Invited Trends Article – Minireview

Siderophores of Fluorescent Pseudomonads

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Z. Naturforsch. **52c**, 713–720 (1997); received August 4, 1997

Fluorescent Pseudomonads, Iron Transport, Pyoverdin Biosynthesis, Siderophores

Introduction

Though iron is fourth in abundance in the earth crust it is not readily available to many microorganisms: In the early days of earth life developed in a reductive atmosphere where iron – necessary for many physiological processes – was abundant in its divalent form. Fe^{2+}-salts are sufficiently water soluble to provide an adequate supply of this element to the cells. But as a consequence of the photolytic cleavage of water, oxygen was set free and soon with rare exceptions only trivalent iron abounded which is present in the soil mainly in the form of its oxide hydrates $[Fe_2O_3 \cdot n\ H_2O]_x$. Due to their low dissociation constants the concentration of free Fe^{3+} is at best 10^{-17} mol/l at physiological pH-values while 10^{-6} mol/l would be needed to provide the necessary supply. To circumvent this problem soil bacteria as well as the ones infecting higher organisms (where the availability of iron is limited for other reasons such as complexation by peptides as, e.g., transferrins) produce a variety of compounds which can form water soluble Fe^{3+}-complexes, so-called siderophores.

Fe^{3+} forms octahedral d^5 high spin complexes which can accomodate three bidentate ligands with oxygen or occasionally nitrogen and sulfur as binding sites. Most frequently encountered are catecholate and hydroxamate units. Mixed systems are not uncommon and other structural types as α–hydroxy-carboxylates are observed occasionally. Three bidentate ligands are often connected by aliphatic segments; this results in an entropic advantage over three non-connected units.

Many members of the genus *Pseudomonas* are soil bacteria, some are plant pathogenes, others are beneficial to their host-plants, and *Pseudomonas aeruginosa* belongs to the most dangerous bacteria exciting "hospitalism" (nosocomial infections) (Botzenhart and Rüden, 1987). The rather large genus *Pseudomonas* is commonly divided into 5 rRNA homolgy groups; no. I of them comprises *i.a.* the so-called "fluorescents" (Palleroni, 1984). Their name derives from an observation made by *Gessard* (Gessard, 1892): When grown under iron deficiency they form yellow-greenishly fluorescent substances today referred to as pseudobactins or more commonly as pyoverdins, the most potent siderophores of the pseudomonads (Budzikiewicz, 1993).

Pyoverdins and related siderophores

Structure and biogenesis

Pyoverdins (**1**) consist of three distinct structural parts, viz. a dihydroxyquinoline chromophore responsible for their fluorescence, a peptide chain bound to its carboxyl group, and a small dicarboxylic acid (or its monoamide) connected amidically to the NH_2-group. The chromophore (1S)-5-amino-2,3-dihydro-8,9-dihydroxy-1*H*-pyrimido-[1,2a]quinoline-1- carboxylic acid is the common structural element of all pyoverdins and one of the binding sites for Fe^{3+}. The peptide chain has a two-fold function: It provides the other two ligands for Fe^{3+} (one ß-hydroxy amino acid and one hydroxamic acid or two hydroxamic acids) in

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0939–5075/97/1100–0713 $ 06.00

the correct position for complexation, and it is responsible for the recognition of the complex at the cell surface. It comprises six to twelve amino acids partially non-proteinogenic and/or structurally modified (Table I). Clear-cut patterns of the aminoacid sequences which could be correlated with the different species of *Pseudomonas* cannot be recognized yet. In the majority of cases a small neutral amino acid (Ala, Ser) is bound to the chromophore followed by a basic amino acid (Dab, Orn, Lys, Arg). About half of the aminoacids are D-configurated; this feature prevents a degradation by proteolytic enzymes. A high percentage of polar amino acids renders the pyoverdins water soluble. Parts of the peptide chains of many pyoverdins form cyclic substructures. Most common is a C-terminal *cyclo*-Orn (3-amino-piperidone-2) ring as in **3**. Larger cycles comprising several amino acids can be formed between the C-terminal carboxyl group and either by an amide bond to the ε-amino group of an in-chain Lys as in **2**, or by an ester bond with Ser or Thr. Occasionally condensation products of the two amino groups of 2,4-diaminobutyric acid (Dab) with the carboxyl group of the neighboring amino acid are encountered yielding a tetrahydropyrimidine ring as in **4**.

Usually several pyoverdins co-occur with identical peptide chains, but differing in the nature of the dicarboxylic acid (R in **1**- **4**) bound to the NH_2-group of the chromophore. Sofar Glu, α-ketoglutaric acid, succinic acid (amide) and malic acid (amide) were found. All these acids belong to the

Table I. List of pyoverdins from *Pseudomonas* spp. for which complete structures were published.

Species	Peptide chain[1,2]	Cycle[3]
aeruginosa	Ser-Arg-Ser-FoOHOrn-Lys-FoOHOrn-Thr-Thr (**2**)	Thr-Lys
	Ser-Dab-FoOHOrn-Gln-Gln-FoOHOrn-Gly (**4**)	Ser/Dab
	Ser-FoOHOrn-Orn-Gly-aThr-Ser-cOHOrn (**3**)	cOHOrn
fluorescens	Lys-OHAsp-Ala-aThr-Ala-cOHOrn	cOHOrn
	Asn(Asp)-FoOHOrn-Lys-Thr-Ala-Ala-FoOHOrn-Lys	Lys-Thr
	Ala-Lys-Gly-Gly-OHAsp-Gln-Ser-Ala-Ala-Ala-Ala-cOHOrn	cOHOrn
	Ala-Lys-Gly-Gly-OHAsp-Gln-Ser-Ala-Gly-aThr-cOHOrn	cOHOrn
	Ser-Lys-Gly-FoOHOrn-Ser-Ser-Gly-Lys-FoOHOrn-Glu-Ser	Ser-Lys
	Ser-Dab-Gly-Ser-OHAsp-Ala-Gly-Ala-Gly-cOHOrn	Ser/Dab,cOHOrn
	Ser-Lys-Gly-FoOHOrn-Lys-FoOHOrn-Ser	Ser-Lys
	Ala-Lys-Gly-Gly-OHAsp-Gln-Dab-Ser-Ala-cOHOrn	Gln/Dab,cOHOrn
	Ser-Lys-OHHis-aThr-Ser-cOHOrn	cOHOrn
	Ser-Ala-AcOHOrn-Gly-Ser-OHAsp-Ser-Thr[4]	---
tolaasii	Ser-Lys-Ser-Ser-Thr-Ser-AcOHOrn-Thr-Ser-cOHOrn	cOHOrn
putida	Asp-Lys-OHAsp-Ser-Thr-Ala-Glu-Ser-cOHOrn	cOHOrn
	Ser-Thr-Ser-Orn-OHAsp-Gln-Dab-Ser-aThr-cOHOrn	Gln/Dab,cOHOrn
	Asp-BuOHOrn-Dab-Thr-Gly-Ser-Ser-OHAsp-Thr	---
	Ser-Ala-AcOHOrn-Gly-Ala-OHAsp-Ser-Thr[4]	---
aptata	Ala-Lys-Thr-Ser-AcOHOrn-cOHOrn	cOHOrn
--?--	Ser-OHAsp-Thr-Ser-AcOHOrn-Ala-Gly-Ser[5]	Ser-Ser
--?--	Ser-Ala-Gly-Ser-Ala-OHAsp-aThr-AcOHOrn	---

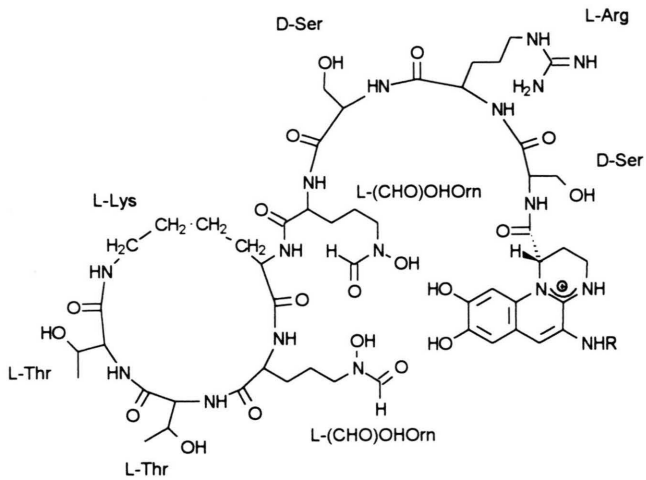
[1] D-aminoacids underlined; broken line: D/L not determined.

[2] Common amino acids: 3-letter code; OHAsp: *threo*-ß-hydroxy-Asp; OH-His: *threo*-ß-hydroxy-His; OHOrn: N^5-hydroxy-Orn; Ac(Fo,Bu)OHOrn: N^5-acetyl (formyl, R-ß-hydroxybutyryl) OHOrn; cOHOrn: cyclo-OHOrn (3-amino-1-hydroxypiperidone as in **3**); aThr: *allo*Thr.

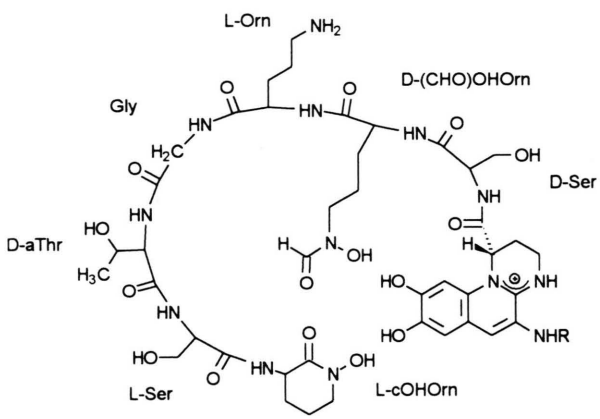
[3] E.g., Thr-Lys indicates a large cycle formed by an amide or ester bond between the carboxyl group of the C-terminal amino acid and an amino- or hydroxy group of an in-chain aminoacid as in **2**; e.g., Ser/Dab refers to a condensation product between Dab and another aminoacid forming a dihydropyrimidine ring as in **4**.

[4] Together with the linear peptide chain an octacyclopeptidic structure was found with an ester bond between the carboxyl group of the C-terminal Thr and the hydroxyl group ot the Ser bound to the chromophore (Khalil-Rizvi *et al.*, 1997).

[5] In the original publication (Yang and Leong, 1984) it is suggested that the cyclooctapeptide ring is linked by an ester bond to the carboxyl group of the chromophore using the hydroxyl group of one of the Ser. As for all other pyoverdins found sofar the peptide chain is bound by an amide bond (α–amino group of an aminoacid or occasionally ε-amino group of Lys) to the chromophore an alternative structure may be envisaged with the macrocycle formed by an ester bond between the C-terminal amino acid and a Ser which in turn is bound amidically to the chromophore (Khalil-Rizvi, 1997).



2



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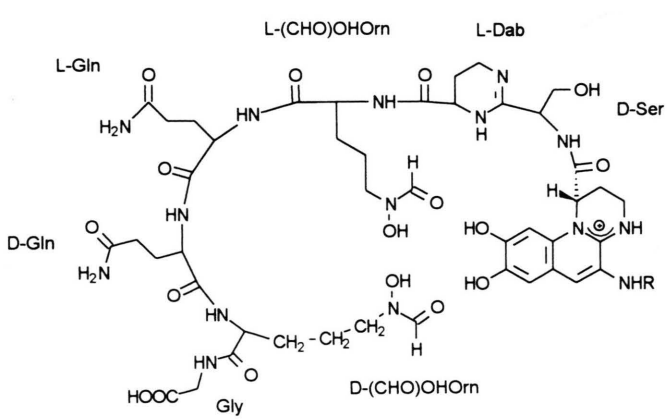
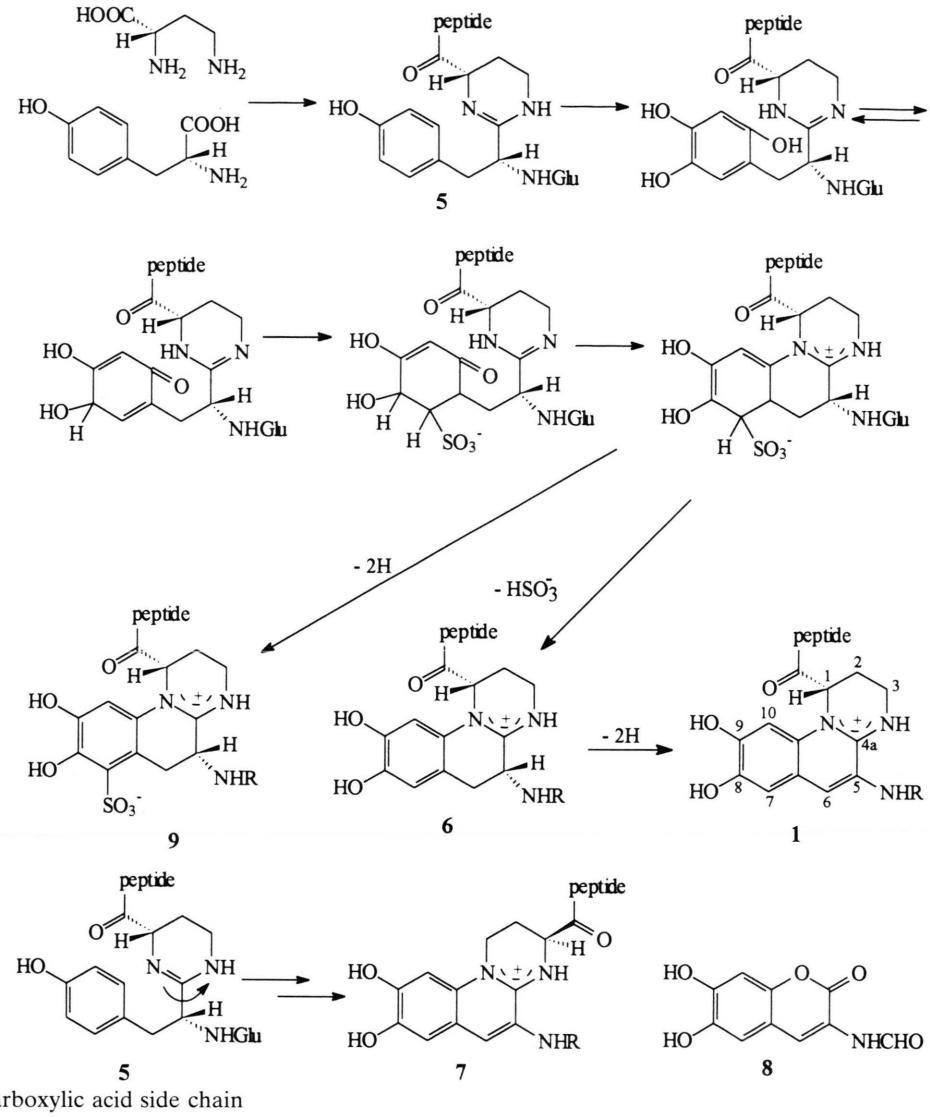


4

R: dicarboxylic acid side chain

Fig. 1. The pyoverdins of the three "siderovars" of *Pseudomonas aeruginosa*.



R: dicarboxylic acid side chain

Fig. 2. Proposed biogenetic scheme for the pyoverdin chromophore.

citric acid cycle followed step-by-step as far as the biogenetic sequence is concerned.

Regarding the biogenesis of the peptide chain evidence has been presented that it occurs not via the ribosomal pathway, but rather through a multienzyme thiotemplate mechanism involving peptide synthetases (Georges and Meyer, 1995). They activate the constituent amino acids as their adenylates (Menhart and Viswanatha, 1990) and may also be responsible for the L/D-isomerisation, but some not entirely conclusive experimental data

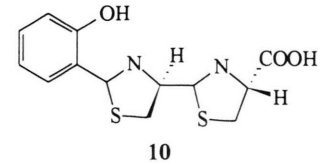


Fig. 3. Pyochelin.

(Maksimova *et al.,* 1992) point towards an independent isomerase activity.

Much light could be shed on the biogenesis of the chromophore by the isolation of compounds co-occurring with pyoverdins in the culture medium. They have the same peptide chain but differ in the structure of the chromophore. The most prominent ones are 5,6-dihydropyoverdins (**6**) and ferribactins (**5**) (Taraz *et al.*, 1991). The pathway depicted in Scheme 1 could be substantiated by the following experiments: The ferribactin chromophore is a condensation product of D-Tyr and L-Dab in agreement with the results obtained by feeding labelled precursors: ^{14}C- and ^{2}H-labels of Tyr (C-2 and C-3) were found in the chromophore as was ^{15}N from 4-[^{15}N]-Dab in the 4-position of the latter (Böckmann *et al.*, 1997). Furtheron, the free rotation of the tetrahydropyrimidine ring of ferribactin should allow ring closure via either of its two N-atoms. The recent discovery of isopyoverdins (**7**) (Jacques *et al.*,1995) confirmed this hypothesis. Apparently a*fter* formation of the ferribactin chromophore two more hydroxyl groups are introduced into the phenyl ring (Phe and Tyr, but not 3,4-dihydroxyphenylalanine – DOPA – are accepted in feeding experiments) (Novak-Thompson and Gould, 1994). This is also suggested by the isolation of **8** from a genetically modified non-fluorescent *Pseudomonas aeruginosa* mutant (Longerich *et al.*, 1993). The isolation of the sulfonic acid derivative **9** makes it likely that ring closure occurs by the mechanism of a *Bucherer* reaction (Schröder *et al.*, 1995).

It should be mentioned here that a different biogenetic scheme was proposed (Maksimova *et al.*, 1993) starting from Phe and dihydroorotic acid which – albeit with some difficulties – could explain the intermediacy of ferribactins, etc. It is ruled out, however, by the observation that [^{15}N] from 4-[^{15}N]-Dab is incorporated in N-4 of the pyoverdin chromophore (in the case of the dihydroorotate mechanism N-4 of the chromophore should come from the carbamyl-NH_2 of N-carbamyl-Asp; for details see Böckmann *et al.*, 1997).

Azotobactins are the typical siderophores of *Azozobacter vinelandii* (Schaffner *et al.,* 1996), but occasionally they were observed also to accompany pyoverdins from *Pseudomonas* spp., in which case they have the same peptide chain as the latter (Hohlneicher *et al.,* 1995). Their chromphore comprises an additional ring formed by the condensation of carbonic acid with both amino groups (4 and C-5 in **1**, urea structure) of the pyoverdin chromophore. How they fit into the biogenetic scheme is still an open question.

Taxonomical questions

The fluorescent *Pseudomonas* spp. are commonly subdivided into the saprophytic group with Arg dihydrolase activity comprising *Ps. aeruginosa, putida, fluorescens* (with *Ps. tolaasii*), *chlororaphis* and *aureofaciens*, and the phytopathogenic group without Arg dihydrolase activity with *Ps. syringae* (of which *Ps. aptata* is a pathovar) and *cichorii* (from this group only the structure of the pyoverdin of a *Ps. aptata* strain was elucidated and partial structures of those produced by two *Ps. syringae* strains were reported; they will not be considered here).

From the first group the human-pathogenic *Ps. aeruginosa* seems to be a well defined species. It can be subdivided into three "siderovars" characterized by different pyoverdins (**2** – **4**) which are not accepted mutually (Meyer *et al*, 1997). *Ps. fluorescens* is a collective species from which *Ps. chlororaphis* and *aureofaciens* were separated because they produce specific phenazine derivatives. The main distinguishing feature of *Ps. putida* is the lack of gelatinase. The differing nutritional patterns of the various biovars of the *Ps. fluorescens/putida* group are less clear-cut. Sofar ca. 20 different pyoverdins are known from *Ps. fluorescens* and 10 from *Ps. putida* strains (those for which complete structures can be found in the literature are listed in Table I). These pyoverdins could be a more reliable classification criterion, especially when two strains produce the same pyoverdin, but had been reported as different species (as, e.g., *Ps. fluorescens* and *chlororaphis,* Hohlneicher *et al.*, 1995; *Ps. fluorescens* and *putida,* Budzikiewicz *et al.*, 1997).

Of greater interest than a new sub-classification of the *Ps. fluorescens/putida* group is the general reclassification of the entire genus *Pseudomonas* and of related species which is going on (cf., e.g., Yabuuchi *et al.*, 1995). Until recently pyoverdins were considered as the siderophors typical for the fluorescent pseudomonads only. The fact that isopyoverdins (see above) were isolated both from

Pseudomonas putida and *Azomonas macrocytogenes* strains (Michalke *et al.*, 1996) and azotobactins from *Azotobacter vinelandii* and from various strains of the *Pseudomonas fluorescens* - group (see above) should be taken as an indication for a closer relationship between the genera or at least species.

Iron transport systems of fluorescent pseudomonads

The variations in the peptide chains of pyoverdins produced even by different strains of the same *Pseudomonas* species – sofar about 40 different sequences are known – suggest that a highly specific recognition mechanism is operating. Associated with the recognition at the cell surface are 80 to 90 kDa outer membrane proteins (Meyer *et al.*, 1979). Yet, relatively little is known with respect to the structural requirements for the recognition of a pyoverdin at the receptor site; only preliminary studies were performed to find out which structural parts of the molecule may be altered without disturbing the transport into the cell (unpublished). ^{55}Fe- and ^{14}C-labelling studies indicate that ferri-pyoverdins do not enter the cytoplasm as an entity. Fe^{3+}-ions are rather set free (probably reductively) in the periplasmatic space (Royt, 1990) and are transferred to a bacterioferritin.

Whether a given strain (siderovar) will recognize only its own pyoverdin, or to what extent those from other strains differing in the peptide chain are accepted as well, this is still an open question. Two tests were developed: One of them uses [^{59}Fe]-ferri-pyoverdins and measures the label uptake by the tested cells in a 15 to 20 minutes kinetic experiment. The other one uses cell cultures in a culture medium containing a strongly Fe^{3+}- binding compound. In the center of a Petri dish the tested ferri-pyoverdin is applied and the bacterial growth around the center is checked over 8 hrs. It was shown by the first test, e.g., that a *Pseudomonas aeruginosa* strain did not accept a *Ps. fluorescens* ferri-pyoverdin (unpublished), by the second one that the growth of certain *Ps. fluorescens* and *putida* strains could be induced by the ferri-pyoverdins of other members of the group (Jacques *et al.*, 1995). Whether the foreign ferri-pyoverdin is mistaken by the receptor for its own pyoverdin due to structural similarities (differences only in non-relevant sections of the peptide chain) or whether a new receptor is developed (see below), could not be decided. After longer periods of time (24 hrs and more) when production of the own pyoverdin has reached an adequate level Fe^{3+} can be secured from a foreign ferri-pyoverdin by trans-complexation (unpublished).

Most if not all fluorescent pseudomonads have a second iron transport system mediated by pyochelin (**10**) (Cox *et al.*, 1981) which is derived biogenetically from salicylic acid and two molecules of Cys. The structure of its Fe^{3+}-complex is not known. In addition, various small molecules such as salicylic acid (Meyer *et al.*, 1992) or N-methyl-N-thioformyl hydroxylamine (fluopsin F) (Shirahata *et al.*, 1970) were identified as siderophores. Also pyridine- and quinoline thiocarboxylic acids (Hildebrand *et al.*, 1985; Neuenhaus *et al.*, 1980) seem to play a role in the iron transport.

While the fluorescent pseudomonads protect the precious iron bound to their pyoverdins by the high complexing constants (between 10^{24} and 10^{26} at pH 7) and the resistence against proteolytic degradation due to the large number of D-amino acids in the peptide chain, they can develop receptor systems for the siderophores of other bacteria. The most notable example is the "alienation" of enterobactin from *Escherichia coli* by *Ps. aeruginosa* (Poole *et al.*, 1990). This is remarkable since fluorescent pseudomonads do not produce catecholate siderophores by themselves. But since enterobactin has a much higher complexing constant for Fe^{3+} (10^{49}) (Loomis and Raymond, 1991) *Ps. aeruginosa* would otherwise not have a chance in competition with *E. coli* e.g. in the human intestines. Another example is ferrioxamine B from *Streptomyces* spp. (Cornelis *et al.*, 1987).

Practical importance of the iron transport systems

Various *Pseudomonas* spp. are associated with higher plants. Some are phytopathogens while the presence of others especially in the rhizosphere helps to suppress plant-deleterious microorganisms (O'Sullivan and O'Gara, 1992; Jacques *et al.* 1993) and/or increases the plant growth directly (Yoshikawa *et al.*, 1993). All fluorescent pseudomonads produce a series of antibiotically active

substances detrimental to their competitors. Of equal importance is apparently the activity of the siderophores: Competing bacteria are deprived of the essential iron. Thus, the growth of the plant pathogen *Ps. solanacearum* is inhibited by *Ps. fluorescens*; both, antibiotic and siderophore activities seem to play a role (Budzikiewicz *et al.*, 1997 and literature cited there). In addition, higher plants seem to have mechansims to use directly Fe^{3+} complexed by bacterial siderophores (Kloepper *et al.*, 1980).

Pseudomonas aeruginosa is a dangerous opportunistic human-pathogenic bacterium responsible for frequently lethal hospital infections. As a soil bacterium it is omnipresent especially due to the effect of the modern air-conditioning systems. It is resistent against many desinfectant agents and – more important – against many of the common antibiotics (Pulverer, 1972; Neu, 1992). It affects severely injured patients and those whose immune system is weakened. An extremely critical situation exists for persons suffering from mucoviscidosis (cystic fibrosis) when *Ps. aeruginosa* (and to a minor extent *Ps. cepacia*) infect the bronchial tubes. Recent studies have shown that essentially only one of the three *Ps. aeruginosa* siderovars (ATCC 27853) could be isolated from CF infections (unpublished). Studies in this laboratory show that it is possible to link ß-lactame- and other antibiotics (for which resistence has been observed; Kallová *et al.*, 1996) to the pyoverdin and thus use the pyoverdin transport mechansim to bring the antibiotics into the cell. *In vitro* antimicrobial activities were observed exceding those of gentamicine which is commonly used for treatment (unpublished).

Acknowledgements

Support of our studies by **Deutsche Forschungsgemeinschaft**, the **European Commission** DG XII under the project "Cell factories for the production of bioactive peptides from *Bacillus subtilis* and *Pseudomonas*" (Bio4-ct95-0176) and by **Association pour la lutte contre la mucoviscidose** is gratefully acknowledged.

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