]. According to the
331 findings from the present study that hydrogen peroxide is involved in the inhibition
332 mechanism of the inhibitory substances of *P. aurantia* and *P. citrea*, it is likely that
333 these substances are also L-AAOs. It is known that diverse media contain distinct
334 types and amounts of amino acids which will have an influence on the activity of
335 L-AAOs [21,22,26]. Gauthier [16] had recognised already such an effect concerning
336 the intensity of the inhibitory activity due to the kind of nutrients supplied to *P. citrea*.
337 This is confirmed also by our study (data not shown). Therefore, these findings
338 additionally confirm the assumption that the inhibitory substances of *P. aurantia* and
339 *P. citrea* are possibly L-AAOs.
340
341 Culture supernatants of both *Pseudoalteromonas* strains were subjected to
342 isoelectric precipitation in order to determine the isoelectric points of the inhibitory
343 substances. As can be seen from Fig. 3, *P. aurantia* produced only one substance
344 with an isoelectric point at 8.7 with inhibitory potential (Fig. 3 A), while *P. citrea*
345 produced two with isoelectric points at pH 8.9 and pH 9.4 (Fig. 3 B). All other
346 fractions showed no activity. Gauthier [16] also found two different polyanionic

347 inhibitory substances produced by *P. citrea* strain NCIMB 1889^T by using an
348 electrophoresis setup with Cellogel strips. Chen et al. [22] determined an isoelectric
349 point of pH 9.4 for a protein of the supernatant of *P. flavipulchra* strain C2 with
350 antibacterial activity. This implies that the inhibitory substances found at pH 9.4 from
351 *P. citrea* (during the present study) and *P. flavipulchra* might have similar properties
352 concerning structure and electrical charge.
353
354 As can be seen from Fig. 4 A, thermostability of the inhibitory substance of
355 *P. aurantia* is much better than that of *P. citrea* when the samples were pre-
356 incubated at the appropriate temperatures for 30 min. The antibacterial activity of the
357 substance produced by *P. aurantia* remained constant until 50°C. Above this
358 temperature, the activity slowly decreased to about 66% at 70°C. No activity was
359 found at 80°C. This is in contrast to the observation with *P. citrea* where the activity
360 decreased enormously to only 25% at 50°C, and no one could be detected above
361 this temperature. The inhibitory substances of both *Pseudoalteromonas* strains
362 tested in the present study kept their full antibacterial activity even after having been
363 stored at - 20°C for 1.5 years (data not shown).
364
365 Chen et al. [22] postulated that the antibacterial protein produced by *P. flavipulchra*
366 strain C2 still had its full inhibitory activity after incubation at 50°C but was lost totally
367 at 70°C, in each case after 30 min incubation. These findings are only partially in
368 accordance with the results obtained in the present study with *P. aurantia*, since its
369 inhibitory substance was still active at 70°C (Fig. 4 A). Thermolability was also
370 reported for the inhibitory substances produced by other species of the genus
371 *Pseudoalteromonas* [15]. The fact that the inhibitory substances produced by

372 *P. aurantia* and *P. citrea* are as thermolabile as those of different
373 Pseudoalteromonads which are proteins, can be taken as a further indication that the
374 inhibitory substances of both these organisms are also of proteinogenous nature.
375
376 Concerning the pH stability of the inhibitory substances produced by both
377 *Pseudoalteromonas* strains used, it could be shown that the intensity of the
378 antibacterial activity was not undergoing significant changes in the tested pH range
379 from 5-10, with the only difference that the antibacterial activity of *P. citrea* was
380 totally lost at pH 5 (Fig. 4 B).
381 This is mainly in accordance with results obtained for *P. flavipulchra* strain C2 whose
382 activity of its antibacterial protein remained unaltered after being kept at pH-values
383 ranging from 2-12 for 2 h [22]. Gauthier and Breittmayer [17] also found similar
384 results for *P. aurantia* when the organism was grown in the pH range from 5-11.
385
386 **4. Conclusions**
387
388 In the present study it could be shown that the production of the inhibitory
389 substances by *P. aurantia* and *P. citrea* occurs during the late exponential to
390 stationary growth phase, respectively. When incubated at 10°C, *P. citrea* is
391 producing inhibitory substances, whereas *P. aurantia* does not. It could be revealed
392 that the inhibitory substances of both Pseudoalteromonads are thermolabile and
393 nearly resistant to acid-alkaline treatment. Various clear hints could be found that the
394 inhibitors are of proteinogenous nature and that the involvement of hydrogen
395 peroxide in the mechanism of antibacterial activity is likely. Referring to the results
396 obtained by isoelectric precipitation, *P. aurantia* is probably producing one inhibitory

substance, whereas *P. citrea* might produce two. Taking into account the results obtained for other species of the genus *Pseudoalteromonas* [22,26], the inhibitory substances produced by *P. aurantia* and *P. citrea* might be L-AAOs also. It might be possible that the inhibitors will find an application as new therapeutic antibiotic agents against MRSA und *S. epidermidis*. However, further comprehensive studies are necessary to clarify this assumed possibility.

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Tables

Table 1 Antibacterial effects of inhibitory substances produced by *Pseudoalteromonas aurantia* NCIMB 2052^T and *Pseudoalteromonas citrea* NCIMB 1889^T upon selected bacterial strains.

Microbial strain	Growth medium	Zones of inhibition (mm)[a]	
		P. aurantia NCIMB 2052^T	*P. citrea* NCIMB 1889^T
Nosocomial strain			
Enterococcus faecalis[b]	LB	2	2
Methicillin-resistant *Staphylococcus aureus*[b]	LB	14	12
Staphylococcus epidermidis[b]	LB	16	14
Laboratory strain			
Bacillus subtilis ATCC 6051^T	LB	0	0
Escherichia coli K12	LB	4	2
Escherichia coli UM255 (catalase negative mutant)	LB	20	20
Cupriavidus metallidurans DSM 2839^T	LB	10	10
Pseudoalteromonas aurantia NCIMB 2052^T	ASW	18	20
Pseudoalteromonas citrea NCIMB 1889^T	ASW	16	19
Pseudomonas fluorescens ATCC 13525^T	LB	16	16
Saccharomyces pastorianus DSM 6580^{NT}	MYG	(8)[c]	0

[a] Mean diameters of inhibition zones were quantified by subtracting the diameter of the well (6 mm). Tests were performed in quadruplicate

[b] clinical isolate, Molzym GmbH, Bremen

[c] Partial inhibition

Table 2 Effect of selected enzymes on antibacterial activity of inhibitory substances produced by *Pseudoalteromonas aurantia* NCIMB 2052^T and *Pseudoalteromonas citrea* NCIMB 1889^T.

Tested enzyme	Effect of enzyme[a]	
	P. aurantia NCIMB 2052^T	*P. citrea* NCIMB 1889^T
α-Amylase	-[b]	-
Catalase	+[b]	+
Peroxidase	+	+
Proteinase K	+	+
Trypsin	-	-

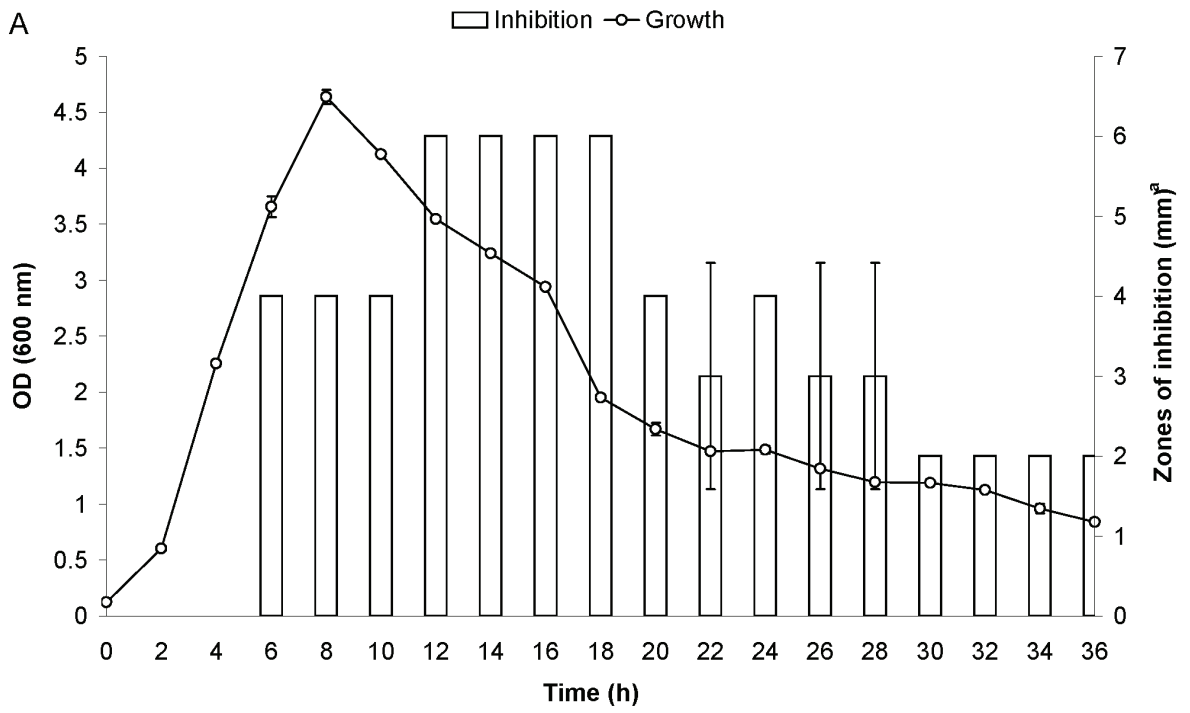
[a] Agar diffusion tests were performed using the catalase negative mutant *Escherichia coli* UM255 as target organism

[b] Antibacterial activity: - = not neutralised; + = neutralised

Figure legends

Fig. 1. Production of inhibitory substances during growth of *Pseudoalteromonas aurantia* NCIMB 2052^T (A) and *Pseudoalteromonas citrea* NCIMB 1889^T (B) under standard incubation conditions (26 respectively 21°C at 150 rpm). Agar diffusion tests were performed using the catalase negative mutant *Escherichia coli* UM255 as target organism.

[a] Mean diameters of inhibition zones were quantified by subtracting the diameter of the well (6 mm)



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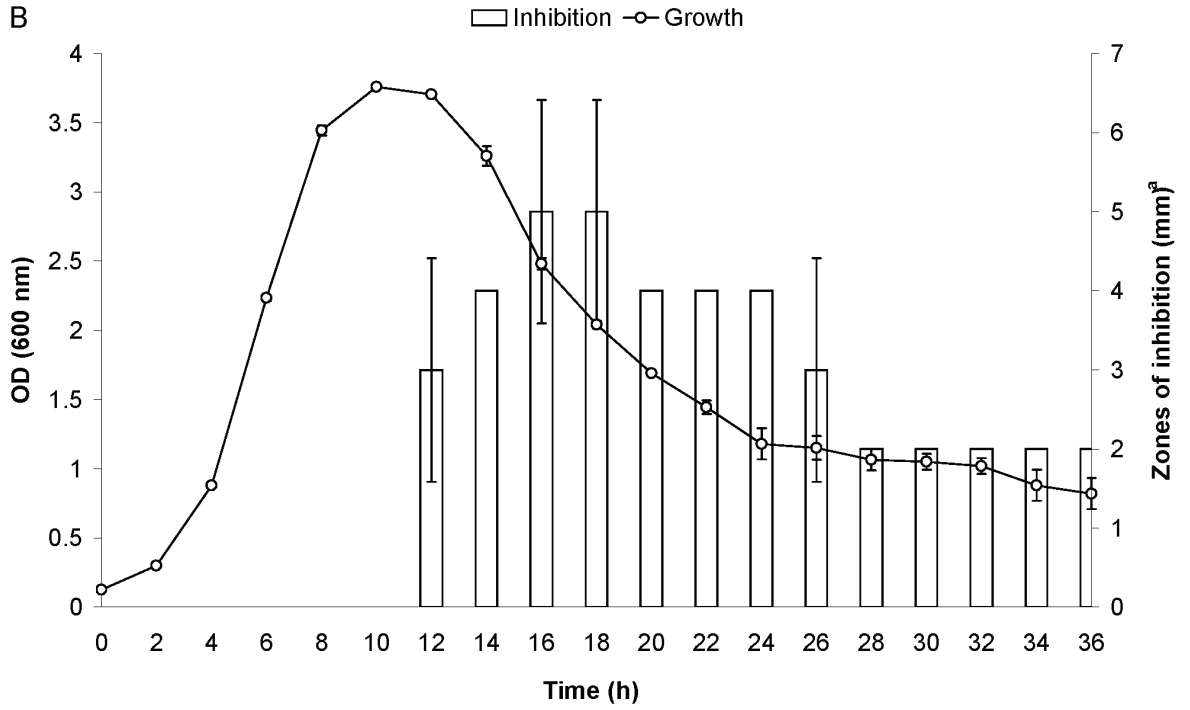


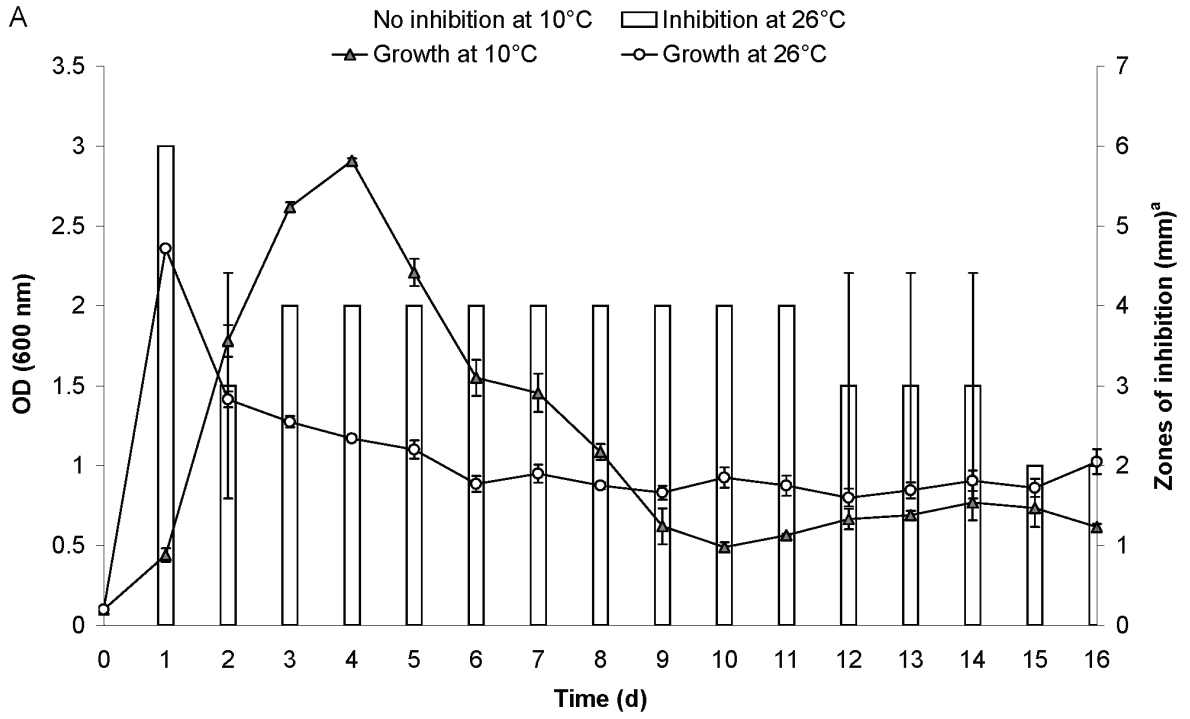
555

556

557

558

559 **Fig. 2.** Production of inhibitory substances during growth of *Pseudoalteromonas*
560 *aurantia* NCIMB 2052^T (A) and *Pseudoalteromonas citrea* NCIMB 1889^T (B) in
561 dependence on temperature without shaking. Agar diffusion tests were performed
562 using the catalase negative mutant *Escherichia coli* UM255 as target organism.
563 [a] Mean diameters of inhibition zones were quantified by subtracting the diameter of
564 the well (6 mm)
565



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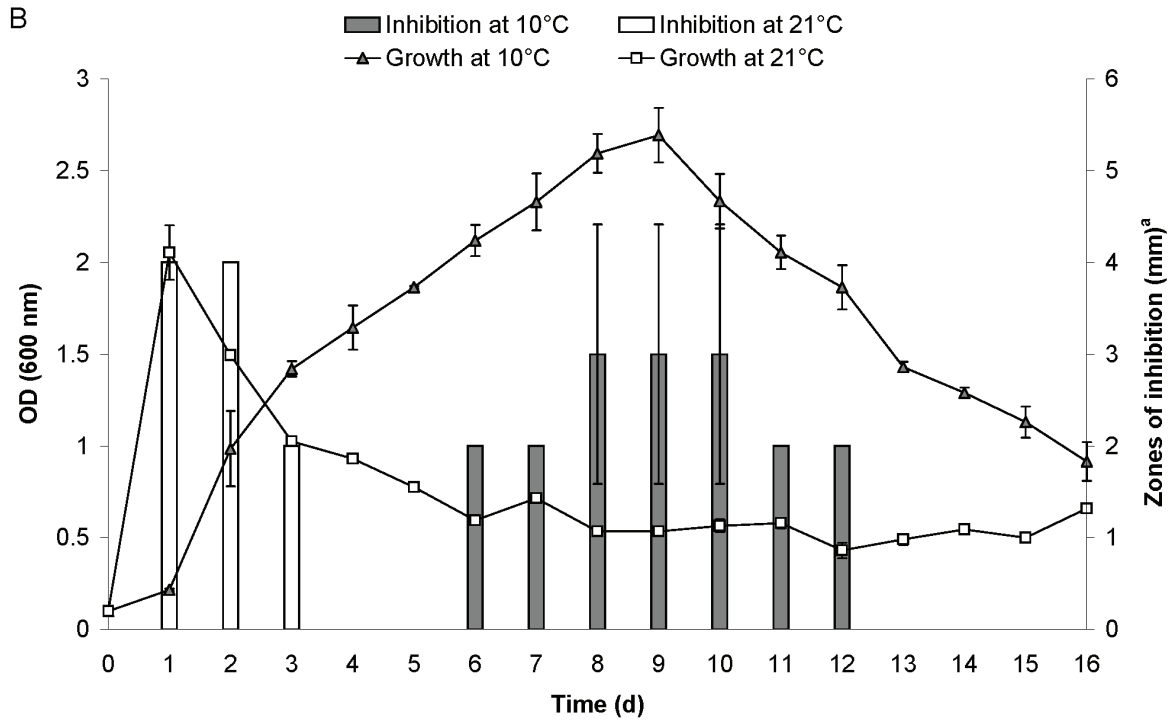

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569

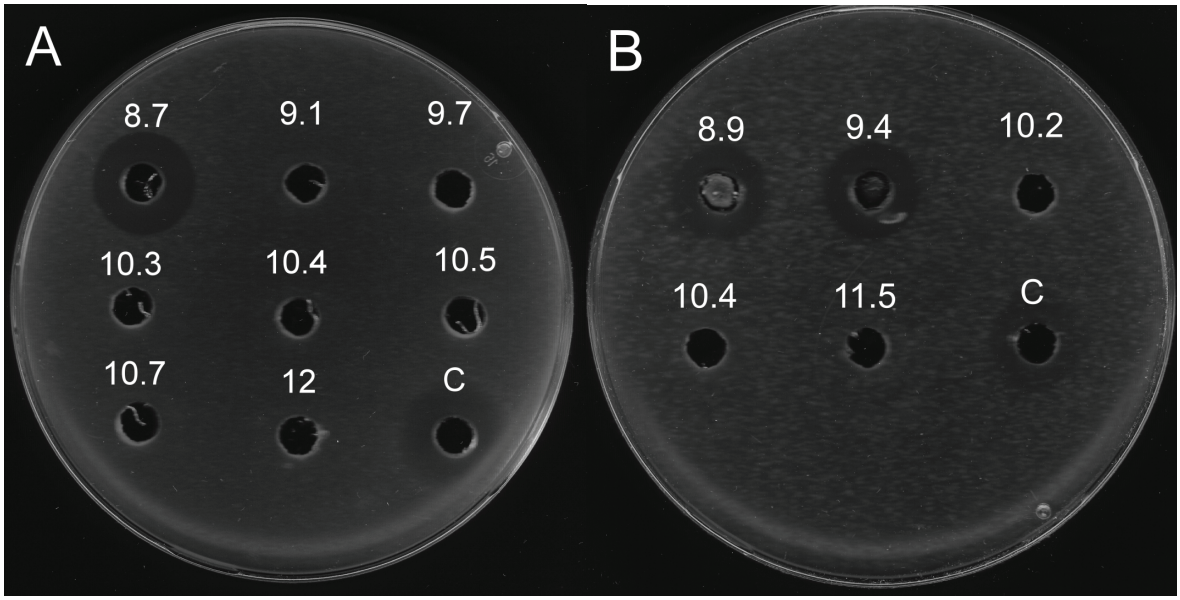
570 **Fig. 3.** Antibacterial activity of samples derived from isoelectric precipitation with

571 culture supernatants of *Pseudoalteromonas aurantia* NCIMB 2052^T (A) and

572 *Pseudoalteromonas citrea* NCIMB 1889^T (B). Agar diffusion tests were performed

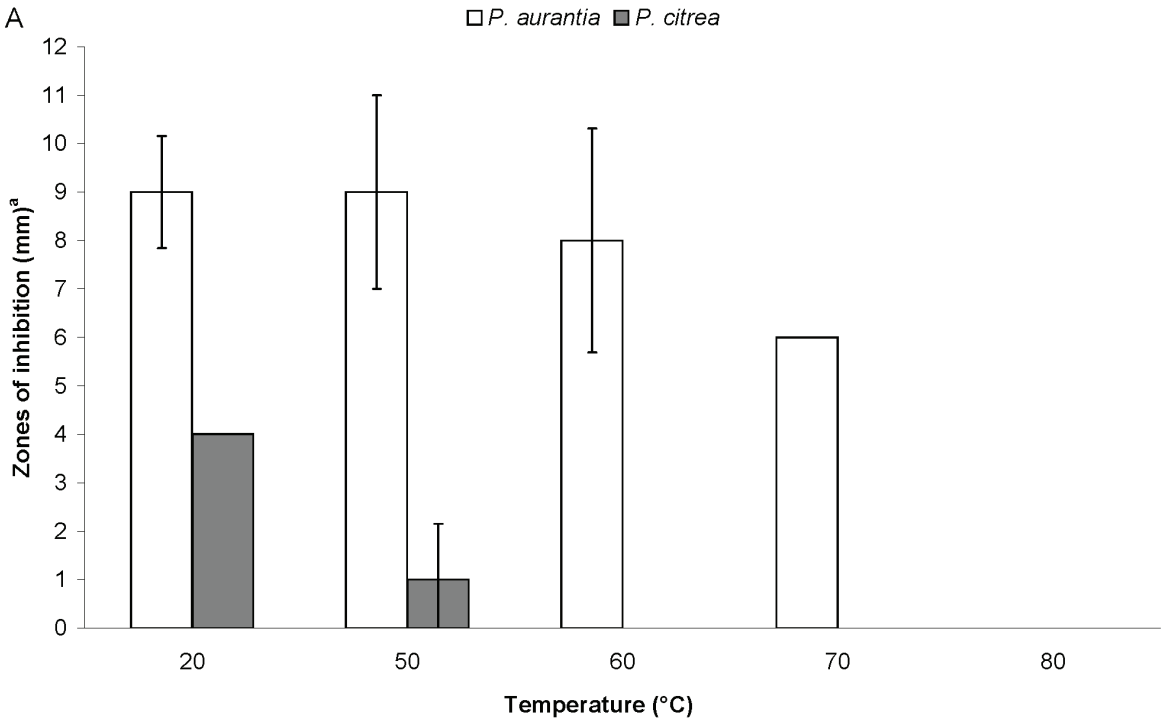
573 using the catalase negative mutant *Escherichia coli* UM255 as target organism.

574 C = control with 0.03% H_2O_2. Numbers = pH values.



575

576 **Fig. 4.** Stability of inhibitory substances produced by *Pseudoalteromonas aurantia*
577 NCIMB 2052^T and *Pseudoalteromonas citrea* NCIMB 1889^T at different temperatures
578 (A) and at different pH values (B). Agar diffusion tests were performed using
579 *S. epidermidis* (clinical isolate, Molzym GmbH, Bremen) as target organism.
580 [a] Mean diameters of inhibition zones were quantified by subtracting the diameter of
581 the well (6 mm)



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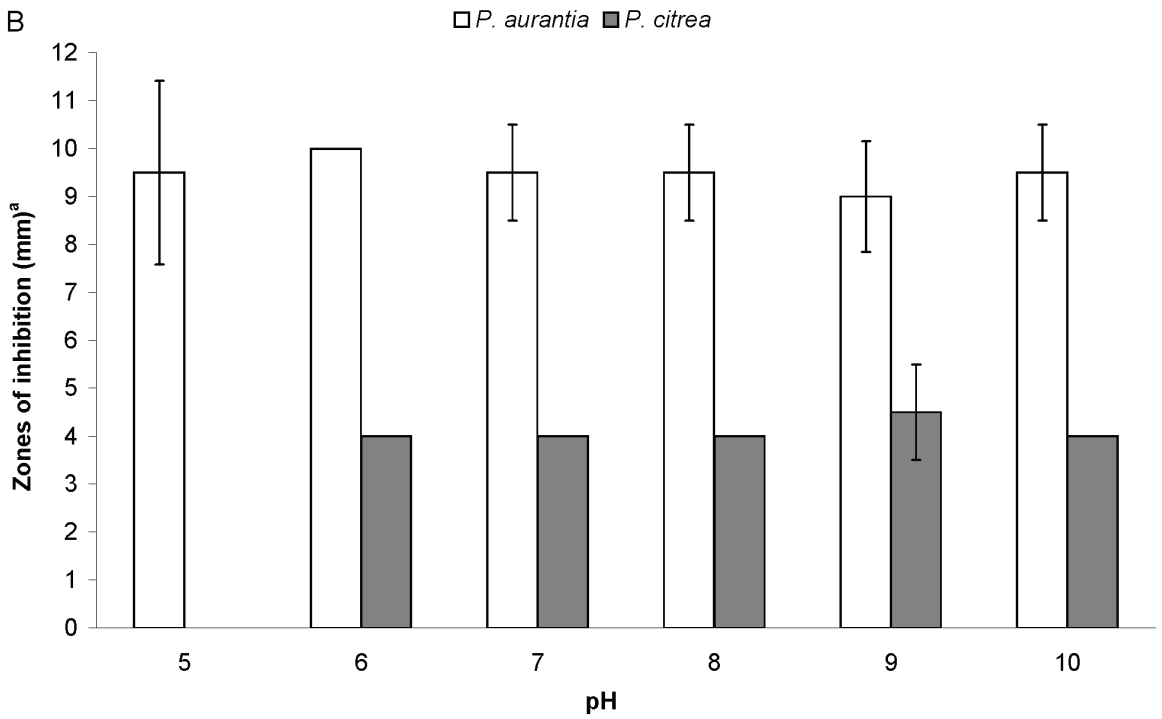
B


P. aurantia
P. citrea
Zones of inhibition (mm)a
0
1
2
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pH

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Manuscript II

3.2 Inhibitory substances from *Pseudoalteromonas aurantia* and *Pseudoalteromonas citrea* are L-amino acid oxidases with broad substrate spectra

Inhibitory substances from *Pseudoalteromonas aurantia* and *Pseudoalteromonas citrea* are L-amino acid oxidases with broad substrate spectra

Jan Erik Rau [1], Olav Grundmann [2], and Ulrich Fischer [1]

[1] Zentrum für Umweltforschung und nachhaltige Technologien, Fachbereich Biologie/Chemie, Abteilung Marine Mikrobiologie, Universität Bremen, Leobener Straße, D-28359 Bremen, Germany

[2] Max-Planck-Institut für Marine Mikrobiologie, Abteilung Mikrobiologie, Celsiusstrasse 1, D-28359 Bremen, Germany

Corresponding author:

Jan Erik Rau

Tel.: +49- (0) 421-218-63409

Fax: +49- (0) 421-218-7222

Email: jerau@uni-bremen.de

Abstract

Inhibitory substances produced by *Pseudoalteromonas aurantia* NCIMB 2052^T and *Pseudoalteromonas citrea* NCIMB 1889^T were examined. The results clearly indicate that these substances are L-amino acid oxidases (L-AAOs), which are resistant to SDS as well as to ß-mercaptoethanol when used in concentrations typical for application during SDS-PAGE. In-gel detection of the L-AAOs using non-denaturing SDS-PAGE linked with antibacterial assays reveals that *P.aurantia* is most probably producing three of these enzymes with molecular masses of 60-65, 225-230, and >260 kDa, whereas *P. citrea* is producing only two with molecular weights of 165-170 and 225-230 kDa. The enzymes of *P.aurantia* and *P. citrea* show high to good hydrogen peroxide producing activities with 11 respectively 10 L-amino acids as substrates (see Fig. 4). Thus, these L-AAOs have certainly broad substrate spectra.

Keywords: L-Amino acid oxidase, antibacterial activity, non-denaturing SDS-PAGE, *Pseudoalteromonas aurantia*, *Pseudoalteromonas citrea*

1. Introduction

An overview about the history, physiological features, and ecological significance of the genus *Pseudoalteromonas* was recently given by Bowman [1]. According to this review, the genus *Pseudoalteromonas* contains numerous marine species synthesizing biologically active substances which act upon a variety of target organisms. This seems to be a unique characteristic for this genus and may greatly benefit *Pseudoalteromonas* cells in their competition for nutrients and colonisation of habitats. Many members of this genus are producing bioactive substances of low and high molecular weight with antimicrobial, anti-fouling, algicidal, and various pharmaceutically-relevant activities [1]. *Pseudoalteromonas aurantia* NCIMB 2052^T and *Pseudoalteromonas citrea* NCIMB 1889^T are also known to produce polyanionic antibiotics [2,3], but their exact substantial and functional nature is still remaining unknown.

L-Amino acid oxidases (L-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.2., L-AAO) are flavoenzymes catalysing the oxidative deamination of L-amino acids to the respective α-keto acids with the release of ammonium and hydrogen peroxide [4] which determines the antimicrobial property of L-AAOs [5]. The enzyme is widely distributed in snake [6,7] and insect venoms [8] but also in algal [9], bacterial [10,11], cyanobacterial [12], fungal [13], gastropodal [14], and vertebratal sources [15,16]. L-AAOs have been described also for some members of the genus *Pseudoalteromonas* such as *P. flavipulchra* [17], *P. luteoviolacea* [18,19], and *P. tunicata* [20,21]. These kinds of compounds have been reported as antitumor [8,13] as well as bacteriostatic and bacteriocidal agents [14]. It is speculated that

Abbreviations:
L-Amino acid oxidase (L-AAO); Fast protein liquid chromatography (FPLC); Size exclusion chromatography (SEC); Tryptic soy agar (TSA)

L-AAOs might be also involved in the amino acid metabolism and the utilization of ammonia as a nitrogen source [22,23]. L-AAOs can be differentiated into enzymes with a more strict substrate specificity [24] and into those which have a broader spectrum [19]. They typically have a dimeric structure. FAD is the cofactor of L-AAOs and binds strongly but not covalently to the protein. A main commercial application for L-AAOs is the production of D-amino acids by enzymatic resolution of racemic amino acid solutions [25,26].

The aim of the present study was to elucidate the structural and functional nature of the inhibitory substances produced by *P. aurantia* NCIMB 2052^T and *P. citrea* NCIMB 1889^T by investigating the mechanism of inhibition, the possible substrate spectra, and the approximate molecular weights and number of these substances.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

P. aurantia NCIMB 2052^T and *P. citrea* NCIMB 1889^T were cultivated using artificial sea water (ASW), modified after Kester et al. [27]. The medium contained (l^{-1} distilled water) 5.0 g Casamino acids, 1.0 g yeast extract, 1.0 g glucose, 17.54 g NaCl, 18.49 g $MgSO_4 \cdot 7H_2O$, 1.12 g KCl, 2.21 g $CaCl_2 \cdot 2H_2O$, 150.0 mg KBr, 50.0 mg Fe(III) citrate, 10.0 mg KH_2PO_4 (pH 7.5). Solid ASW contained (l^{-1} distilled water) 15.0 agar, additionally. The culture medium was adjusted to pH 7.6. In dependence of the assay to be performed and if not otherwise stated, cultivation was routinely performed at 26°C (*P. aurantia*) respectively 21°C (*P. citrea*) for 1-4 d on either solid

or in liquid ASW by shaking at 150 rpm. In the latter case, an OD_{600} of 0.1 was used to inoculate the bacterial cultures. Cultivation of the target organism and catalase negative mutant *E. coli* UM255 [28] was routinely performed at 37°C using LB as described for the Pseudoalteromonads. Microorganisms were maintained at -80°C in appropriate medium with 30% (v/v) glycerol.

2.2 Preparation of concentrated supernatants

P. aurantia and *P. citrea* were grown in 1 l ASW for 24 h in 2 l Erlenmeyer flasks as described. The bacterial culture supernatants were collected by centrifugation at 10,000 rpm and 4 °C for 60 min and sterile filtered. 100-fold concentration of the sterile supernatants was performed using cross-flow filtration with a cut off value of 10 kDa. Storage was done by freezing at -20°C.

2.3 Spectrophotometrical identification of inhibitory substances and determination of substrate spectra

A modified peroxidase-coupled assay, based on the spectrophotometrical measurement of hydrogen peroxide described by Chen et al. [17], was carried out in a 1 ml cuvette with 500 µl acetone precipitated supernatants derived from *P. aurantia* and *P. citrea* cultures per standard assay mixture test (final volume: 1 ml). Acetone precipitation was performed by adding 5 volumes ice-cold acetone to 20 ml culture supernatant each and homogenised by gentle shaking. The mixtures were stored for 24 h at -20 °C before centrifugation (4,000 x *g*, 4 °C, 30 min). The pellets were resuspended in 20 ml distilled water each.

116

117 2.4 Effect of SDS and ß-mercaptoethanol on inhibitory substances

118

119 Sterile-filtrated, 100-fold concentrated culture supernatants derived from both
120 *Pseudoalteromonas* strains were exposed to SDS (0.01, 0.1, 1, 2%; final
121 concentrations), SDS plus ß-mercaptoethanol (1.5 respectively 4%; final
122 concentrations) as well as 1-fold concentrated sample buffer (0.0625 M Tris-HCl,
123 pH 6.8, 1.5% SDS, 4% ß-mercaptoethanol, 10% glycerol, and 0.01% bromophenol
124 blue; final concentrations). The initial concentration of the concentrated supernatants
125 was divided by half trough mixing with the above mentioned solutions. The resultant
126 solutions were used in agar diffusion tests, based on the method described by Bauer
127 et al. [29] with the following modifications. Circular wells with a diameter of 6 mm
128 were cut into the surface of an LB agar plate. Approximately 15 ml agar medium
129 were used per plate. Culture of the target organism *E. coli* UM255 (60 µl;
130 OD_{600} = 0.1) was plated onto the agar surface using a Drigalski spatula. 50 µl of the
131 different solutions respectively control were added into the wells, and zones of
132 inhibition were determined after 24 h of incubation at 37 °C. SDS (0.01, 0.1, 1, 2%),
133 SDS plus ß-mercaptoethanol (1.5 respectively 4%), 1-fold concentrated sample
134 buffer, 50-fold concentrated culture supernatants, and a 0.03% H_2O_2 solution were
135 used as different controls. Mean diameters of inhibition zones were quantified by
136 subtracting the diameter of the well (6 mm).

137

138 2.5 Determination of number and molecular weights of inhibitory substances

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140 2.5.1 Ultrafiltration by using different pore sizes

An initial volume of 15 ml sterile filtrated culture supernatants of *P. aurantia* and *P. citrea* each was subjected to a sequence of several pressure filtration runs using ultrafiltration units (Amicon), beginning with the largest pore size. The different cut-off values were 10, 30, 50, and 100 kDa. Centrifugation steps were performed at 4,000 x *g* and 4 °C for 20 min. Each time, the concentrate as well as 150 µl of the flow-trough were collected and stored at 4 °C until usage. The remaining of the flow-trough was subjected to another ultrafiltration unit with the next smaller pore size and centrifuged again. This procedure was repeated until the smallest pore size with a cut-off value of 10 kDa was reached, resulting overall in 8 fractions (4 times each concentrate respectively flow-through) of different kDa ranges (see Fig. 3). These fractions were used in agar diffusion tests, which were performed as described, except that the target organism was not plated onto the solid agar, because the LB agar medium had been pre-inoculated with an OD_{600} of 0.001. These plates were stored at most for 7 d.

2.5.2 In-gel detection of inhibitory substances using SDS-PAGE linked with antibacterial assays

SDS-PAGE and in-gel detection of inhibitory substances were performed as described previously [30] with the following modifications: i) 100-fold concentrated culture supernatants derived from both *Pseudoalteromonas* strains were used, ii) the stacking gel had an acrylamide concentration of 4% and the separation gel of 6%, respectively, iii) the Spectra™ Multicolor Broad Range Protein Ladder (Fermentas) was used for the protein standards, iv) the Coomassie-stained lane was not treated

with the modified assay mixture solution, whereas the first non-stained lane was treated, and v) the second non-stained lane was placed onto an LB agar plate containing the catalase negative mutant *E. coli* UM255 (OD600: 0.001) as target organism and incubated at 37 °C for 24 h to detect possible inhibitory activities of the protein bands.

All tests were performed in duplicates.

3. Results and Discussion

3.1 Identification and functional characterisation of inhibitory substances

The inhibitory substances produced by *P. aurantia* and *P. citrea* seem to have a relatively broad substrate spectrum (Fig. 4). A significant production of hydrogen peroxide could be detected with concentrated culture supernatants of both organisms when the L-amino acids Met, Leu, Gln, Phe, Tyr, Lys, Arg, His, Trp, Ala, and Asn were added as sole substrates. As can be seen in Fig. 1, the H_2O_2 production was always weaker with the supernatant of *P. citrea*. Using L-Gln as substrate, it could be shown that the H_2O_2 production was high only with the culture supernatant of *P. aurantia*, while it was less than the control with the one from *P.citrea*. When the D-amino acids Met, Leu, and Lys were used as substrates, the H_2O_2 production was clearly weaker than that of the substances alone (data not shown). Therefore, the inhibitory substances produced by *P. aurantia* and *P. citrea* are actually L-AAOs with broad substrate spectra.

L-AAOs from other species of the genus *Pseudoalteromonas* are described to have either relatively low substrate specificity [17,19], or a very high one [20,21]. Comparing the substrate spectra of the L-AAOs produced by *P. flavipulchra* strain C2 [17] and strains of *P. luteoviolacea* [19] with those from *P. aurantia* and *P. citrea*, a lot of similarities can be found. The L-amino acids Met, Leu, Gln, Tyr, Phe, Trp, and Lys served nearly in all cases as substrates for the L-AAOs produced by the mentioned organisms.

3.2 Substantial characterisation of inhibitory substances

As can be seen clearly in Figure 2 A and B, the antibacterial activity of 50-fold concentrated supernatant from *P. aurantia* was not significantly affected in dependence on different SDS-concentrations (0.01-2%; Fig. 2 A) as well as by a combination of SDS (1.5%) plus ß-mercaptoethanol (4%) and by 1-fold concentrated sample buffer, respectively (Fig. 2 B, I and III). Use of both latter mentioned solutions showed only weak inhibition (Fig. 2 B, II and IV) which was unambiguously weaker than that of both solutions together with concentrated supernatant (Fig. 2 B, I and III). In all these approaches, the antibacterial activity was similar to that of untreated 50-fold concentrated supernatant from *P. aurantia* alone (data not shown). Growth of the target organism *E. coli* UM255 was not influenced when only SDS was used in the same concentrations as controls (data not shown). Similar results as described for *P. aurantia* were also obtained with *P. citrea* (data not shown).

The obtained data have shown that the antibacterial activity of the inhibitory enzymes produced by *P. aurantia* and *P. citrea* is relatively resistant to SDS as well

as ß-mercaptoethanol. This is in accordance to results reported by Chen et al. [11,17]. The authors found the activities of antibacterial proteins from *Rheinheimera* sp. strain GR5 respectively *P. flavipulchra* strain C2 to be resistant to SDS as well as ß-mercaptoethanol (final concentrations 1% each).

Culture supernatants derived from both *Pseudoalteromonas* strains were applied to a sequence of ultrafiltration runs in order to determine the approximate molecular weights and number of their inhibitory substances. The resultant fractions were used in agar diffusion tests. As illustrated in Figure 3 A, antibacterial activity was found in three concentrated fractions (>100, >30 <50, and >50 <100 kDa in order of descending activities) from the supernatant of *P. aurantia*, but only in one of that from *P. citrea* (>100 kDa; see Fig. 3 B). None of the flow-through fractions (not concentrated) exhibited antibacterial activity, even when the corresponding concentrated ones showed activity. Most probably too low inhibitory substance concentrations in the not concentrated fractions were the reason for these findings. High molecular weights of produced inhibitory substances have also been reported for other species of the genus *Pseudoalteromonas* [17-20,31,32].

Non-denaturing SDS-PAGE was used to maintain enzymatic activity and to estimate the approximate number and molecular weights of the inhibitory enzymes investigated in the present study. Using Coomassie brilliant blue staining, the main proteins, present in sterile-filtrated, 100-fold concentrated culture supernatants derived from *P. aurantia* and *P. citrea*, were detected (Fig. 4 A and B). Coloured versions of the figures are accessible as supplementary material (Supplementary Fig. S1, available in EMT Online).

240 The bands of antibacterial as well as H_2O_2 producing activity and the corresponding
241 Coomassie-stained protein bands on SDS-PAGE from *P. aurantia* were present in
242 the range i) between 60-65 kDa, ii) between 225-230 kDa, and iii) larger than
243 260 kDa in size (Fig. 4 A), while corresponding bands of *P. citrea* were found in
244 ranges between i) 165-170 kDa and ii) 225-230 kDa in size (Fig. 4 B). Thus, the
245 inhibitory substances produced by *P. aurantia* and *P. citrea* are L-AAOs with high
246 molecular weights.
247 This is in agreement with several reports concerning other L-AAO producing strains
248 from the genus *Pseudoalteromonas*. Gómez et al. [19] also found the antimicrobial
249 macromolecules synthesised by 3 different *P. luteoviolacea* strains to be L-AAOs
250 and estimated a molecular weight of approximately 110 kDa for the L-AAO of strain
251 CPMOR-2, while Chen et al. [17] reported a molecular mass of 60 kDa for the L-AAO
252 of *P. flavipulchra* strain C2. *P. tunicata* strain D2 is also known to produce a 190 kDa
253 L-AAO [20,21].
254 Additionally, the L-AAOs produced by *P. aurantia* and *P. citrea* also have been
255 investigated using size exclusion chromatography (SEC) via fast protein liquid
256 chromatography (FPLC). The resulting fractions were examined for antibacterial
257 activity using tryptic soy agar (TSA) to pour the plates and *Staphylococcus*
258 *epidermidis* as target organism, revealing that *P. aurantia* is producing 3 L-AAOs of
259 approximately 100, 240, and 480 kDa in size (data not shown). These findings are
260 mainly in accordance with the results obtained in the SDS-PAGE study, whereby
261 *P. aurantia* is also producing 3 L-AAOs (see above). Gauthier and Breittmayer [3]
262 reported only the presence of one polyanionic inhibitory substance produced by
263 *P. aurantia*.

For *P. citrea*, the SEC together with the tested antibacterial activity showed that only one L-AAO of approximately 120 kDa in size was produced (data not shown). This is in contrast to the SDS-PAGE study, where *P. citrea* was producing 2 L-AAOs (see above). Using an electrophoresis setup with Cellogel strips, Gauthier [2] also found 2 different polyanionic inhibitory substances for *P. citrea*. Two facts might be considered to explain the differences between the results of the SDS-PAGE and SEC studies as well as those obtained by Gauthier [2] and by Gauthier and Breittmayer [3]. First, *Staphylococcus epidermidis* was used as target organism for the agar diffusion tests with fractions obtained during SEC, by Gauthier [2] and by Gauthier and Breittmayer [3]. This organism is less sensitive to the inhibitory enzymes produced by *P. aurantia* and *P. citrea* (data not shown), in comparison to the catalase negative mutant *E. coli* UM255, which was used for the SDS-PAGE study. Second, tryptic soy agar (TSA) was used for the agar diffusion tests during the SEC study, by Gauthier [2] and by Gauthier and Breittmayer [3], instead of LB, which was used for the SDS-PAGE study. It is known that the type and the amount of amino acids present in the incubation medium influence the activity level of L-AAOs [11,17,19]. Thus, both mentioned characteristics might be an explanation for the difference in the number of inhibitory enzymes obtained with the in-gel assays after SDS-PAGE and by SEC in the present study as well as reported by Gauthier [2] and by Gauthier and Breittmayer [3].

To prove that the L-AAOs from *P. aurantia* of high molecular weights (225-230 and >260 kDa) might be polymers of the 60-65 kDa L-AAO, acetone-precipitated 100-fold concentrated culture supernatant was subjected to a 100 kDa ultrafiltration unit (Amicon) to separate the 60-65 kDa L-AAO from the others. Both fractions obtained

were then undergone the described peroxidase-coupled assay. The results showed that the substrate spectra of both fractions are nearly identical (see Supplementary Fig. S2, available in EMT Online).

From these observations, it can be assumed that *P. aurantia* does not produce 3 different L-AAOs but only one structural unit of 60-65 kDa in size. The 225-230 kDa substance is possibly a tetramer of the 60-65 kDa unit, whereas the substance >260 kDa, respectively 480 kDa with regard to the results obtained by SEC, might be the corresponding octamer. This assumption is supported by findings with respect to isoelectric point studies of the inhibitory substances from *P. aurantia*. We found out that only the precipitate derived from pH 8.7 had inhibitory activity, but none of the fractions obtained at other pH values (data not shown). In addition, if the 100-fold concentrated culture supernatant of *P. aurantia* was incubated together with sample buffer for 30 min at various temperatures (20, 40, 50, and 60°C) before performing SDS-PAGE, it could be demonstrated that the bands corresponding to L-AAOs of 225-230 and >260 kDa in size clearly disappeared before that of the 60-65 kDa L-AAO (see Supplementary Fig. S3, available in EMT Online). Both of these findings (isoelectric points and temperature treatment) contribute to the hypothesis of the occurrence of only one structural unit of 60-65 kDa in size with inhibitory activity. This assumption is additionally supported by studies on the green microalga *Chlamydomonas reinhardtii* strains CW15, a class-C mutant characterised by the absence or greatly reduced quantity of cell wall compared to wildtype cells. Piedras et al. [33] found the L-AAO of the mentioned strain to have a molecular weight of ~470 kDa, consisting of eight identical or similarsized subunits of ~60 kDa each, as also speculated in the present study for the L-AAO of *P. aurantia*. Vallon et al. [34] investigated strain CW15 as well and described an L-AAO of

~900-1000 kDa and another form of ~1200-1300 kDa, consisting of ~13-16 identical subunits of 66 kDa in size (with L-AAO activity), and, in the case of the heavier form, additionally of 3-4 copies of a 135 kDa polypeptide (without L-AAO activity). If the L-AAO reported by Piedras et al. [33] is part of the L-AAO found by Vallon et al. [34] or if both are different enzymes sharing some similar properties, could not be clarified [34].

4. Conclusions

In the present study it could be shown that the antibacterial activity of the inhibitory substances produced by *P. aurantia* and *P. citrea* is maintained during treatment with SDS as well as ß-mercaptoethanol when used in concentrations typical for application during SDS-PAGE. The results of the in-gel detection of inhibitory substances using non-denaturing SDS-PAGE linked with antibacterial assays have indicated that *P.aurantia* is most probably producing three inhibitory substances (60-65, 225-230, and >260 kDa) and *P. citrea* two (165-170, 225-230 kDa). Further on, it can be assumed that the three inhibitory substances of *P. aurantia* might be all composed of one structural unit of 60-65 kDa in size. Due to the specificity of the used H_2O_2 detecting assay, it could be clearly shown that the inhibitory substances of *P. aurantia* and *P. citrea* are L-AAOs whose antibacterial activities are caused by the production of H_2O_2 in the presence of appropriate amino acids. These L-AAOs had broad substrate spectra. When comparing the L-AAO spectra of both organisms, it could be shown that there are some differences, but generally both spectra are rather similar. Thus, it could be presumed that the structure of the active parts of the L-AAOs might be identical, supported by findings concerning other

Pseudoalteromonas species [17,19]. It would be desirable to gain more information about the L-AAOs produced by *P. aurantia*, *P. citrea*, and other Pseudoalteromonads due to their possible use in antibiotic therapy as well as in the biotechnological field.

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Figure legends

Fig. 1. Hydrogen peroxide production (ppm) formed in a peroxidase-coupled assay with acetone-precipitated culture supernatants of *P. aurantia* NCIMB 2052^T and *P. citrea* NCIMB 1889^T depending on the L-amino acid added. Further details are given in material and method section. Control without any amino acid, C.

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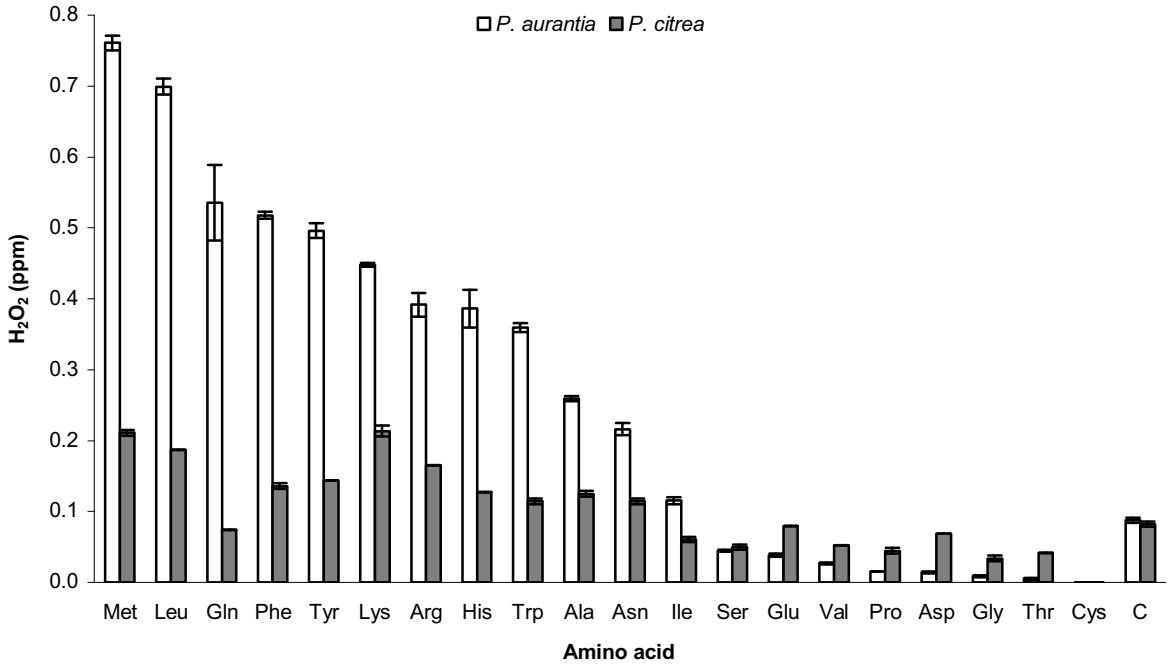


488
489

490 **Fig. 2.** Influence of SDS, ß-mercaptoethanol, and sample buffer on the antibacterial
491 activity of 50-fold concentrated culture supernatant of *P. aurantia* NCIMB 2052^T.
492 A: Antibacterial activity in dependence on different SDS-concentrations.
493 B: Antibacterial activity in the presence of (I) 1.5% SDS plus 4% ß-mercaptoethanol
494 and (III) 1-fold concentrated sample buffer (see Materials and Methods).
495 II and IV: Same solutions as in (I) respectively (III) but without concentrated culture
496 supernatant. Agar diffusion tests were performed using the catalase negative mutant
497 *Escherichia coli* UM255 as target organism. 0.03% H_2O_2 served as control, C.

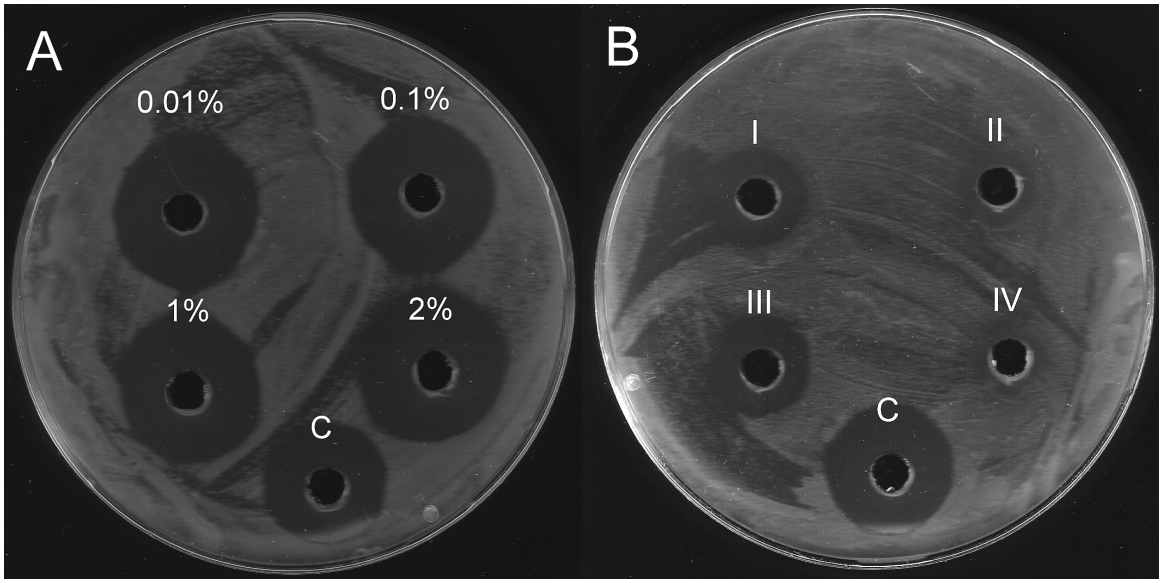


Fig. 3. Antibacterial activity in fractions (concentrate respectively flow-through) of different molecular weights obtained by using a sequence of ultrafiltration runs from culture supernatants of *P. aurantia* NCIMB 2052^T (A) and *P. citrea* NCIMB 1889^T (B). Agar diffusion tests were performed using the catalase negative mutant *Escherichia coli* UM255 as target organism. Further details are given in the material and method section. 0.03% H_2O_2 served as control, C. Numbers refer to kilodalton (kDa). Concentrated fractions are marked (+).

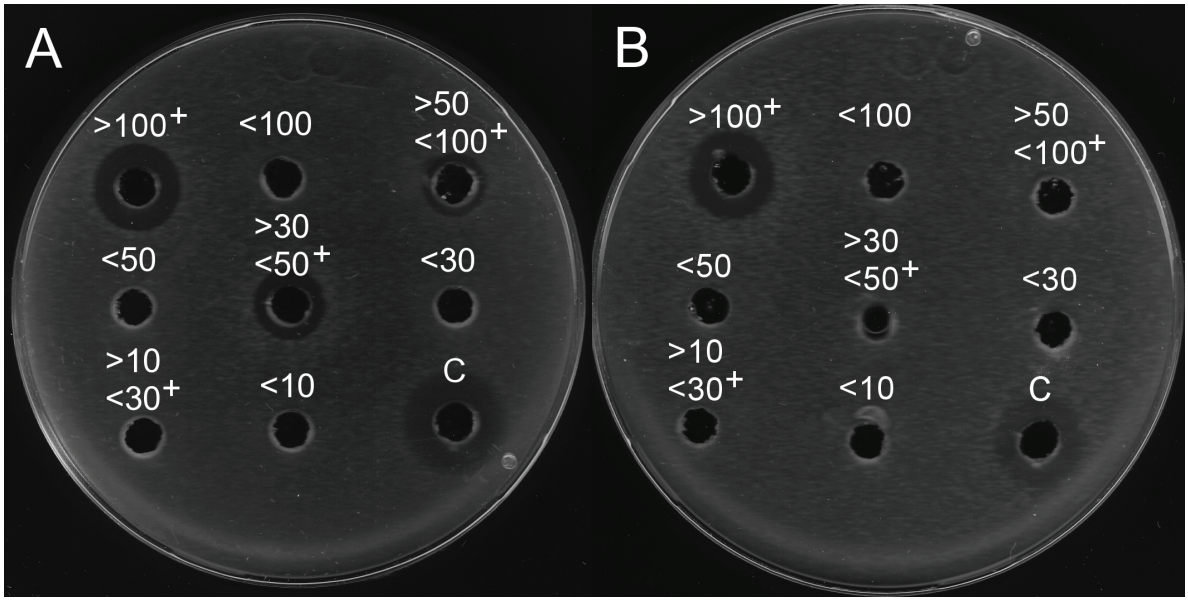


Fig. 4. SDS-PAGE and 2 different in-gel assay techniques for detecting H_2O_2 production and antibacterial activity of 100-fold concentrated culture supernatants from *P. aurantia* NCIMB 2052^T (A) and *P. citrea* NCIMB 1889^T (B). Triplicate lanes loaded with identical non-heated samples were handled separately after gel electrophoresis. Lane I: stained with CBB. Lane II: in-gel detection of the inhibitory substances using the H_2O_2 detecting assay. Lane III: inhibitory plate test on LB agar with the catalase negative mutant *E. coli* UM255 as target organism (zones of inhibition were visualised). M: molecular masses (kDa) of standard proteins (Spectra™ Multicolor Broad Range Protein Ladder; Fermentas). Coloured versions of the figures are accessible as supplementary material (Supplementary Fig. S1, available in EMT Online).

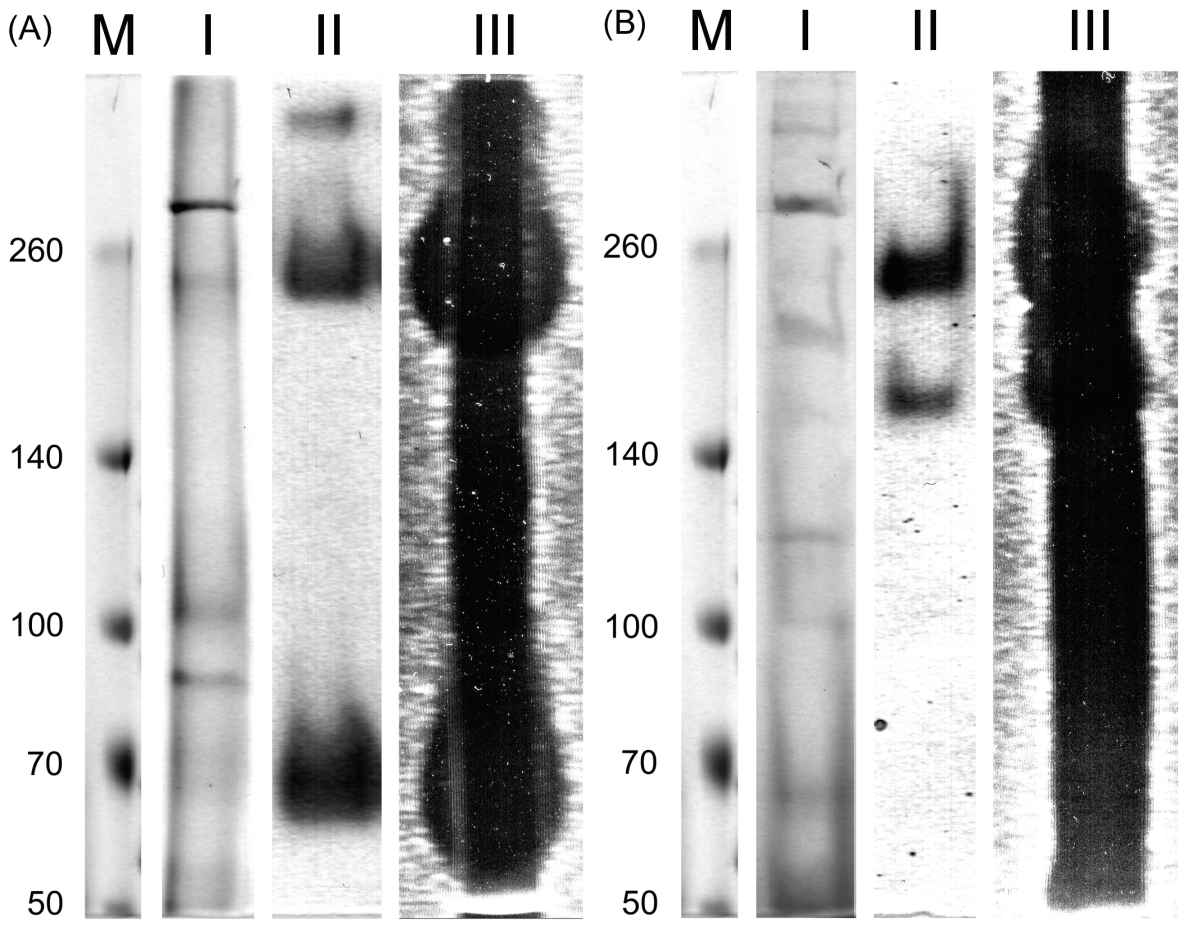
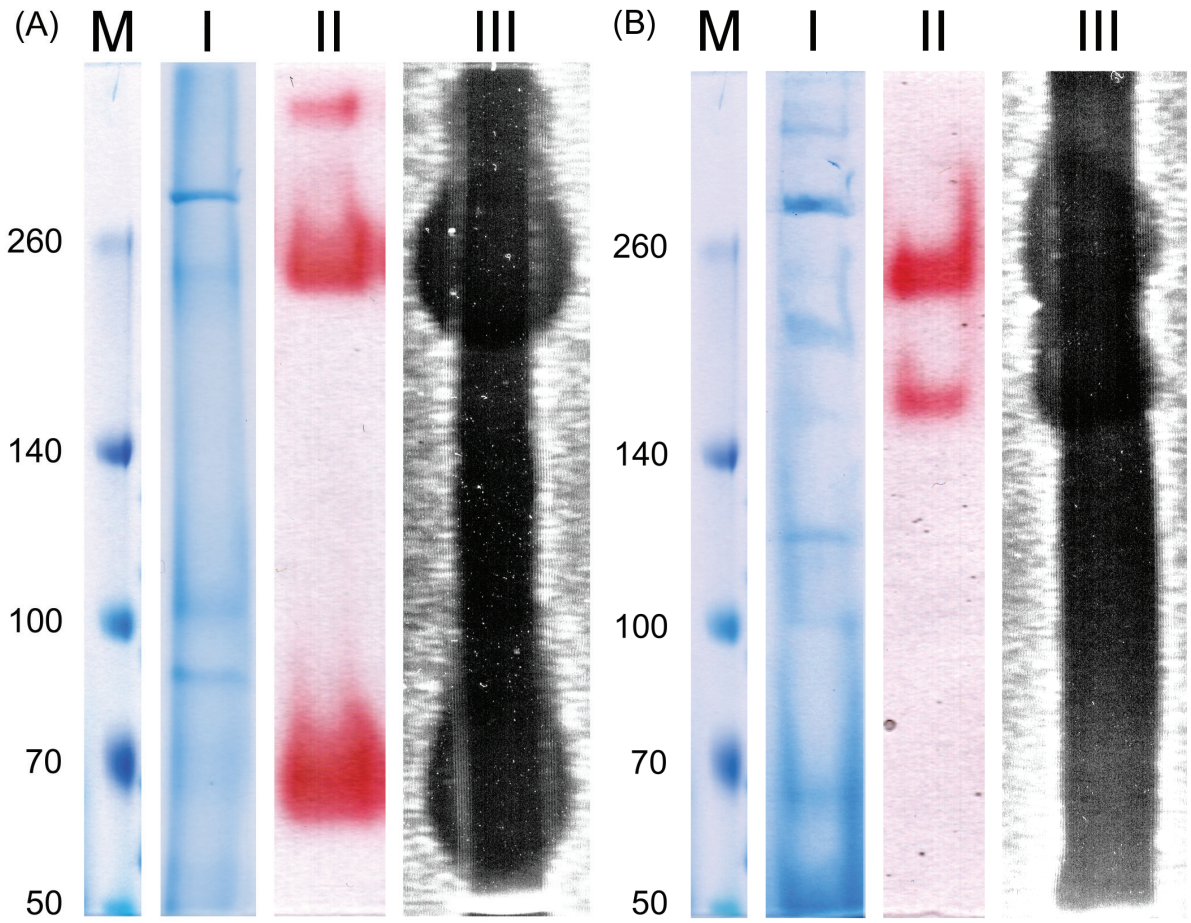
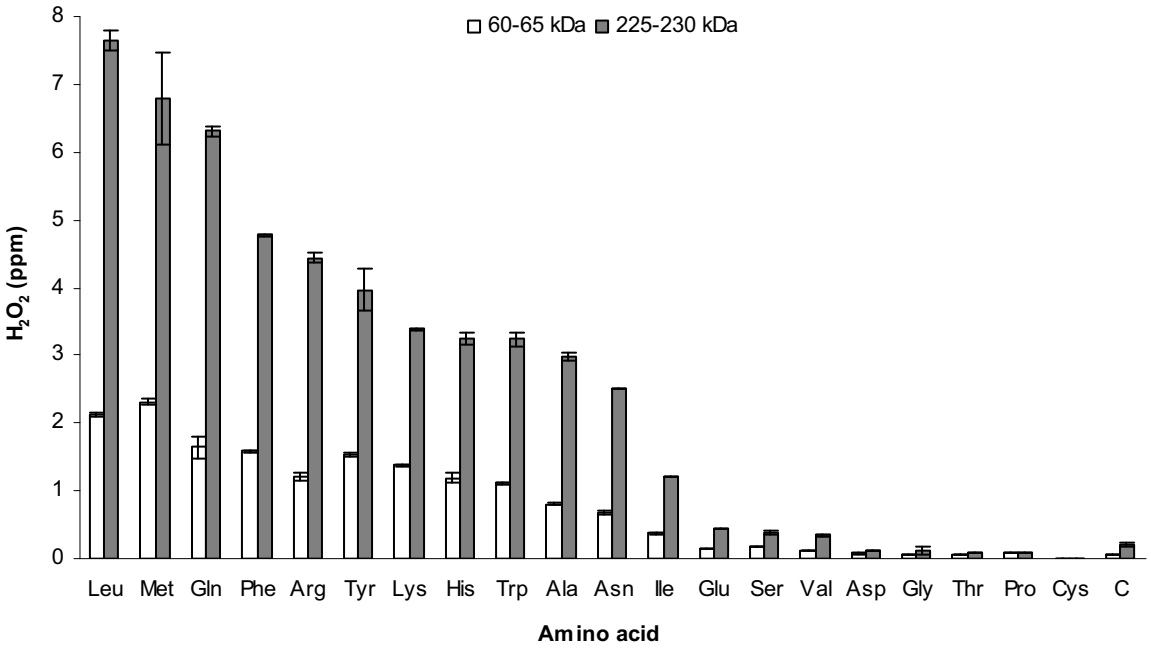


Fig. S1. SDS-PAGE and 2 different in-gel assay techniques for detecting H_2O_2 production and antibacterial activity of 100-fold concentrated culture supernatants from *P. aurantia* NCIMB 2052^T (A) and *P. citrea* NCIMB 1889^T (B). Triplicate lanes loaded with identical non-heated samples were handled separately after gel electrophoresis. Lane I: stained with CBB. Lane II: in-gel detection of the inhibitory substances using the H_2O_2 detecting assay. Lane III: inhibitory plate test on LB agar with the catalase negative mutant *E. coli* UM255 as target organism (zones of inhibition were visualised). M: molecular masses (kDa) of standard proteins (Spectra™ Multicolor Broad Range Protein Ladder; Fermentas).

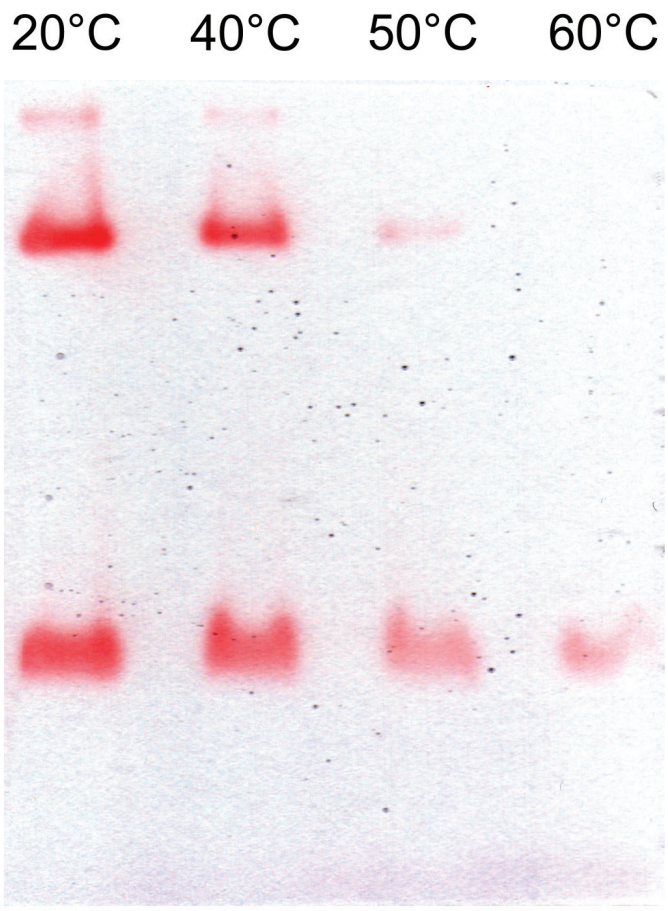


534 **Fig. S2.** Hydrogen peroxide production (ppm) formed in a peroxidase-coupled assay
535 with fractions containing the inhibitors of either 60-65 or 225-230 kDa in size derived
536 from acetone-precipitated 100-fold concentrated culture supernatant of *P. aurantia*
537 NCIMB 2052^T depending on the L-amino acid added. Further details are given in the
538 material and method section. Control without any amino acid, C.
539



540
541

542 **Fig. S3.** SDS-PAGE and in-gel assay technique for detecting H_2O_2 producing activity
543 of 100-fold concentrated culture supernatant from *P. aurantia* NCIMB 2052^T after
544 having been incubated for 30 min at different temperatures. Samples applied were
545 incubated together with 1-fold concentrated sample buffer (see Materials and
546 Methods).
547



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Manuscript III

3.3 L-lysine oxidase: an inhibitory substance produced by the marine filamentous cyanobacterium *Geitlerinema* strain Flo1

Running title:

L-lysine oxidase from *Geitlerinema* strain Flo1

Title:

L-lysine oxidase: an inhibitory substance produced by the marine filamentous cyanobacterium *Geitlerinema* strain Flo1

Jan Peter Schrübbers[a]

Jan Erik Rau[a]

Ulrich Fischer[a]

[a]Zentrum für Umweltforschung und nachhaltige Technologien, Fachbereich Biologie/Chemie, Abteilung Marine Mikrobiologie, Universität Bremen, Leobener Straße, D-28359 Bremen, Germany

Corresponding author:

Jan Erik Rau

Tel.: +49- (0) 421-218-63409

Fax: +49- (0) 421-218-7222

Email: jerau@uni-bremen.de

Abstract

An inhibitory substance is secreted by the marine filamentous cyanobacterial strain Flo1 which inhibits the growth of several microorganisms. The inhibitory effect of culture supernatant derived from strain Flo1 is caused by an L-amino acid oxidase (L-AAO). The enzyme shows high hydrogen peroxide producing activity only with the substrate L-lysine and can therefore be regarded as an L-lysine oxidase. This enzyme uses L-lysine, oxygen, and water as substrates resulting in the respective α-keto acid with the release of ammonium and hydrogen peroxide. In-gel detection of the enzyme using non-denaturing SDS-PAGE linked with antibacterial assays reveals that strain Flo1 produces an L-lysine oxidase with an approximate molecular weight of 55 kDa. Phylogenetic studies show that strain Flo1 is taxonomically most closely related to the genus *Geitlerinema*. However, the closest relative of strain Flo1 at 16S rDNA level, *Geitlerinema* strain PCC 7105, does not produce a substance of this kind. This is the first report of a marine cyanobacterium using an L-lysine oxidase for generating hydrogen peroxide that inhibits microbial growth.

Keywords: Antibacterial activity, *Geitlerinema* strain Flo1, L-lysine oxidase, non-denaturing SDS-PAGE

Abbreviations: L-amino acid oxidase (L-AAO); Artificial seawater of half salinity supplemented with nitrate (ASN III/2)

Introduction

The marine filamentous cyanobacterial strain Flo1 has been studied recently due to its high biotechnological potential. This organism produces and secretes at least one biologically active substance into the environment which inhibits growth of the yeast *Saccharomyces cerevisiae*, of several heterotrophic marine bacteria as well as of fungi (Heyduck-Söller and Fischer, 2000; Caicedo et al., 2010). However, the substance has not been identified so far by the mentioned studies. Additionally, strain Flo1 has been used as model organism for quantitative analysis of bioproduction of antimicrobial compounds (Caicedo et al., 2010).

Cyanobacteria are commonly known as effective producers of secondary metabolites of biotechnological and pharmaceutical interest. Many different products with a broad range of biological activity have been reported (Burja et al., 2001). These substances can have toxic, antibacterial, antiviral, fungicide, immunosuppressive, and enzyme inhibiting activities (Burns et al., 2005).

A further class of bioactive substance with allelopathic effects are the L-amino acid oxidases (L-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.2., L-AAO). L-AAOs are flavoenzymes catalysing the oxidative deamination of L-amino acids to the respective α-keto acids with the release of ammonium and hydrogen peroxide (Wellner and Meister, 1961) which determines the antimicrobial property of L-AAOs (Stiles et al., 1991). The enzyme is widely distributed in snake (Du and Clemetson, 2002; Alves et al., 2008) and insect venoms (Ahn et al., 2000) but also in algal (Fujisawa et al., 1982), bacterial (Tong et al., 2008; Chen et al., 2010a), cyanobacterial (Gau et al., 2007), fungal (Kusakabe et al., 1980), gastropodal (Yang et al., 2005), and vertebratal sources (Sun et al., 2002; Kasai et al., 2010). L-AAOs

have been reported as antitumor (Kusakabe et al., 1980; Ahn et al., 2000) as well as bacteriostatic and bacteriocidal agents (Yang et al., 2005). L-AAOs can be differentiated into enzymes with a more strict substrate specificity (Lucas-Elío et al., 2006) and into those which have a broader spectrum (Gómez et al., 2008). They typically have a dimeric structure. FAD is the cofactor of L-AAOs and binds strongly but not covalently to the protein. A main commercial application for L-AAOs is the production of D-amino acids by enzymatic resolution of racemic amino acid solutions (Parikh et al., 1958; Takahashi et al., 1997).

The aim of the present study was i) to elucidate the structural and functional nature of an inhibitory substance produced by the marine filamentous cyanobacterial strain Flo1 by investigating the mechanism of inhibition, the possible substrate spectrum, and the approximate molecular weight of this substance as well as ii) to analyse the phylogenetic position of strain Flo1 amongst closely related cyanobacteria. As a reference, the same experiments were performed with *Geitlerinema* strain PCC 7105, the closest relative to strain Flo1.

Material and methods

Culture and growth conditions

Strain Flo1 was originally isolated from a mangrove wood in Florida near Key Biscane and cultivated in the culture collection of the Department of Marine Microbiology at the University of Bremen (Germany). The reference strain *Geitlerinema* PCC 7105 was provided by the Pasteur Culture Collection (Paris, France). Both strains were axenic, as proven by light microscopy. Organisms were

cultivated for 3 to 4 weeks in glass-columns (52 cm length and 5 cm in diameter, closed by a rubber stopper) containing 600 ml of artificial seawater of half salinity supplemented with nitrate (ASN III/2; modified after Rippka et al., 1979). The medium contained (l^{-1} distilled water) 12.5 g NaCl,1.0 g $MgCl_2 \cdot 6H_2O$, 0.25 g KCl,1.75 g $MgSO_4 \cdot 7H_2O$, 0.25 g $CaCl_2 \cdot 2H_2O$, 0.75 g $NaNO_3$, 0.12 g Na_2CO_3, 0.01 g $K_2HPO_4 \cdot H_2O$, 1.5 mg Fe-NH_4-citrate, 5 mg vitamin B_{12}, and 0.5 ml of a trace metal mix solution (modified after Rippka et al., 1979). This solution contained 3.0 g Na_3-citrate$\cdot 2H_2O$, 5.0 g Na_2-EDTA$\cdot 2H_2O$, 2.86 g H_3BO_3, 1.81 g $MnCl_2 \cdot 4H_2O$, 0.22 g $ZnSO_4 \cdot 7H_2O$, 0.39 g $Na_2MoO_4 \cdot 5H_2O$, 0.08 g $CuSO_4 \cdot 5H_2O$, and 0.05 g $Co(NO_3)_2 \cdot 6H_2O$. Cultures were kept under a constant photon flow density of 5 µE m^{-2} s^{-1} photosynthetically active radiation and a constant aeration with sterile-filtered air to avoid shading and precipitating of the cells. Cells were harvested by centrifugation at 5,000 rpm for 10 min. Cultivation of the target organisms was routinely performed at the optimal growth temperature of the particular strains for 1-3 d on either solid LB or MYG media or in liquid ones (see also 2.2) by shaking at 120 rpm. In the latter case an OD600 of 0.1 was used to inoculate the bacterial cultures.

Antibiogram assay

The antibacterial potential of culture supernatant from strains Flo1 and PCC 7105 were investigated using the agar diffusion test, based on the method described by Bauer and co-workers (1966) with the following modifications. Circular wells with a diameter of 6 mm were stamped into the surface of an LB agar plate (for *Saccharomyces pastorianus* DSM 6580^{NT} MYG agar was used) by the upper end of

a sterile Pasteur pipette. Approximately 15 ml agar medium were used per plate. Cultures of test strains (60 µl; OD600 = 0.1) were plated onto the agar surface using a Drigalski spatula. 50 µl of sterile filtered cyanobacterial culture supernatant were added to the wells and zones of inhibition were determined after 24 h of incubation at the optimal growth temperature of the respective target organism. 50 µl 0.03% H_2O_2 were used as a control. Mean diameters of inhibition zones were quantified by subtracting the diameter of the well (6 mm). Tests were performed in quadruplicate. Antibacterial activities of the inhibitory substance of strain Flo1 were tested by using the following organisms: *Bacillus subtilis* ATCC 6051^T, *Escherichia coli* K12, catalase negative mutant *Escherichia coli* UM255 (Mulvey et al., 1988), *Pseudomonas fluorescens* ATCC 13525^T, *Staphylococcus epidermidis* (clinical isolate, Molzym GmbH, Bremen), and the yeast *Saccharomyces pastorianus* DSM 6580^{NT}.

Identification of the inhibitory substance and determination of its substrate spectrum

A modified peroxidase-coupled assay, based on the spectrophotometrical measurement of hydrogen peroxide was carried out as described by Chen and co-workers (2010b) in order to identify the inhibitory substance produced by strain Flo1 and to determine its substrate spectrum. 500 µl sterile filtrated culture supernatant of strain Flo1 were used in a 1 ml cuvette per standard assay mixture test (final volume: 1 ml). Tests were performed in duplicate.

SDS-PAGE and in-gel antibacterial assays

143 SDS-PAGE and in-gel detection of inhibitory substances were performed as
144 described previously (Rau and Fischer, 2011), but with the following modifications:
145 37.5-fold concentrated culture supernatants derived from strains Flo1 and PCC 7105
146 were used. The Coomassie-stained lane was not treated with the modified assay
147 mixture solution, whereas the first non-stained lane was. The second non-stained
148 lane was placed onto an LB agar plate containing the catalase negative mutant
149 *E. coli* UM255 (OD600 0.001) as target organism and incubated at 37 °C for 24 h to
150 detect possible inhibitory activities of the protein bands. Spectra™ Multicolor Broad
151 Range Protein Ladder (Fermentas) was used as protein standard. The
152 corresponding figures 3 and S1 were digitally enhanced by adjusting colour and
153 contrast using the freeware program GIMP (version 2.6.11).
154
155 DNA extraction
156
157 Cells were disrupted by using N-Lauroylsarcosine (final conc. 1% for 1 h in the dark
158 at 4 °C), lysozyme (0.5 mg/ml), SDS (final conc. 1%), and proteinase K (100 µg/ml;
159 derived from *Tritirachium album*, AppliChem) for 1h at 37 °C. DNA was extracted
160 with phenol/chloroform/isoamylalcohol (25:24:1 (v/v/v)) and precipitated with
161 isopropanol.
162
163 Amplification of 16S rDNA
164
165 Amplification of 16S rDNA was performed using 2.5 mM $MgCl_2$, 0.2 mM dNTP
166 solution, 0.5 U polymerase (TrueStart Polymerase; Fermentas), and 0.8 µM each of

the primers 8F (AGAGTTTGATCCTGGC; Lane, 1991) and 1494R (GTACGGCTACCTTGTTACGAC; Taton et al., 2003).

The PCR thermal cycle included an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, primer annealing for 30 sec at 58 °C followed by a cycle extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. Amplification reactions were performed using a T3 Thermocycler (Biometra). PCR products were visualised with RedSafe (iNtRON Biotechnology) on 1% agarose gels. Products were purified (DNA Clean and Concentrator sample Kit; Zymo Research) and sequenced (GATC, Konstanz, Germany).

Phylogenetic analysis

The sequences obtained from amplification were merged together with Chromas Pro version 1.34. The closest relatives of strain Flo1 were determined by performing BLAST database searches. Subsequent analyses of the 16S rDNA sequence of the isolate, as well as of closely related sequences were performed using the ARB software package (version 5.2; Ludwig et al., 2004) and the corresponding SILVA SSURef 104 database (release October 2010; Pruesse et al., 2007). Nearly the complete 16S rDNA sequence of strain Flo1 (1,348 bp) was aligned automatically using the PT server implemented in the ARB software package and the alignment was checked manually. Phylogenetic trees were reconstructed according to the neighbour-joining (Saitou and Nei, 1987), maximum-parsimony (Fitch, 1971), and maximum-likelihood (Felsenstein, 1981) methods. The stability of the groupings was estimated by bootstrap analysis (Felsenstein, 1985) of the neighbour-joining and maximum-parsimony methods based on 1,000 replications. The 16S rDNA sequence

of strain Flo1 is deposited in the NCBI database (GenBank accession no. FJ042947).

Results and discussion

Antibiogram of the inhibitory substance

The antibiogram showed diverse inhibitory effects on the target organisms used. Gram-negative as well as Gram-positive bacteria were affected by the culture supernatant without any purification or concentration, indicating the high potential of the inhibitory substance. In particular, the culture supernatant of strain Flo1 showed no inhibitory effect upon the yeast *Saccharomyces pastorianus* and upon *Bacillus subtilis*, whereas distinct inhibition zones of 8 to 19 mm were found with strains *E. coli* K12*, P. fluorescens,* and *S. epidermidis* in ascending order. The highest inhibitory effect, resulting in an inhibition zone of 22 mm, was obtained with the catalase negative mutant *E. coli* UM255. The supernatant of the used reference strain PCC 7105 exhibited no inhibitory effect upon none of the target organisms tested (Table 1).

The inhibitory potential of substances produced by strain Flo1 was investigated previously by two other studies. Heyduck-Söller and Fischer (2000) reported that the cyanobacterial culture medium, pre-cultivated with strain Flo1 (basonym: *Oscillatoria limnetica* strain Flo1), showed growth inhibitory effects against the yeast *Saccharomyces cerevisiae* as well as against several Gram-negative and

Gram-positive bacteria, including *Escherichia coli*. Interestingly, these authors could not detect any inhibitory growth effect against *Bacillus subtilis*.

Caicedo and co-workers (2010) found two different Amberlite-extracts of the culture supernatant from strain Flo1 to show respectively antifungal and antibacterial activities against several fungi as well as against *B. subtilis*. Both studies (Heyduck-Söller and Fischer, 2000; Caicedo et al., 2010) made only minor attempts to identify the inhibitory substance(s). Caicedo and co-workers (2010) figured out that the addition of bromide ions to the culture medium resulted in a higher inhibitory activity, but only against the terrestrial bacteria tested and not against the marine bacterial strain Bo 53-31.

In the present study, we found a few contrary results to the above mentioned ones regarding the antimicrobial effects of culture supernatant from strain Flo1. Compared to the findings of Caicedo and co-workers (2010), *B. subtilis* was not inhibited by culture supernatants of strain Flo1 in the present study and in that by Heyduck-Söller and Fischer (2000). In addition, no inhibitory effect upon yeasts from the genus *Saccharomyces* was observed in the present work, contrary to the previous studies (Heyduck-Söller and Fischer, 2000; Caicedo et al., 2010). These differences might be explained by the use of different approaches to determine the antimicrobial activity of the culture supernatant from strain Flo1. As in the present study, Heyduck-Söller and Fischer (2000) used agar diffusion tests, but with the following differences: i) ASN III/2 agar medium supplemented with 0.1% (w/v) yeast extract was taken, ii) 1 ml of 10^6 cells of the respective target organism cultures was mixed with 15 ml of the agar medium, iii) paper disks were applied, and iv) only 10 µl of culture supernatant were applied. Caicedo and co-workers (2010) did not use agar diffusion tests. The authors determined the antimicrobial potential by following the

241 growth of the target organisms in microtiter plates by measurement of the turbidity at
242 570 nm. Instead of untreated culture supernatant, the authors evaluated the
243 inhibitory potential of different Amberlite-extracts derived from culture supernatant.
244 50 µl of the respective extract were mixed with 50 µl of 10^4 cells of the appropriate
245 target organism cultures per well of the microtiter plate.
246 As shown in the present study, the growth inhibitory effect of the culture supernatant
247 from strain Flo1 was found to be highest against the catalase negative mutant *E. coli*
248 UM255. This organism lacks catalase encoding genes and, therefore, is unable to
249 degrade present hydrogen peroxide. This indicates that catalase and, thereby, also
250 hydrogen peroxide could play an important role in the mechanism of inhibition of one
251 of the inhibitory substances produced by strain Flo1. However, the influence of
252 bromide ions was not investigated in the present study.
253
254 Identification and determination of the substrate spectrum
255
256 The antibacterial substance derived from concentrated culture supernatant of strain
257 Flo1 was used in a peroxidase-coupled assay with the 20 standard amino acids to
258 identify it and to elucidate its substrates (Fig. 1). When L-lysine was offered as sole
259 substrate, a significant production of hydrogen peroxide could be measured which
260 was unambiguously higher than that of the compound alone. When D-lysine was
261 used, the H_2O_2 production was clearly lower than that of the compound alone.
262 Therefore, the inhibitory compound produced and excreted into the medium by strain
263 Flo1 is actually an L-AAO with very high substrate specificity and can be regarded as
264 L-lysine oxidase.

Regarding the presence of L-AAOs in other cyanobacteria, these enzymes, to our knowledge, have been detected so far only in the order *Chroococcales* and, in particular, in the *Synechococcus elongatus* strains PCC 6301 and PCC 7942 as well as in *Synechococcus cedrorum* strain PCC 6908 (Gau et al., 2007). According to the latter mentioned study, the L-AAOs produced by the particular *Synechococcus* strains can catalyse the oxidative deamination of basic L-amino acids (L-Arg > L-Lys > L-Orn > L-His) into their respective α-keto acids, whereas other amino acids are not converted. In contrast to these observations, the L-AAO derived from strain Flo1 shows activity only with the substrate L-lysine. To our knowledge, substrate specificity restricted to only one L-amino acid was not observed for any other cyanobacterial strain so far. Therefore, the finding of the present study can be regarded as a novum concerning this kind of organisms.

Furthermore, it is known that different kinds of media contain distinct types and amounts of amino acids which have an influence on the activity of L-AAOs (Gómez et al., 2008; Chen et al., 2010a,b). Caicedo and co-workers (2010) also mentioned an influence of the used growth medium on the strength of the inhibitory activity of different Amberlite-extracts derived from strain Flo1 culture supernatants. This can be regarded as additional proof that one of the inhibitory substances of Flo1 is an L-AAO.

Further analysis of the antibacterial substance

The antibacterial enzyme investigated in this study did not loose its inhibitory activity when in contact with SDS and ß-mercaptoethanol, hence non-denaturing SDS-PAGE was used to maintain enzymatic activity and to estimate the approximate

290 molecular weight of this substance. Using Coomassie brilliant blue staining, the main
291 proteins present in sterile-filtrated, 37.5x concentrated culture supernatant derived
292 from strain Flo1 were visualized. Two different in-gel assay techniques were applied
293 to detect single bands of antibacterial, H_2O_2 producing activity which belonged to a
294 salient protein band (Fig. 2, II). The antibacterial, H_2O_2 producing activity bands
295 (Fig. 2B, II and C, II) and the corresponding stained one on SDS-PAGE (Fig. 2A, II)
296 appeared in each case as a separate band of approximately 55 kDa in size for the
297 L-lysine oxidase of strain Flo1.
298 The same experiments were also performed with the reference strain *Geitlerinema*
299 PCC 7105. However, this strain neither produced a 55 kDa protein in significant
300 amounts nor gave a positive response in the H_2O_2-detecting assay or showed any
301 inhibition in the antibacterial plate assay (Fig. 2, I).
302
303 Gau and co-workers (2007) investigated putative L-AAOs in different cyanobacteria
304 by evaluating 24 genomes for the presence of the *aoxA* gene encoding a protein with
305 similarity to this kind of enzymes. The results revealed that in several cyanobacterial
306 genera this gene is present, including *Gloeobacter*, *Nostoc*, *Synechococcus*,
307 *Synechocystis*, and *Trichodesmium*. Furthermore, the same authors calculated
308 possible molecular weights of 30-59 kDa (mainly 51-54 kDa) for the putative L-AAOs
309 encoded by the *aoxA* gene. These results are in accordance to the findings from the
310 present study, where a molecular weight of approximately 55 kDa for the L-AAO
311 produced by strain Flo1 by using in-gel detection after non-denaturing SDS-PAGE
312 was determined.
313 Apart from that, Schriek and co-workers (2009) found *Synechocystis* sp. PCC 6803
314 to produce an L-amino acid dehydrogenase of approximately 404 kDa in size.

315 However, compared with L-AAOs, this enzyme is not causing any production of H_2O_2
316 by its activity and, thereby, has no inhibitory effects.
317
318 Phylogenetic analysis
319
320 Phylogenetic trees were reconstructed to give an overview of strain Flo1 and its
321 taxonomic position amongst closely related cyanobacteria.
322 Nearly the complete 16S rDNA sequence (1,348 bp) of Flo1 was used for an initial
323 BLAST search against GenBank, resulting in a sequence similarity of 99.6-100.0% to
324 *Geitlerinema* PCC 7105 (GenBank accession no. AB039010 and AF132780,
325 respectively). These unambiguous sequence identities clearly demonstrate the
326 taxonomic position of strain Flo1 within the genus *Geitlerinema*. The affiliation of
327 strain Flo1 to this genus and, in particular, to *Geitlerinema* PCC 7105 (AB039010
328 and AF132780) is also documented in the phylogenetic tree (Fig. 3). Strain Flo1
329 formed a phyletic cluster together with the former mentioned strain as well as with
330 *Geitlerinema* MBIC10006 (AB058204) and *Geitlerinema* A28DM (FJ410907). The
331 overall tree topology was supported by all tree-making methods used in this study.
332 However, the branching patterns varied little depending on the tree-making method
333 used.
334
335 **Conclusions**
336
337 In the present study we report for the first time of an inhibitory effect caused by an
338 L-AAO produced by the marine filamentous cyanobacterium *Geitlerinema* strain
339 Flo1. The enzyme described in the present study shows very high substrate

specificity to L-lysine which is a novelty concerning a cyanobacterial strain. The function of the L-AAOs in the overall metabolism of cyanobacteria is still unclear (Gau et al., 2007), however, it is likely that L-AAOs are involved in the utilization of ammonia as a nitrogen source (MacHeroux et al., 2001). Additional studies are planned to gain more information about the L-lysine oxidase produced by *Geitlerinema* strain Flo1 concerning its possible application as an antibiotic or in biotechnology.

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Tables

Table 1 Antibacterial activity of cyanobacterial supernatants from strains Flo1 and PCC 7105 upon different target organisms. Zones of inhibition were determined after 24 h of incubation. Further details are given in the material and method section.

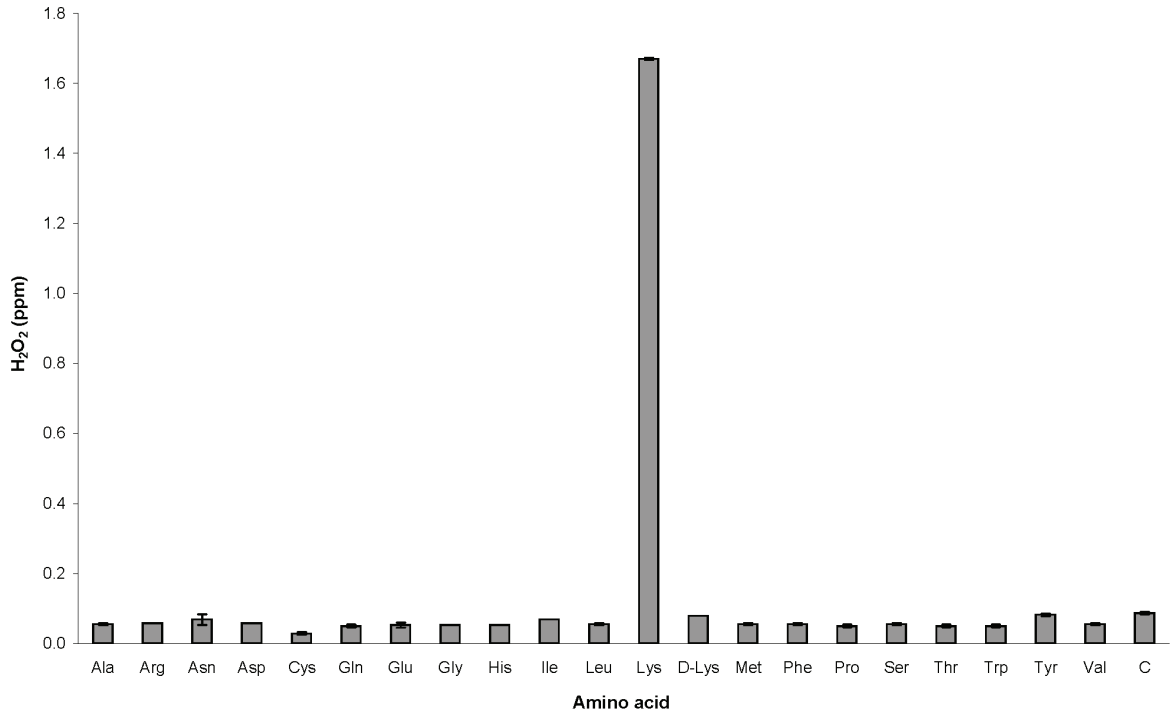
Microbial strain	Growth medium	Zones of inhibition (mm)[a]	
		Geitlerinema strain Flo1	*Geitlerinema* strain PCC 7105
Nosocomial strain			
Staphylococcus epidermidis[b]	LB	19	0
Laboratory strain			
Bacillus subtilis ATCC 6051^T	LB	0	0
Escherichia coli K12	LB	6	0
Escherichia coli UM255 (catalase negative mutant)	LB	22	0
Pseudomonas fluorescens ATCC 13525^T	LB	14	0
Saccharomyces pastorianus DSM 6580^{NT}	MYG	0	0

[a] Mean diameters of inhibition zones were quantified by subtracting the diameter of the well (6 mm). Tests were performed in quadruplicate

[b] clinical isolate, Molzym GmbH, Bremen

Figure legends

Fig. 1. Hydrogen peroxide production (ppm) formed in a peroxidase-coupled assay with concentrated culture supernatant of strain Flo1 in dependence of L-amino acids and D-lysine offered. Tests were performed in duplicate. C: control without any amino acid.



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530

531 **Fig. 2.** H_2O_2 production and antibacterial activities exhibited by strains *Geitlerinema*
532 PCC 7105 (I) and Flo1 (II) documented by SDS-PAGE (A) and two different in-gel
533 assay techniques (Panels B and C). Triplicate lanes loaded with identical non-heated
534 samples were handled separately after gel electrophoresis. Panel A was stained with
535 Coomassie brilliant blue, the two others were used for in-gel detection of the
536 inhibitory substances using an H_2O_2 detecting assay (B) and an inhibitory plate test
537 on LB agar with catalase negative mutant *E. coli* UM255 as target organism (C).
538 M: molecular masses (kDa) of standard proteins (Spectra™ Multicolor Broad Range
539 Protein Ladder; Fermentas). A coloured version of the figure is accessible as
540 supplementary material (Supplementary Fig. S1, available in EMT Online).

541

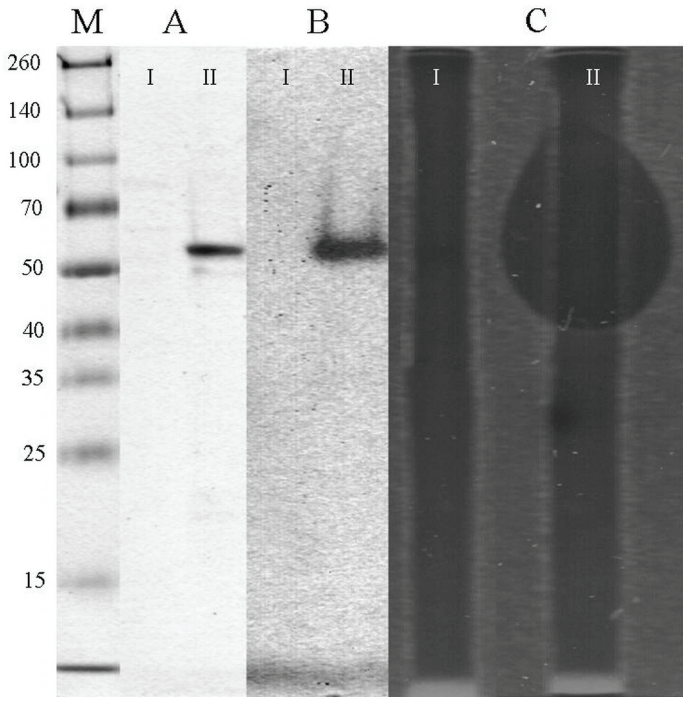
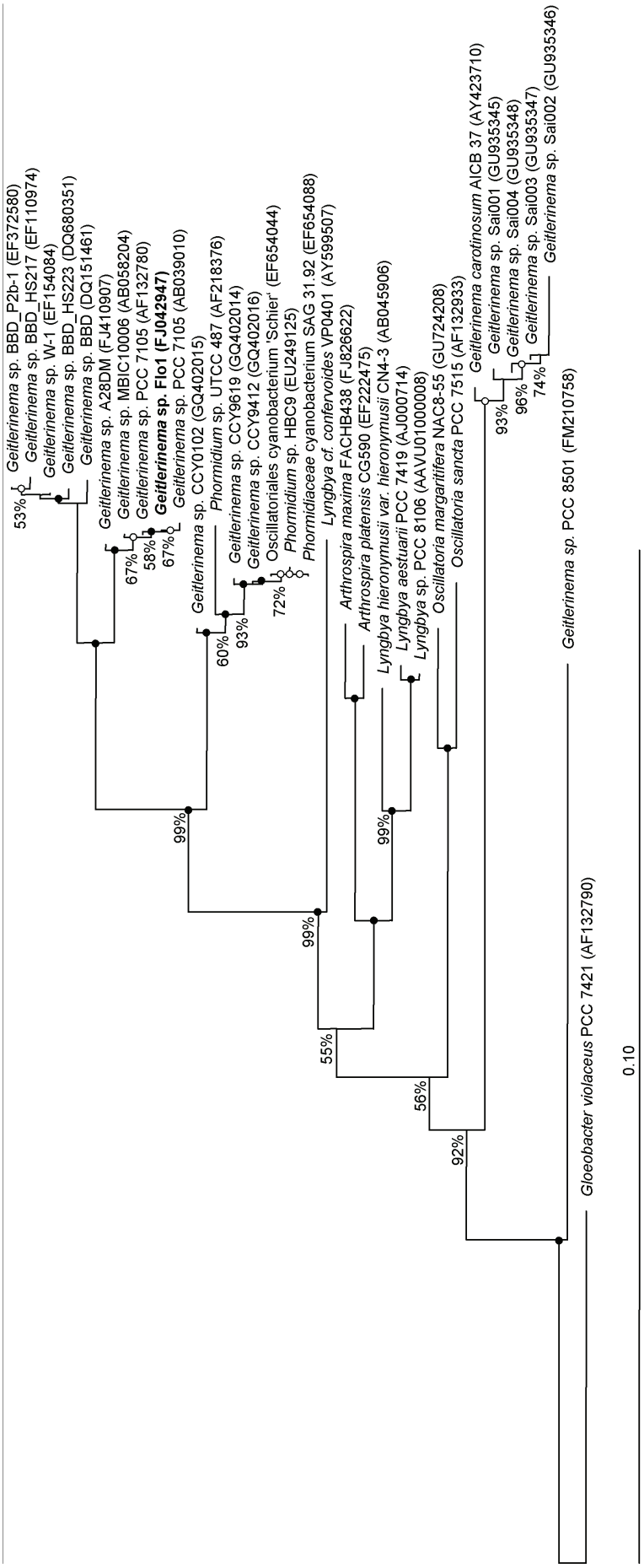


Fig. 3. Neighbour-joining phylogenetic tree based on 16S rDNA sequences of strain Flo1 and closely related cyanobacteria. Bootstrap values (>50 %) based on 1,000 resamplings are shown. Filled circles: correspond also to nodes recovered in trees generated with the maximum-parsimony and maximum-likelihood algorithms. Open circles: correspond also to nodes recovered with either the maximum-parsimony or the maximum-likelihood algorithm. The sequence of *Gloeobacter violaceus* PCC 7421 (GenBank accession no. AF132790) was used as an outgroup. Bar, 0.1 substitutions per nucleotide position.


Geitlerinema sp. BBD_P2b-1 (EF372580)
Geitlerinema sp. BBD_HS217 (EF110974)
Geitlerinema sp. W-1 (EF154084)
Geitlerinema sp. BBD_HS223 (DQ680351)
Geitlerinema sp. BBD (DQ151461)
Geitlerinema sp. A28DM (FJ410907)
Geitlerinema sp. MBIC10006 (AB058204)
Geitlerinema sp. PCC 7105 (AF132780)
Geitlerinema sp. Flo1 (FJ042947)
Geitlerinema sp. PCC 7105 (AB039010)
Geitlerinema sp. CCY0102 (GQ402015)
Phormidium sp. UTCC 487 (AF218376)
Geitlerinema sp. CCY9619 (GQ402014)
Geitlerinema sp. CCY9412 (GQ402016)
Oscillatoriales cyanobacterium 'Schier' (EF654044)
Phormidium sp. HBC9 (EU249125)
Phormidiaceae cyanobacterium SAG 31.92 (EF654088)
Lyngbya cf. confervoides VP0401 (AY599507)
Arthrospira maxima FACHB438 (FJ826622)
Arthrospira platensis CG590 (EF222475)
Lyngbya hieronymusii var. hieronymusii CN4-3 (AB045906)
Lyngbya aestuarii PCC 7419 (AJ000714)
Lyngbya sp. PCC 8106 (AAVU01000008)
Oscillatoria margaritifera NAC8-55 (GU724208)
Oscillatoria sancta PCC 7515 (AF132933)
Geitlerinema carotinosum AICB 37 (AY423710)
Geitlerinema sp. Sai001 (GU935345)
Geitlerinema sp. Sai004 (GU935348)
Geitlerinema sp. Sai003 (GU935347)
Geitlerinema sp. Sai002 (GU935346)
Geitlerinema sp. PCC 8501 (FM210758)
Gloeobacter violaceus PCC 7421 (AF132790)
53%
67%
58%
67%
60%
93%
72%
99%
99%
55%
99%
56%
92%
93%
96%
74%
0.10

552

553 **Supplementary figures**

554

555 **Fig. S1.** H_2O_2 production and antibacterial activities exhibited by strains *Geitlerinema*
556 PCC 7105 (I) and Flo1 (II) documented by SDS-PAGE (A) and two different in-gel
557 assay techniques (Panels B and C). Triplicate lanes loaded with identical non-heated
558 samples were handled separately after gel electrophoresis. Panel A was stained with
559 Coomassie brilliant blue, the two others were used for in-gel detection of the
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562 M: molecular masses (kDa) of standard proteins (Spectra™ Multicolor Broad Range
563 Protein Ladder; Fermentas).

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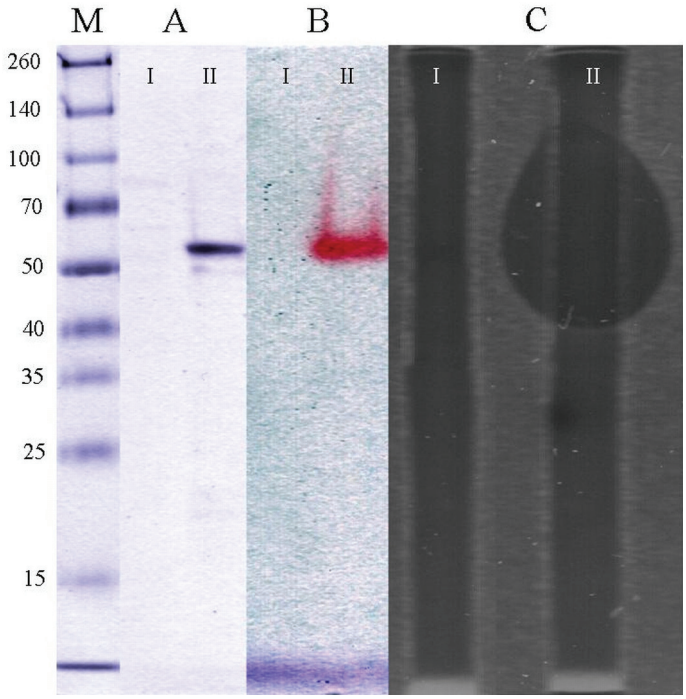

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Manuscript IV

3.4 In-gel detection of L-amino acid oxidases based on the visualisation of hydrogen peroxide production

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Note

In-gel detection of L-amino acid oxidases based on the visualisation of hydrogen peroxide production

Jan Erik Rau *, Ulrich Fischer

Zentrum für Umweltforschung und nachhaltige Technologien, Fachbereich Biologie/Chemie, Abteilung Marine Mikrobiologie, Universität Bremen, Leobener Straße, D-28359 Bremen, Germany

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ABSTRACT

A simple and practicable method for in-gel detection of bacterial produced L-amino acid oxidases (L-AAOs) after non-denaturing SDS-PAGE based on the visualisation of occurring hydrogen peroxide production is described. Advantages of this screening method for L-AAOs are the determination of their numbers and approximate molecular weights additionally in one approach.

L-Amino acid oxidases (L-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.2., L-AAO) are flavoenzymes catalysing the oxidative deamination of L-amino acids to the respective α-keto acids with the release of ammonium and hydrogen peroxide (Wellner and Meister, 1961) which determines the antimicrobial property of L-AAOs (Stiles et al., 1991). The enzyme is widely distributed in snake (Alves et al., 2008; Du and Clemetson, 2002) and insect venoms (Ahn et al., 2000) but also in algal (Fujisawa et al., 1982), bacterial (Tong et al., 2008; Chen et al., 2010a), cyanobacterial (Pistorius and Voss, 1980), fungal (Kusakabe et al., 1980), gastropodal (Yang et al., 2005), and vertebratal sources (Kasai et al., 2010; Sun et al., 2002). L-AAOs have been reported as antitumor (Ahn et al., 2000; Kusakabe et al., 1980) as well as bacteriostatic and bacteriocidal agents (Yang et al., 2005) and can be differentiated in enzymes with a more strict substrate specificity (Lucas-Elío et al., 2006) and in those which have a broader spectrum (Gómez et al., 2008). They typically have a dimeric structure. FAD is the cofactor of L-AAOs and binds strongly but not covalently to the protein. A main commercial application for L-AAOs is the production of D-amino acids by enzymatic resolution of racemic amino acid solutions (Takahashi et al., 1997; Parikh et al., 1958).

The aim of the present paper is to describe a new application of an already published peroxidase-coupled assay for the detection of hydrogen peroxide production by L-AAO activity. Chen et al. (2010b) used this method spectrophotometrically to determine the substrate specificity of an L-AAO produced by *Pseudoalteromonas flavipulchra* strain C2. In the present study, we used basically the same assay mixture for direct in-gel detection of L-amino acid oxidases after SDS-PAGE based on the visualisation of occurring hydrogen peroxide production.

Sterile-filtrated, 30× concentrated, possibly L-AAO-containing culture supernatant derived from a marine bacterium was mixed in a 4:1 (v/v) ratio with 4-fold concentrated sample buffer (0.25 M Tris–HCl, pH 6.8, 6% SDS, 20% ß-mercaptoethanol, 40% glycerol, and 0.04% bromophenol blue). Concentrated samples were used in this study to even detect very low amounts of possibly present L-AAOs. The samples were placed in the polyacrylamide gel without heating (1 min at RT). The stacking gel had an acrylamide concentration of 4% and the separation gel of 10%, respectively. Protein separation was performed by SDS-PAGE, as described by Laemmli (1970). Triplicate lanes were loaded with identical samples and handled separately after PAGE. Two lanes were stained with Coomassie brilliant blue (CBB) according to Kang et al. (2002), the other was washed three times for 5 min with distilled water. The non-stained lane as well as one of the Coomassie-stained lanes were used for in-gel detections of the inhibitory substances using the assay mixture described by Chen et al. (2010b) with some modifications. Originally, the standard assay mixture in a 1 ml cuvette contained 500 µl antibacterial protein solution, 60 mM Tris–HCl, pH 7.8, 5 mM L-amino acid (one of the 20 standard amino acids), 400 mM 4-aminoantipyrine, 1 mM phenol, and 4 units of peroxidase (Sigma). The dye is produced by peroxidase from hydrogen peroxide, 4-aminoantipyrine, and phenol. Chen et al. (2010b) detected it spectrophotometrically at 505 nm. In the present study, instead of a defined amount of one of the standard amino acids, a casamino acid solution (final concentration 1%) was used as amino acid source. Alternatively, a well-defined mixture of the 20 standard amino acids (5 mM each) can also be used (data not shown). Furthermore, no solution containing antibacterial compounds was added. For direct in-gel detection of L-AAO activity, 2 ml of the enzyme assay mixture was put on the appropriate gel lane. Using a scanner

Abbreviations: L-AAO, L-Amino acid oxidase; CBB, Coomassie brilliant blue.

* Corresponding author. Tel.: +49 421 218 63409; fax: +49 421 218 7222.
E-mail address: jerau@uni-bremen.de (J.E. Rau).

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(Canon MX320), the formation of the dye was documented immediately over a period of 15–45 min in intervals of 2 min; depending on the L-AAO concentration. Protein bands which produce H_2O_2 in the presence of the assay mixture appear pale pink. Results are shown in Fig. 1.

The L-AAOs used in the present study were resistant to SDS and ß-mercaptoethanol, therefore, the SDS-PAGE could be performed under non-denaturing conditions to maintain enzymatic activity and to estimate the approximate molecular weight of these substances. Considering the molecular weight standards (Page Ruler Prestained Protein Ladder; Fermentas), the bands of H_2O_2 producing activity and the corresponding Coomassie-stained protein bands on SDS-PAGE were present i) in the range between 55 and 70 kDa and ii) in the range larger than 170 kDa in size (Fig. 1A, B and C). The L-AAOs still kept their activity, even after having been exposed to the staining procedure with CBB. Therefore, the assay mixture in one approach was directly used with the Coomassie-stained gel to visualise H_2O_2 production (Fig. 1B). If this is not the case, a non-stained lane should be incubated with the assay mixture, and the result has to be compared with a Coomassie-stained lane loaded with an identical sample to detect active L-AAOs (Fig. 1C). When no activity is detected during non-denaturing SDS-PAGE, the same experiments can be repeated by using native PAGE, but without any information concerning the molecular weight of the protein bands (data not shown). The visible smear of some bands is due to the highly concentrated samples in combination with the sensitivity of the used L-AAO detecting assay. To get sharply bounded bands, it seems to be necessary to lower the concentration of L-AAOs in the samples. Regarding the sensitivity of the H_2O_2 detecting assay mixture, we found out that bands, unseen after staining with CBB according to Kang et al. (2002), appeared well visible after having a sufficient contact with the assay mixture (data not shown).

The results obtained in the present study have clearly shown that the described in-gel L-AAO detecting application is simple, sensitive, rapid, and reproducible. Therefore, many samples can be screened for L-AAO presence in the course of a day. In addition, if a sample shows H_2O_2 producing activity when being in contact with the assay mixture, the number and the approximate molecular weight of the L-AAOs can be determined in one step as well, which are clear advantages in comparison to common spectrophotometric L-AAO detecting methods. Knowing directly numbers and approximate molecular weights of the L-AAOs can be very helpful for further purification and characterisation of this kind of enzymes.

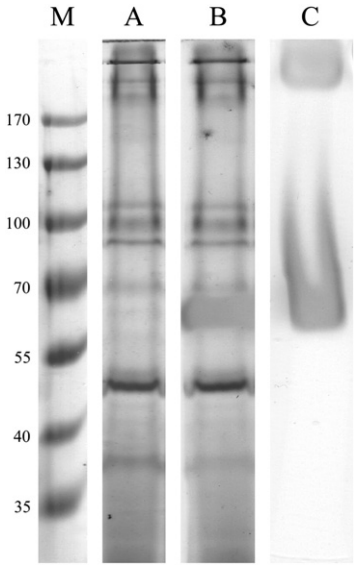

Fig. 1. SDS-PAGE and an in-gel assay technique for detecting H_2O_2 production. Triplicate lanes loaded with identical non-heated samples were handled separately after gel electrophoresis. Lanes A and B: stained with CBB. Lane B was additionally treated with an H_2O_2 detecting assay mixture. Lane C: in-gel detection of the inhibitory substances using the H_2O_2 detecting assay. M: molecular masses (kDa) of standard proteins (Page Ruler Prestained Protein Ladder; Fermentas). A coloured version of the figure is accessible as supplementary material (Supplementary Fig. S1, available in JMM Online).

Supplementary data to this article can be found online at doi: 10.1016/j.mimet.2011.03.002.

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4. General discussion

In this chapter, the main results obtained in the present study are discussed and compared with those reported for other *Pseudoalteromonas* and *Geitlerinema* species as well as some further distantly related organisms. Finally, some general conclusions are made and an outlook regarding promising continuative experiments is given.
The purpose of this chapter should not be to repeat the results and discussion as well as conclusion parts from the manuscripts in detail, but to provide a more general and coherent overview about the achieved results, conclusions, and assumptions.

4.1 Inhibitory substances from *P. aurantia* and *P. citrea*

Several experiments were performed to characterise the inhibitory substances from *P. aurantia* and *P. citrea* concerning their properties as well as the growth conditions when they are produced (see Manuscript I, pages 33-61 and Manuscript II, pages 62-88).

It could be shown that *P. aurantia* and *P. citrea* are producing their inhibitory substances under standard incubation conditions during the late exponential to stationary growth phase, respectively (for details see Manuscript I, pages 44-45 and 56-57, Figure 1 A and B). Similar results were reported by Chen and coworkers (2010b) for the *Pseudoalteromonas* species *P. flavipulchra* strain C2, which is producing its inhibitory protein at the beginning of the stationary growth phase. *P. luteoviolacea* strain 9K-V10 is also producing and secreting an antibacterial protein during the late exponential to stationary growth phase (McCarthy et al., 1994). The *Bifidobacterium* strains RBL 68 and RBL 85 are starting to produce bacteriocin-like inhibitory substances in their late exponential growth phase and reached a maximum inhibitory activity during and after the stationary growth phase (Zouhir et al., 2011), whereas the antibacterial protein marinocine from *Marinomonas mediterranea* can first be detected at the death phase of growth (Lucas-Elío et al., 2005). Bowman (2007) speculated that the time when the synthesis of inhibitory substances by *Pseudoalteromonas* species is starting, is triggered possibly by quorum sensing systems. This assumption is supported by several studies

concerning the role of quorum sensing in antibiotic compound production (El-Sayed et al., 2001; McGowan et al., 2005; Liu et al., 2007; Veselova et al., 2008; Duerkop et al., 2009).

The results of the present study revealed that *P. citrea* is producing inhibitory substances when incubated at 10°C without shaking, whereas *P. aurantia* did not (for details see Manuscript I, pages 45-46 and 58-59, Figure 2 A and B). In contrast to that, Gauthier and Breitmayer (1979) found the production of inhibitory substances from *P. aurantia* not to be influenced by the incubation temperature. In addition, Gauthier (1977) reported that *P. citrea* formed higher levels of inhibitory substances at low temperatures, whereas the inhibitor production in the present study was not differing significantly in dependence of the incubation temperature. Only when *P. citrea* was grown by shaking at 150 rpm, the production was higher at 21 rather than at 26°C. The observed differences concerning the influence of the incubation temperature by Gauthier (1977), Gauthier and Breitmayer (1979), and the present study are possibly caused by the use of different growth media, artificial seawater in the present study, and marine broth (Difco) in that of both other studies mentioned before. Generally, the antibiotic production of bacteria often is strongly influenced by the incubation temperature (Paul and Banerjee, 1983; Larsen et al., 1993; Todorov et al., 1999).

The present study has shown that the inhibitory activities of the inhibitors from both *Pseudoalteromonas* species are still detectable after contact with the enzymes α-amylase and trypsin, whereas the activity is totally lost after exposure of the inhibitory substances to catalase, peroxidase as well as proteinase K (for details see Manuscript I, pages 46-47 and 55-56, Table 2). Since the conclusions from these findings were already described in detail in the mentioned manuscript, only the most important ones are discussed here, namely the influences of the last three mentioned enzymes. The neutralisation of the inhibitory activities by catalase and peroxidase leads to the assumption that hydrogen peroxide is involved in the mechanisms of inhibition, whereas the neutralising effect of proteinase K supports the concept of a proteinogenous nature for the inhibitory substances. The neutralising effect of catalase respectively the involvement of H_2O_2 in the inhibitory mechanism was reported previously not only for *P. aurantia* and *P. citrea*, but also

for other Pseudoalteromonads (Gauthier, 1977; Gauthier and Breittmayer, 1979; Gómez et al., 2008; Chen et al., 2010b). Interestingly, Gómez and coworkers (2008) as well as Chen and coworkers (2010b) found the inhibitory substances produced by different *P. luteoviolacea* strains respectively *P. flavipulchra* strain C2 to be L-AAOs and, therefore, are also of proteinogenous nature. Thereby, it has to be kept in mind that the activity of L-AAOs leads to the accumulation of H_2O_2. Thus, these findings in conclusion with the results from the present study gave the first hints that the inhibitory substances of *P. aurantia* and *P. citrea* could also be L-AAOs. Further details are discussed in the results and discussion part of Manuscript I (page 47).

It could be shown that the inhibitory substances from both Pseudoalteromonads were thermolabile (those from *P. citrea* were more heat-sensitive than those from *P. aurantia*), but resistant against acid-alkaline treatment (for details see Manuscript I, pages 48-49 and 60-61, Figure 4 A and B). Other species of the genus *Pseudoalteromonas* are known also for the production of heat-sensitive proteinogenous inhibitory substances (Bowman, 2007). Thus, the presence of thermolabile inhibitory substances in *P. aurantia* and *P. citrea* is additionally supporting the assumption that both species are producing inhibitory proteins. The pH stability of the inhibitors produced by both Pseudoalteromonads is in accordance with the results from other studies dealing with members of this genus, which are also producing such substances more or less resistant to pH value variations (Gauthier and Breittmayer, 1979; Chen et al., 2010b). L-AAOs normally are also relatively heat-sensitive and resistant against acid-alkaline treatment (Piedras et al., 1992; Arima et al., 2003; Yang et al., 2005; Chen et al., 2010b; Lee et al., 2011). Hence, the heat-sensitivity as well as the pH stability of the inhibitors from *P. aurantia* and *P. citrea* had also supported the idea that these substances are possibly L-AAOs, but at least proteins.
These assumptions were additionally confirmed by the fact that isoelectric points of pH 8.7 (*P. aurantia*) respectively 8.9 and 9.4 (*P. citrea*) could be detected for the inhibitors from both species in the studies performed (for details see Manuscript I, pages 47-48 and 59, Figure 3 A and B).

Regarding the antimicrobial potential of the inhibitory substances produced by *P. aurantia* and *P. citrea*, it could be shown by antibiogram studies that they strongly

inhibit the growth of MRSA, *S. epidermidis* and of several other bacterial strains. In the case of *P. aurantia*, the yeast *S. pastorianus* was inhibited, too (for details see Manuscript I, pages 42-43 and 55, Table 1). Other members of the genus *Pseudoalteromonas* are known also for the production of inhibitory substances active against MRSA and other clinical relevant strains. Some of these substances are of low molecular weight, but also high molecular weight substances, such as L-AAOs and other antibacterial proteins, are known (Bowman, 2007; Chen et al., 2010b). Interestingly, the inhibitory substances from *P. aurantia* and *P. citrea* had the strongest effect against the growth of the catalase negative mutant *Escherichia coli* UM255. This finding again showed that catalase and, thus, also hydrogen peroxide are playing an important role in the mechanism of inhibition and is supporting the assumption that the inhibitors from both Pseudoalteromonads are possibly L-AAOs.

In addition, the antibiogram studies have indicated autoinhibition by *P. aurantia* and *P. citrea*, which is caused by the activity of their L-AAOs and the corresponding production of hydrogen peroxide (for details see Manuscript I, pages 42-43 and 55, Table 1). Holmström and coworkers (2002) also reported that *P. citrea* does inhibit its own growth, while *P. rubra* and *P. tunicata* exhibit only weak autoinhibitions, among 10 different *Pseudoalteromonas* species tested. Further studies have shown that the latter mentioned organism also produces an L-AAO, which could be identified as the autoinhibiting compound (Mai-Prochnow et al., 2006, 2008). Two *P. luteoviolacea* strains (Gómez et al., 2008) and *P. flavipulchra* (Chen et al., 2010b) were found to produce L-AAOs as well. Thus, due to the hydrogen peroxide producing activity of these enzymes, the latter mentioned organisms probably also inhibit their own growth, but this was not investigated so far. In addition, two other *P. luteoviolacea* strains are known for their production of autotoxic substances (Gauthier and Flatau, 1976; Sakata et al., 1986). With regard to the above mentioned findings from the present and also from other studies, it can be assumed that probably the substances from both *P. luteoviolacea* strains as well as the high molecular weight polyanionic substance with autotoxic property from *P. rubra* (Gauthier, 1976b) are L-AAOs as well.

The influence of *P. tunicata* on the formation of biofilms and the ecological significance of its autotoxicity has been investigated in several studies (Mai-Prochnow et al., 2004, 2006; Burmølle et al., 2006; Rao et al., 2010). The organism

was reported to exhibit a wide range of bioactive activities (Mai-Prochnow et al., 2004; see also pages 130-132, Table 4.1), but, as already mentioned before, is also inhibiting its own growth. Mai-Prochnow and coworkers (2004) reported that AlpP, a 190 kDa L-AAO, is responsible for this autoinhibition. The same authors described the autotoxicity of AlpP as a mechanism similar to programmed cell death. Webb and coworkers (2003) previously described an equal mechanism during biofilm dispersal for *Pseudomonas aeruginosa*. In the case of *P. tunicata*, the process of programmed cell death due to L-AAO activity within substructures of the biofilm seems to provide nutrients for a subpopulation of cells, which is resistant to AlpP (Mai-Prochnow et al., 2004, 2008). As all *Pseudoalteromonas* cells, also the resistant ones are motile by swimming and it is therefore likely that they are dispersed in the surrounding environment. Thus, these survivors can recolonise new surfaces by again forming biofilms (Mai-Prochnow et al., 2004, 2006). Investigations on other bacteria lead to the assumption that programmed cell death due to the presence of antibiotic substances could be relatively common amongst biofilm forming organisms (Bayles, 2007). Hence, most probably also other autotoxic compound producing *Pseudoalteromonas* species, including *P. aurantia* and *P. citrea*, are showing the same effects on biofilm formation and dispersal.

To finally prove the above mentioned assumption that the inhibitory substances from both Pseudoalteromonads are possibly L-AAOs, their presence was verified with a specific assay. L-AAOs can be detected using various spectrophotometric as well as fluorimetric methods. In all cases not the enzymes themselves, but the resulting products of the oxidative deamination of L-amino acid substrates by the activity of L-AAOs are detected. Some of these methods are indirectly measuring occurring hydrogen peroxide production i) by fluorimetric detection of H_2O_2-oxidised Amplex red (10-acteyl-3,7-dihydoxyphenoxazine) using a horseradish peroxidase-coupled assay (Palamakumbura and Trackman, 2002; Gómez et al., 2006), ii) by a spectrophotometric detection of a dye produced by peroxidase from H_2O_2, 4-aminoantipyrine and phenol (Chen et al., 2010b) as well as iii) by a spectrophotometric detection of a dye produced by peroxidase from H_2O_2 and *o*-dianisidine (Geueke and Hummel, 2002). In another approach, specific for L-lysine oxidases, the production of the α-keto acid formed is indirectly followed by the spectrometric measurement of the formation of a semicarbazone derivative obtained

by the reaction of semicarbazide with the α-keto acid obtained after oxidative deamination of L-lysine (Danson et al., 2002). Furthermore, L-AAO activity can be spectrophotometrically determined by a coupled lactate dehydrogenase assay using different L-amino acids as substrates (Geueke and Hummel, 2002). The authors measured the formation of lactate produced by the enzyme from NADH and pyruvate.

In the present study, the inhibitory substances from *P. aurantia* and *P. citrea* were identified using the spectrophotometric assay reported by Chen and coworkers (2010b). Applying this, it could be clearly shown that both *Pseudoalteromonas* species are producing amino acid oxidases (for details see Manuscript II, pages 70-71 and 82-83, Figure 1). With the same assay also the substrate spectra of those enzymes were determined, revealing that the amino acid oxidases from both species can use several L-amino acids as substrates, but none of the tested D-amino acids. This led to the conclusion that both species are producing L-AAOs with broad substrate spectra. Among the members of the genus *Pseudoalteromonas* species are known to produce L-AAOs with low as well as with very high substrate specificities (Gomez et al., 2008; Mai-Prochnow et al., 2008; Chen et al., 2010b; see also chapter 1, page 24, Table 1.1).

To get a rough idea of the number and molecular weights of the L-AAOs from *P. aurantia* and *P. citrea*, culture supernatants derived from both species were applied to a sequence of ultrafiltration runs and the resultant fractions were screened for inhibitory activity using agar diffusion tests (for details see Manuscript II, pages 72 and 84, Figure 3 A and B). The results have shown that in the supernatant of *P. aurantia* inhibitory activity could be detected in three fractions (>100, >30 <50, and >50 <100 kDa in order of descending activities), but only in one of that from *P. citrea* (>100 kDa). In conclusion, *P. aurantia* is forming at least one inhibitory substance of 30-100 kDa and one larger than 100 kDa, whereas *P. citrea* is producing at least one larger than 100 kDa.

To determine the number and molecular weights of the L-AAOs produced by *P. aurantia* and *P. citrea* more precisely, the assay mixture for the spectrophotometric test used by Chen and coworkers (2010b) and in the present

study was directly applied to a polyacrylamid gel, loaded with samples possibly containing L-AAOs, both after native PAGE and non-denaturing SDS-PAGE (for details concerning the method see Manuscript IV, pages 117-118). The described in-gel L-AAO detecting approach was found to be simple, sensitive, rapid, and reproducible. In comparison to common spectrophotometric or fluorimetric methods the clear advantages of this new technique are not only the validation of L-AAO presence in a sample, but also the determination of their number and approximate molecular weights. Non-denaturing SDS-PAGE could be used due to the finding that the activity of the inhibitory substances produced by *P. aurantia* and *P. citrea* had shown to be relatively resistant to SDS and ß-mercaptoethanol (for details see Manuscript II, pages 71-72 and 83-84, Figure 2 A and B).

Using the new approach, it could be determined that *P. aurantia* most probably produces three L-AAOs of 60-65, 225-230, and >260 kDa in size, whereas *P. citrea* forms two, with molecular masses of 165-170 and 225-230 kDa (for details see Manuscript II, pages 72-73 and 85, Figure 4 A and B). *P. aurantia* was first described by Gauthier and Breittmayer (1979), who reported the presence of only one polyanionic inhibitory substance, whereas Gauthier (1977) found 2 different polyanionic inhibitory substances for *P. citrea*. Other members of the genus *Pseudoalteromonas* are known also for the production of high molecular weight L-AAOs (Gomez et al., 2008; Mai-Prochnow et al., 2008; Chen et al., 2010b; see also chapter 1, page 24, Table 1.1).

Size exclusion chromatography (SEC) via fast protein liquid chromatography (FPLC) was also used to analyse the L-AAOs produced by both *Pseudoalteromonas* species studied. The resulting fractions were screened for inhibitory activity showing that *P. aurantia* produces 3 L-AAOs of approximately 100, 240, and 480 kDa in size, whereas *P.citrea* forms only one of approximately 120 kDa (for details see Manuscript II, pages 73-74). Concerning *P. aurantia*, these findings are mainly in accordance with the results obtained in the SDS-PAGE study (see above), but, regarding *P. citrea*, there is a discrepancy in the number of L-AAOs (one instead of two; see above). The facts which might explain the differences between the findings from the SDS-PAGE and SEC studies as well as those obtained by Gauthier (1977)

and by Gauthier and Breittmayer (1979) are discussed in detail in the Manuscript II (page 74).

In the present study, various hints could be found that the three L-AAOs from *P. aurantia* might be all composed of one structural unit of 60-65 kDa in size. In this context, the 225-230 kDa L-AAO might be a tetramer of this small unit, whereas the L-AAO >260 kDa, respectively 480 kDa with regard to the results obtained by the SEC study, is possibly the corresponding octamer. These assumptions are supported by several other findings from the present study and by those reported by other authors and are discussed comprehensively in Manuscript II (pages 74-76).

4.2 Inhibitory substance from *Geitlerinema* strain Flo1

The filamentous cyanobacterial strain Flo1 was investigated regarding its taxonomic position amongst closely related organisms. In addition, an inhibitory substance produced by this strain was identified and characterised (see Manuscript III, pages 89-115).

By using the basic local alignment search tool (BLAST) at GenBank and by re-constructing phylogenetic trees with the nearly complete 16S rDNA sequence (1,348 bp) of strain Flo1 and those of closely related strains, it could be revealed that Flo1 is clustering amongst species from the genus *Geitlerinema*. The morphological and physiological characteristics of the strain (e.g. filamentous, unsheated, non-heterocystous, mostly bright blue-green with cylindrical, straight trichomes which are motile by gliding) also fit to those of other members of this genus. Thus, the affiliation of strain Flo1 to the genus *Geitlerinema* could be unambiguously clarified (for details see Manuscript III, pages 103 and 113-114, Figure 3 as well as chapter 6, pages 158-159, Figures S4 and S5).

The antimicrobial potential of culture supernatants from strain Flo1 and from the reference strain *Geitlerinema* PCC 7105 was determined by using agar diffusion tests. Thus, it could be shown that the culture supernatant of strain Flo1 had inhibitory activity against several heterotrophic bacteria, including the clinical relevant strain *S. epidermidis*, whereas the growth of the tested yeast was not affected. In contrast to that, the culture supernatant of strain PCC 7105 exhibited no inhibitory

activity against any of the organisms tested (for details see Manuscript III, pages 98 and 111, Table 1). These results lead to the conclusion that strain Flo1 is producing at least one inhibitory substance with antibacterial activity, but strain PCC 7105 does not. By taking a closer look at the inhibition pattern of the substance from Flo1, noticeable similarities with the results from the antibiogram studies performed with *P. aurantia* and *P. citrea* could be observed. In all cases growth of *B. subtilis* was not inhibited and that of the yeast *S. pastorianus* only partially by the supernatant from *P. aurantia*. The growth of *E. coli* K12, *P. fluorescens*, *S. epidermidis*, and *E. coli* UM255 was inhibited in all cases, whereas the catalase-negative mutant *E. coli* UM255 was affected most (for details see Manuscripts I, page 55, Table 1 and Manuscript III, page 111, Table 1). Heyduck-Söller and Fischer (2000) as well as Caicedo and coworkers (2010) also investigated the antimicrobial potential of culture supernatants from strain Flo1 and found, in some respects, contrary results. These findings in detail and possible reasons for the differences are broadly discussed in Manuscript III (pages 98-100).
The above mentioned inhibition pattern similarities have led to the assumption that the inhibitory potential of the culture supernatant derived from strain Flo1 is possibly due to an L-AAO presence, as could be verified for both *Pseudoalteromonas* species (for details see Manuscript II, page 70 and chapter 4.1, page 124).

To clarify this possibility, the same spectrophotometric approach (Chen et al., 2010b), used to identify the inhibitory substances from *P. aurantia* and *P. citrea*, was performed (compare chapter 4.1, pages 123-124). Thus, it could be clearly shown that strain Flo1 is producing an amino acid oxidase, in particular, a specific L-lysine oxidase (for details see Manuscript III, pages 100-101 and 111-112, Figure 1). Other cyanobacterial species have also been reported to produce L-AAOs. Accordingly, Gau and coworkers (2007) mentioned that different *Synechococcus* strains are producing L-AAOs specific for basic L-amino acids, but, to my knowledge, a specific L-lysine oxidase was not described so far. Therefore, the finding from the present study can be regarded as a novum concerning cyanobacteria.

Using the newly described in-gel L-AAO detecting technique (see Manuscript IV, pages 117-118 and chapter 4.1, pages 124-125), it could be shown that strain Flo1 is producing one L-AAO of approximately 55 kDa in size, whereas PCC 7105 does

not produce any substance of this kind (for details see Manuscript III, pages 101-102 and 112-113, Figure 2). Gau and coworkers (2007) analysed putative L-AAOs in different cyanobacteria by evaluating 24 genomes for the presence of the *aoxA* gene encoding a protein with L-AAO similarity. The authors reported that in members of several cyanobacterial genera this gene is detectable, including *Gloeobacter*, *Nostoc*, *Synechococcus*, *Synechocystis* as well as *Trichodesmium* species. In addition, the same authors evaluated possible molecular weights of mainly 51-54 kDa for the putative L-AAOs. Hence, these results are in accordance with the finding from the present study of a 55 kDa L-AAO produced by strain Flo1.
Schriek and coworkers (2009) reported that *Synechocystis* sp. PCC 6803 is producing an L-amino acid dehydrogenase of approximately 404 kDa in size, called Slr0782. In comparison to L-AAOs, the dehydrogenase is not causing H_2O_2 production by its activity and, thus, has no inhibitory effects. Apart from that, the authors found out that Slr0782 is functioning as an L-arginine dehydrogenase which is mediating the electron transfer from L-arginine into the respiratory electron transport chain utilizing oxygen as electron acceptor via cytochrome oxidase. The same authors concluded that Slr0782 is acting as an additional substrate dehydrogenase being able to interact with the electron transport chain of the thylakoid membrane of strain PCC 6803.

If and, when, to what extent cyanobacterial produced L-AAOs, like the one from *Geitlerinema* strain Flo1, are playing a role in the pathogenicity of the BBD (compare chapter 1.2.2, pages 21-22) is not known so far, but it can be speculated that they probably have influences in this regard.

4.3 Bioactive substances from *Pseudoalteromonas* and *Geitlerinema* species: an overview

Species from the genera *Pseudoalteromonas* and *Geitlerinema* are known for the production of various bioactive substances with diverse activities, including L-AAOs. *P. aurantia* strain NCIMB 2052^T, *P. citrea* strain NCIMB 1889^T, and *Geitlerinema* strain Flo1, as could be shown in the present study, are also producing this type of bioactive substances with inhibitory activity.

To provide a comprehensive overview about the different producers and their associated substances, corresponding findings concerning *Pseudoalteromonas* and *Geitlerinema* species are summarised in Table 4.1 (pages 130-132).

Interestingly, the genus *Pseudoalteromonas* can be relatively accurately taxonomically separated into pigmented and non-pigmented species clades (Bowman, 2007; see also chapter 6, page 157, Figure S3). Accordingly, Egan and coworkers (2002) found that pigmentation is linked with the ability of *P. tunicata* to produce its bioactive natural products. Previous studies of Holmström and coworkers (1996) as well as of Holmström and Kjelleberg (1999) confirmed this finding also for other members of the genus. Several other pigmented Pseudoalteromonads are also known to produce bioactive compounds (Gauthier, 1976; Holmström et al., 1998; Egan et al., 2001; Ivanova et al., 2002a; Isnansetyo and Kamei, 2003a,b; Gómez et al., 2008; Chen et al., 2010b etc.), whereas non-pigmented do not seem to have the same extensiveness of synthesis of this kind of substances (Bowman, 2007; see also Table 4.1, pages 130-132). The two *Pseudoalteromonas* species *P. aurantia* and *P. citrea*, investigated during the present study, are also pigmented and are producing bioactive compounds.

Tab. 4.1 Sources, pigmentation, and bioactive substances as well as their properties of members from the genera *Pseudoalteromonas* and *Geitlerinema*. Species and results from the present study are in bold type.

Species	Source	Pigmentation	Bioactive substance	Property
P. agarivorans [1]	ascidians, sea water	-	-	degrades algal polysaccharides
P. aliena [2]	sea water	+ (melanin)	unknown compound(s)	anti-tumour
P. antarctica [3,4]	muddy soils, sea-ice, sea water, sediment	-	unknown compound(s)	antibacterial; novel polysaccharides, cold-active enzymes
P. arctica [5]	arctic sea water	+ (slightly orange)	-	pullulanase, pectinase
P. atlantica [6]	surface of macroalgae, sea water	+ (beige to pale yellow orange)	-	degrades algal polysaccharides
P. aurantia [7-9]	sea water, surface of *Ulva lactuca*	+ (yellow to orange)	**L-AAO(s)**; possibly other unknown compounds	**antibacterial**; anti-fungal; inhibits settlement of invertebrate larvae; inhibits algal spore settlement
P. byunsanensis [10]	tidal flat sediment	+ (purple)	-	gelatinase
P. carrageenovora [6]	surface of macroalgae, sea water	+ (beige to pale yellow orange)	-	degrades algal polysaccharides
P. citrea [4,7,9,11,12]	ascidians, mussels, sea water, sponges	+ (yellow)	**L-AAO(s)**; possibly other unknown compounds	**antibacterial**; inhibits settlement of invertebrate larvae; cytotoxic against sea urchin; degrades algal polysaccharides
P. denitrificans [13-19]	sea water	+ (red)	cycloprodigiosin HCl	anti-malarial; anti-tumour; inhibits T cell/lymphocyte proliferation; induces settlement of sea urchin
P. distincta [20]	sponge	± (melanin)	-	-
P. donghaensis [21]	sea water	-	-	-
P. elyakovii [22,23]	macroalga, mussels	-	-	-
P. espejiana [24]	sea water	-	-	-
P. flavipulchra [25,26]	coral, sea water	+ (yellow to orange)	L-AAO	antibacterial
P. haloplanktis [27-30]	sea water	-	novel diketopiperazines	cold-active enzymes; probiotic benefits to shellfish
P. issachenkonii [4,31]	marine alga	-	isatin; unknown reddish brown compound	antibacterial; anti-fungal; hemolytic
P. lipolytica [32]	sea water	-	-	lipolytic
P. luteoviolacea [4,9,19,33-36]	marine alga, seawater	+ (purple, yellow)	L-AAO; low and high molecular weight inhibitory substances, including pyrrole-containing compounds	antimicrobial; anti-algal; anti-fungal; inhibits algal spore settlement; inhibits settlement of invertebrate larvae; cytotoxic against sea urchin; induces settlement of sea urchin
P. maricaloris [25,37]	sponges	+ (yellow)	dibromo- and bromoaltero-chomides	antimicrobial; cytotoxic against sea urchin
P. marina [38]	tidal flat sediment	+ (pale yellow)	-	-
P. mariniglutinosa [39]	diatoms	-	-	-
P. nigrifaciens [40,41]	mussels, salted foods, sea water	± (melanin)	-	-
P. paragorgicola [42]	sponge	-	-	-
P. peptidolytica [4,43]	sea water	+ (yellow)	unknown compound(s)	antibacterial; hemolytic
P. phenolica [44,45]	sea water	+ (brown)	brominated biphenyl compound (MC21-A)	antibacterial

P. piscicida [4,9,46-49]	estuarine waters, fish	+ (yellow)	toxic protein; unknown anti-algal compounds	antimicrobial; anti-algal
P. prydzensis [50]	sea ice	-	-	-
P. rubra [9,14-18,51-54]	sea water	+ (red)	high molecular weight polyanionic substance; cycloprodigiosin HCl; rubrenoic acids	antibacterial; anti-algal; anti-fungal; anti-malarial; anti-tumour; bronchodilatatoric; inhibits T cell/lymphocyte proliferation; inhibits settlement of invertebrate larvae; inhibits algal spore settlement
P. ruthenica [4,55-57]	mussels, saltern, sea water	+ (pale orange)	unknown compound(s)	antibacterial; hemolytic
P. spongiae [58,59]	sponge	+ (pale orange)	unknown compound(s)	induces settlement of invertebrate larvae
P. tetraodonis [60,61]	puffer fish	-	tetrodotoxin	neurotoxic effects
P. translucida [42]	sea water	-	-	-
P. tunicata [9,62-67]	marine algae, tunicates	+ (dark-green, purple, white, yellow)	unknown purple pigment; tambjamine-like alkaloid (YP1); toxic protein AlpP (L-AAO); unknown compound(s)	antibacterial; anti-fungal; anti-algal; inhibits settlement of invertebrate larvae; inhibits algal spore settlement; inhibits protists
P. ulvae [9,64,68]	marine alga	+ (dark-purple)	unknown dark-purple pigment; unknown compound(s)	antibacterial; inhibits settlement of invertebrate larvae and algal spores; inhibits algal spore germination
P. undina [4,9,24,29,30]	fish, sea water	-	unknown compound(s)	antibacterial; hemolytic; probiotic benefits; possible opportunistic fish pathogen
Geitlerinema BBD strains [69-71]	microbial mats on corals	+ (blue-green)	microcystins	induces coral tissue lysis and death
Geitlerinema cf. sp. [72]	sea water	+ (blue-green)	yellow cytotoxic linear lipopeptide (mitsoamide)	anti-tumour; toxic against brine shrimp
***Geitlerinema* strain Flo1** [7,73,74]	sea water (mangrove wood)	+ (blue-green)	**L-AAO**; possibly other unknown compounds	**antibacterial**; antimicrobial
G. splendidum [75]	freshwater	+ (blue-green)	microcystins	antimicrobial; anti-algal; induces morphological and ultrastructural alterations of cyanobacteria and microalgae

+ = present; - = not present or not determined; [1] Romanenko et al., 2003a; [2] Ivanova et al., 2004; [3] Bozal et al., 1997; [4] Kalinovskaya et al., 2004; [5] Al Khudary et al., 2008; [6] Akagawa-Matsushita et al., 1992; [7] **data from the present study**; [8] Gauthier and Breittmayer, 1979; [9] Holmström et al., 2002; [10] Park et al., 2005; [11] Gauthier, 1977; [12] Urvantseva et al., 2006; [13] Enger et al., 1987; [14] Magae et al., 1996; [15] Kawauchi et al., 1997; [16] Kim et al., 1999; [17] Campàs et al., 2003; [18] Pérez-Tomás et al., 2003; [19] Huggett et al., 2006; [20] Romanenko et al., 1995; [21] Oh et al., 2011; [22] Ivanova et al., 1996a; [23] Sawabe et al., 2000; [24] Chan et al., 1978; [25] Ivanova et al., 2002a; [26] Chen et al., 2010b; [27] Baumann et al., 1972; [28] Mitova et al., 2005; [29] Riquelme et al., 1996; [30] Maeda et al., 1997; [31] Ivanova et al., 2002b; [32] Xu et al., 2010; [33] Gauthier and Flatau, 1976; [34] Kamei et al., 1986; [35] Bowman, 2007; [36] Gómez et al.,

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4.4 Conclusions

Taking advantage of the broad-spectrum antibacterial activity of the L-AAOs investigated in the present study and, in the case of the *Pseudoalteromonas* L-AAOs, their alkaline stability, these enzymes could possibly be used as antibiotics in medical therapy. Although, L-AAOs in general are reported to exhibit some toxic activities (Samel et al., 2008), it has been also shown that this kind of enzymes have only very low lethal toxicity in mice (Tan and Fung, 2010). Ciscotto and coworkers (2009) described a so-called “bactericidal window” where concentrations of hydrogen peroxide and other reactive oxygen species sufficient to inhibit bacterial growth are no thread to the host (human) cells. Sorg (2004) also pointed out that microorganisms are normally more sensitive to reactive oxygen species than human tissues. But, due to the proteinaceous nature of L-AAOs and relatively high production costs, the application of these enzymes in medical therapy as antibiotic agents will probably always be limited. Anyhow, further investigations with respect to the principles of L-AAO activities possibly will contribute to the development of new pharmaceutical compounds or to the implementation of novel therapeutic applications.

Regarding the functions of the L-AAOs in the *Pseudoalteromonas* and *Geitlerinema* species investigated in the present study, it only can be speculated. It would be possible that they play a role in the utilization of L-amino acids as a nitrogen source. Furthermore, it is likely that they display defence and/or pathogenic functions. In

addition, it would be conceivable that the produced L-AAOs are influencing the formation and the dispersal of microbial biofilms.

4.5 Outlook

In the present study the inhibitory substances from *P. aurantia* and *P. citrea* were characterised concerning various aspects. In addition, also a substance with inhibitory activity produced by *Geitlerinema* strain Flo1 was investigated. Although, in all cases the substances could be identified, several questions could not be answered so far.

Hence, it would be interesting to investigate the effects of different growth parameters, e.g. pH, temperature (a broader range than already determined), supply of nutrients, salts, trace elements, and vitamins on the respective L-AAO production rates.

Furthermore, only the antimicrobial potential of the examined L-AAOs was evaluated in the present study. Thus, little to nothing is known, what kind of effects these enzymes from *P. aurantia*, *P. citrea*, and from the cyanobacterial strain Flo1 have upon algae, blood components (platelets), cells (including cancer cells), dinoflagellates, fungi, and viruses. In addition, it would be interesting to further investigate, if the L-AAOs are able to inhibit the settlement of invertebrate larvae and algal spores (from other species than already reported).

Additionally, it could be investigated, what kind of physiological roles the L-AAOs have in their producers and in interaction with the environment. In this respects, the influences of L-AAOs during biofilm formation would be of special interest.

Another promising approach would be the search for new biotechnological applications for L-AAOs. Accordingly, it would be desirable to determine the amino acid sequences of the investigated L-AAOs, for instance using matrix-assisted laser desorption ionization-time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry top-down sequence analysis. Another step could be the cloning of the respective L-AAO encoding genes into an appropriate microorganism, e.g. into *E. coli* or into a yeast, to produce the L-AAOs on large scale.

Last but not least, further *Pseudoalteromonas* and *Geitlerinema* species as well as those from other promising genera could be spectrophotometrically screened for the presence of L-AAOs and the newly developed in-gel L-AAO detecting approach

could be used to determine their number and approximate molecular weights. Promising *Pseudoalteromonas* candidates could be *P. aliena*, *P. antarctica*, *P. issachenkonii*, *P. peptidolytica*, *P. piscicida*, *P. rubra*, *P. ruthenica*, *P. spongiae*, *P. ulvae*, and *P. undina*, due to the presence of so far unidentified bioactive substances.

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6. Annex

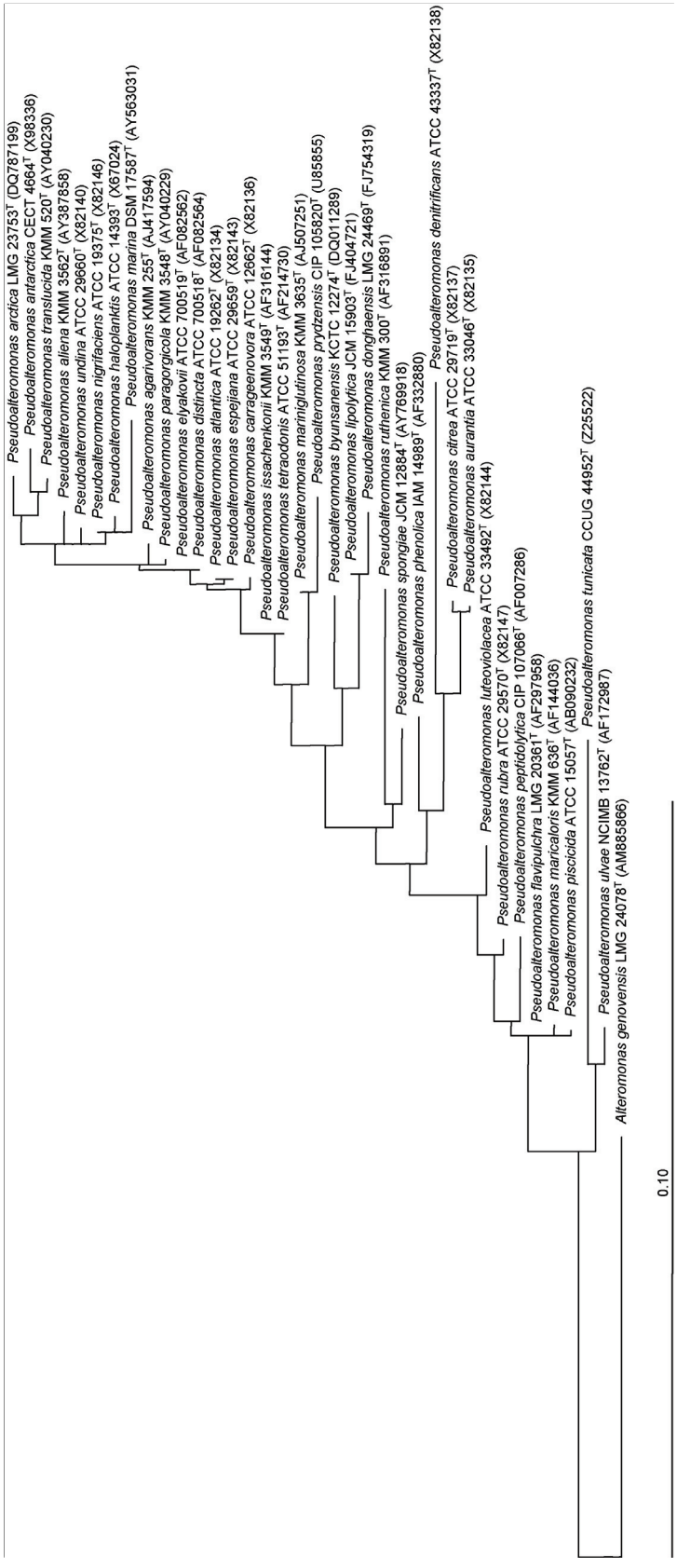

Fig. S1 Maximum-likelihood phylogenetic tree based on the 16S rRNA gene sequences of all validly published *Pseudoalteromonas* species. The sequence of *Alteromonas genovensis* LMG 24078^T (GenBank accession no. AM885866) was used as an outgroup. Bar, 0.1 substitutions per nucleotide position.

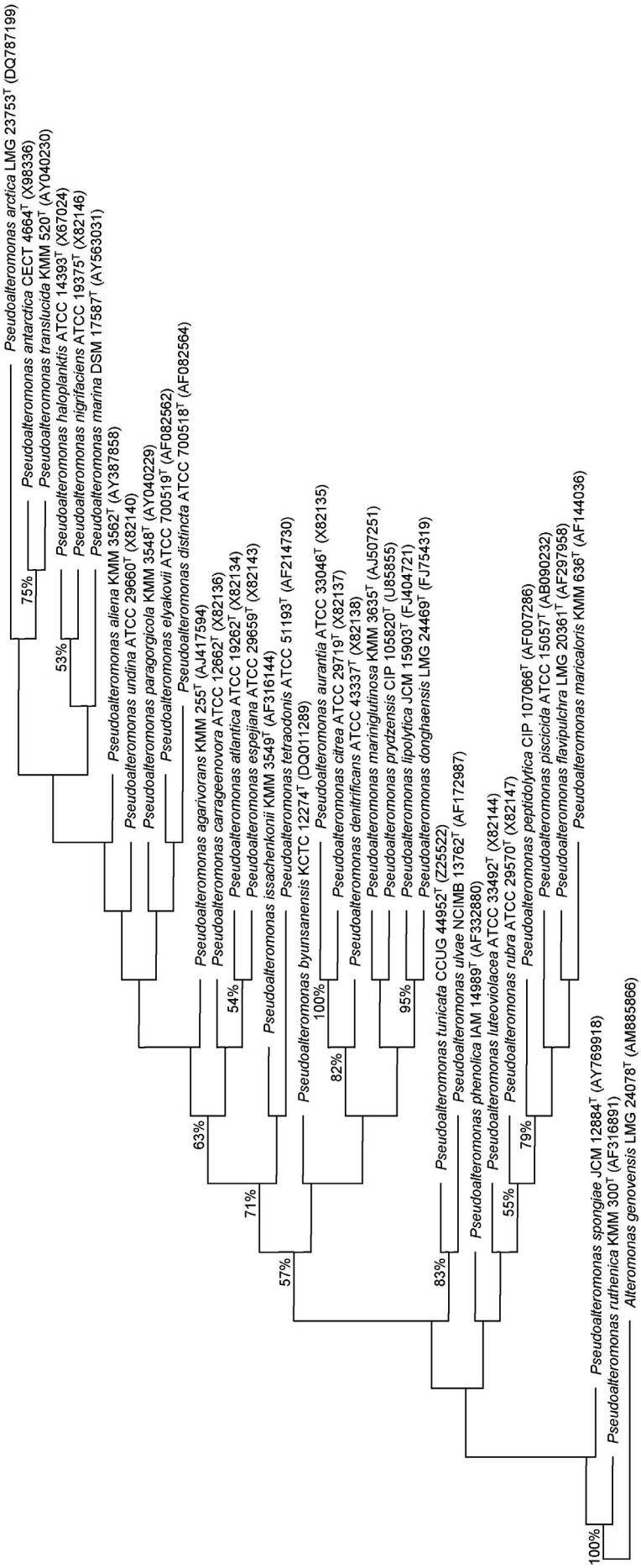

Fig. S2 Maximum-parsimony phylogenetic tree based on the 16S rRNA gene sequences of all validly published *Pseudoalteromonas* species. Bootstrap values (>50%) based on 1,000 resamplings are shown. The sequence of *Alteromonas genovensis* LMG 24078^T (GenBank accession no. AM885866) was used as an outgroup. Bar, 0.1 substitutions per nucleotide position.

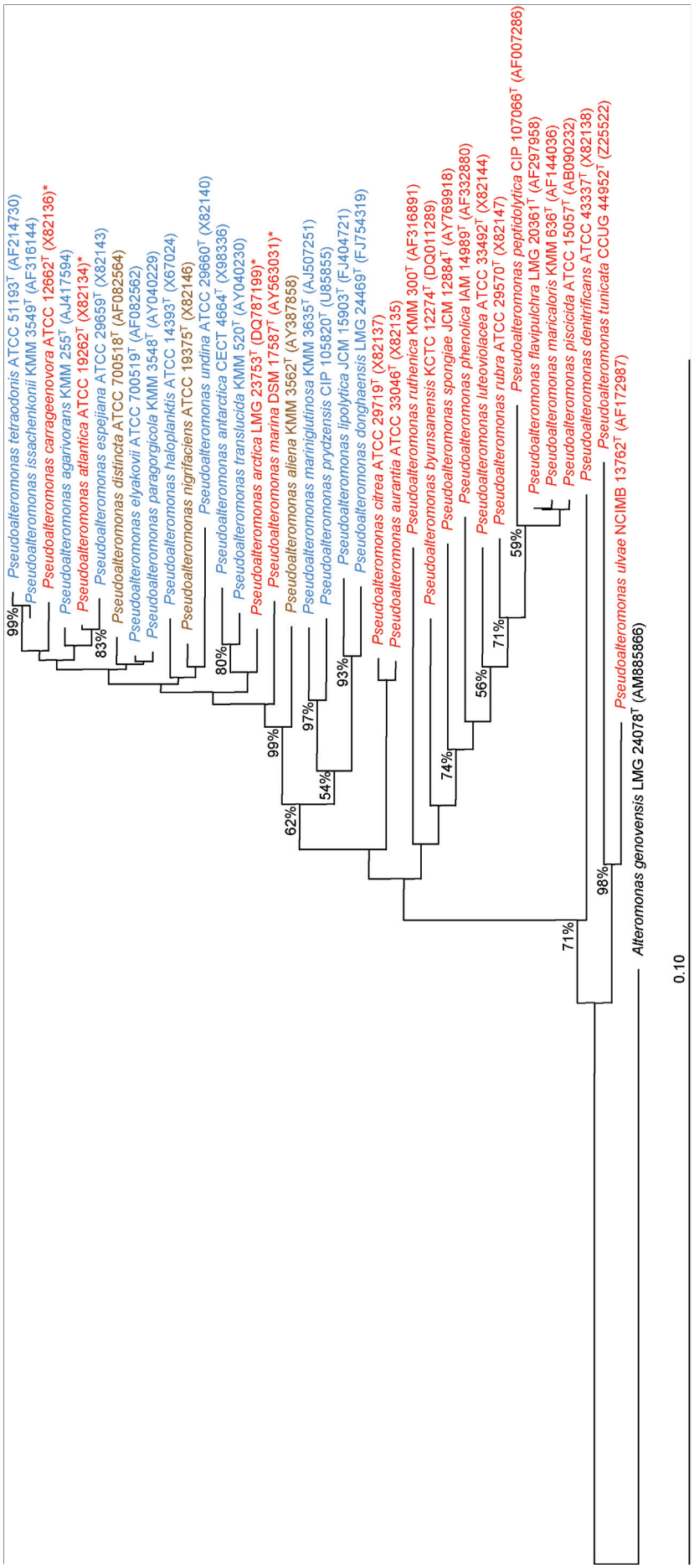

Fig. S3 Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences of all validly published *Pseudoalteromonas* species. Bootstrap values (>50%) based on 1,000 resamplings are shown. Species given in red are pigmented, those given in blue are not (species marked with * are only weakly pigmented). Species given in brown are producing melanin-like pigments. The sequence of *Alteromonas genovensis* LMG 24078^T (GenBank accession no. AM885866) was used as an outgroup. Bar, 0.1 substitutions per nucleotide position.

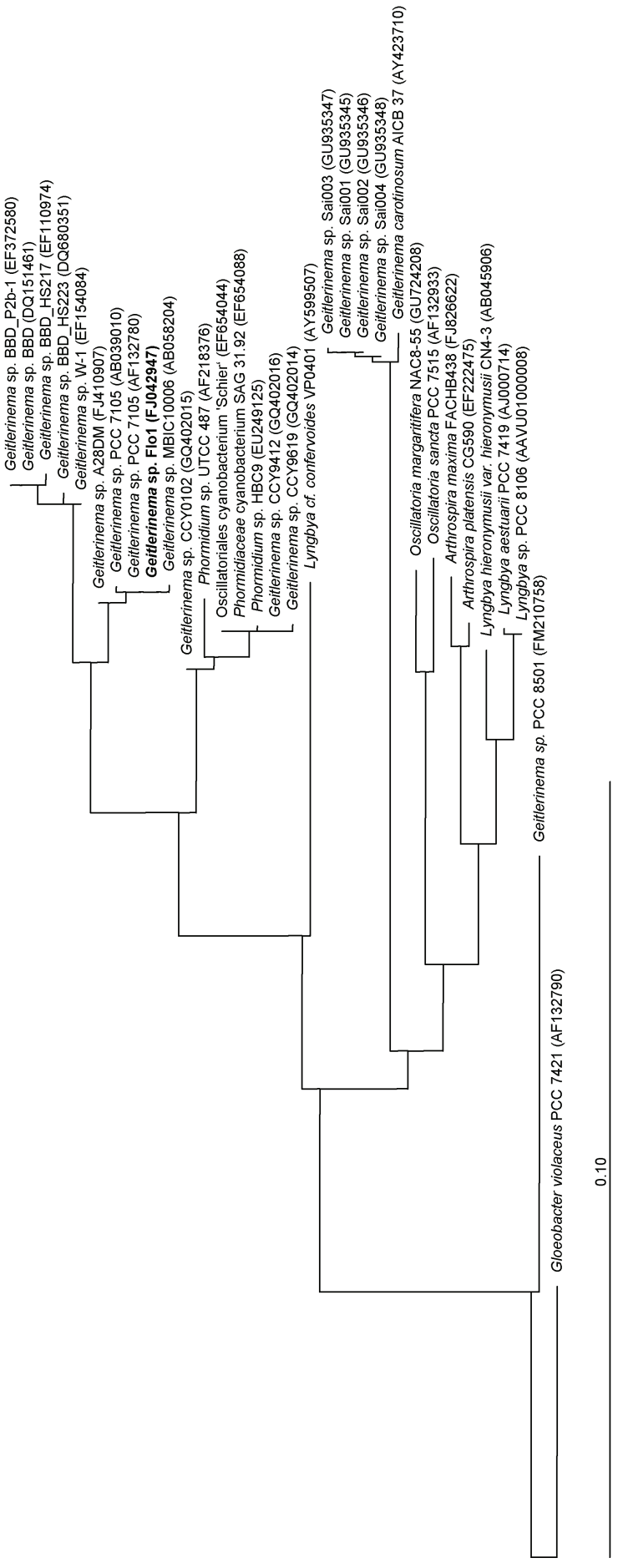

Fig. S4 Maximum-likelihood phylogenetic tree based on 16S rDNA sequences of strain Flo1 and closely related cyanobacteria. The sequence of *Gloeobacter violaceus* PCC 7421 (GenBank accession no. AF132790) was used as an outgroup. Bar, 0.1 substitutions per nucleotide position.

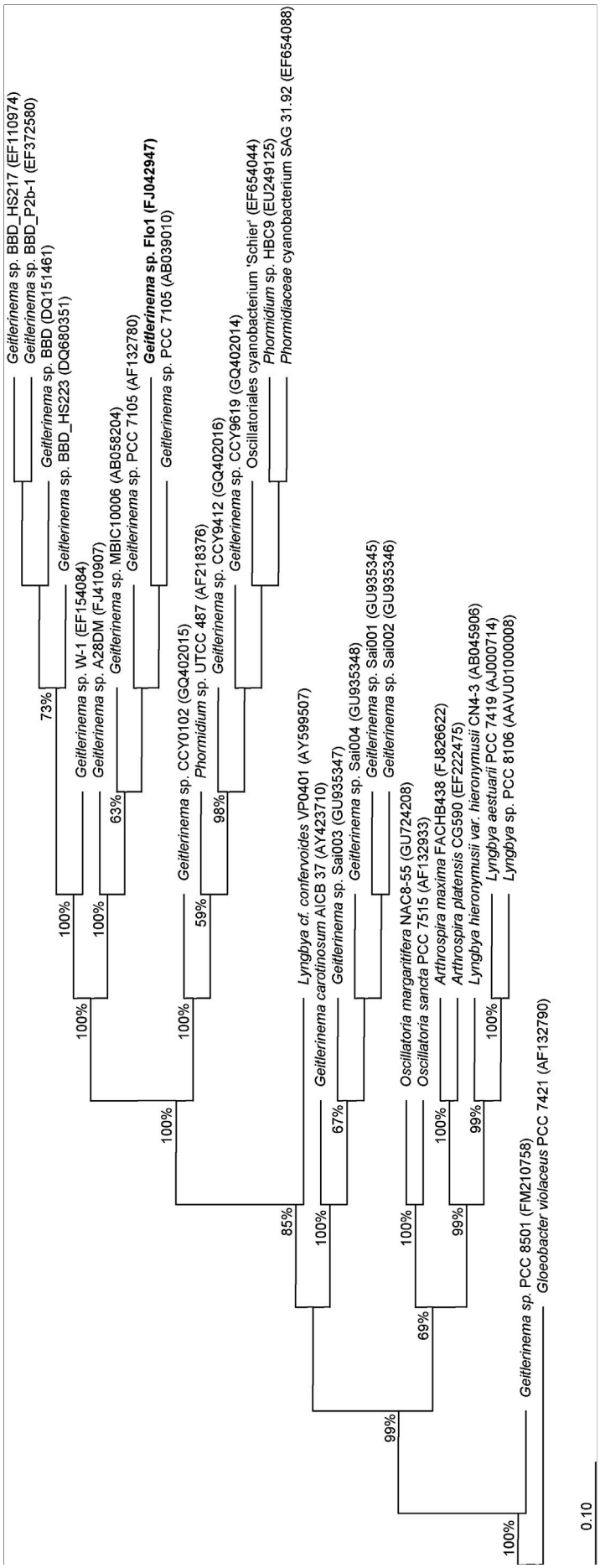

Fig. S5 Maximum-parsimony phylogenetic tree based on the 16S rRNA gene sequences of strain Flo1 and closely related cyanobacteria. Bootstrap values (>50%) based on 1,000 resamplings are shown. The sequence of *Gloeobacter violaceus* PCC 7421 (GenBank accession no. AF132790) was used as an outgroup. Bar, 0.1 substitutions per nucleotide position.

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Erklärung gemäß § 11 Abs. 2 der Promotionsordnung der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche

Die vorliegende Dissertation wurde in folgenden Punkten überarbeitet:

1.) Seite 2: Tag des Promotionskolloquiums (14.07.2011) wurde eingetragen

2.) Seite 13, Absatz 2, Zeile 2: „introduded“ wurde korrigiert in „introduced“

3.) Seite 27, Absatz 1, Zeile 2: „as evaluated light microscopical studies” wurde korrigiert in „as evaluated by light microscopical studies”

4.) Seite 30, Absatz 3, Zeilen 3-4: „Submitted to “Archives of Microbiology” on April 1, 2011” wurde geändert in „Intended for publication in “Enzyme and Microbial Technology””, da das Manuskript I von Gutachtern des Journals „Archives of Microbiology” abgelehnt wurde und nun eine Veröffentlichung im Journal „Enzyme and Microbial Technology” angestrebt wird

5.) Seite 31, Absatz 2, Zeile 3: „Submitted to “Enzyme and Microbial Technology” on May 31, 2011” wurde geändert in „Submitted to “Zeitschrift für Naturforschung C” on June 7, 2011”, da das Manuskript III, wie von einem Editor des Journals „Enzyme and Microbial Technology” mitgeteilt, nicht in das Spektrum des Journals passt und daher auch keinem weiteren Begutachtungsprozess unterzogen wurde. Das entsprechende Manuskript wurde nun zur Veröffentlichung beim Journal „Zeitschrift für Naturforschung C“ eingereicht

6.) Seite 33-61: Manuskript I wurde umformatiert für eine Veröffentlichung in „Enzymes and Microbial Technology“, inhaltlich wurden keine Änderungen vorgenommen

7.) Seite 89-115: Manuskript III wurde umformatiert für eine Veröffentlichung in „Zeitschrift für Naturforschung C“, inhaltlich wurden keine Änderungen vorgenommen. Die Arbeit ist daher insgesamt um eine Seite länger, was zusätzlich eine Anpassung der Seitenzahlenangaben im Inhaltsverzeichnis sowie bei den Verweisen auf bestimmte Kapitel oder einzelne Seiten erforderte

8.) Seite 161, Absatz 1, Zeilen 1-2: „I am grateful for the financial support of the University of Bremen” geändert in „I am grateful for the financial support of the “Zentrale Kommision für Forschung und wissenschaftlichen Nachwuchs” of the University of Bremen“

Alle Änderungen wurden im Einvernehmen mit Prof. Dr. Ulrich Fischer (erster Gutachter und Vorsitzender der zuständigen Prüfungskommission) und Dr. Olav Grundmann (zweiter Gutachter) vorgenommen.

Erklärung gemäß § 6 Abs. 5 (Nr. 1-3) der Promotionsordnung der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche

Hiermit versichere ich, dass ich die vorliegende Dissertation mit dem Titel „Characterisation of inhibitory substances produced by two *Pseudoalteromonas* species and the cyanobacterial strain Flo1“

1) ohne unerlaubte fremde Hilfe angefertigt habe
2) keine anderen, als die von mir angegebenen Quellen und Hilfsmittel benutzt habe und
3) die den benutzten Werken wörtlich und inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Bremen, 1. Juni 2011 _____ (Jan Erik Rau)