Biol Metals (1991) 4:176-180

BIOLOGY OF METALS

Production of siderophores by strains of the genus *Trichoderma*

Isolation and characterization of the new lipophilic coprogen derivative, palmitoylcoprogen

Heidrun Anke[1], **Josiane Kinn**[1], **Karl-Erik Bergquist**[2], **and Olov Sterner**[2]

[1] Department of Biotechnology, University of Kaiserslautern, Paul-Ehrlich-Str. 23, W-6750 Kaiserslautern, Federal Republic of Germany
[2] Organic Chemistry 2, Lund Institute of Technology, P.O.B. 124, S-22100 Lund, Sweden

Received March 6, 1991

Summary. The production of siderophores by nine strains (six species) of the genus *Trichoderma* was investigated. Under conditions of iron deficiency, the culture filtrate of all strains contained coprogen, coprogen B, and ferricrocin. In addition, *T. longibrachiatum* and *T. pseudokoningii* produced siderophores of the fusigen type. The yields of siderophores varied according to the strains examined from 270 mg/l (strain HA 34-88) to 2080 mg/l (strain HA 113-84). In extracts of the mycelia of *T. longibrachiatum* and *T. pseudokoningii* no siderophores could be detected. From the mycelia of the other strains three siderophores, namely coprogen, ferricrocin and a new coprogen derivative which carried a palmitoyl instead of an acetyl group, were obtained. Palmitoylcoprogen is the first fungal siderophore which is solely found in the cells and is not excreted into the culture broth.

Key words: *Trichoderma* - Siderophores - Fusigen - Ferricrocin - Palmitoylcoprogen

Introduction

Many microorganisms produce and excrete low-molecular-mass ferric-ion-chelating siderophores when grown under iron stress (Neilands 1981, 1984; Winkelmann 1986). In microbial interactions in the rhizosphere these compounds play a crucial role. Plant growth-promoting activities of beneficial bacteria, for example pseudomonads, are due to the production of siderophores. Suppression of disease-causing fungi such as *Fusarium oxysporum* or *Gaeumannomyces graminis* can be achieved not only by addition of pseudomonads to conductive soils but also by adding the proper siderophores (Kloepper et al. 1980a, b; Neilands and Leong 1986; Schippers et al. 1987; Sneh et al. 1984).

Offprint requests to: H. Anke

Protection of plants against soil-borne fungal diseases can also be achieved by certain fungi. Strains of *Trichoderma harzianum* and *T. hamatum* have been used to control damping off of bean, tomato and eggplant seedlings or black-root rot of strawberries (Chet 1987; Cook and Baker 1983). Since most *Trichoderma* species are mycoparasites, production of siderophores by these fungi has not so far been investigated. Since, for growth and survival in the rhizosphere, the ability to compete successfully for iron might play an important role for these beneficial fungi, the production of siderophores by nine *Trichoderma* strains, some with high antagonistic potential towards *Pythium ultimum*, was investigated.

Materials and methods

Organisms and culture conditions. Trichoderma strain HA 35-84 was isolated from a fruiting body of a basidiomycete; strain HA 113-84 was isolated from a soil sample collected in Ethiopia and HA 34-88 was selected for growth at low temperatures ($\leq 10°$ C). The following strains were obtained from CBS: *T. hamatum*, CBS 691.74; *T. harzianum*, CBS 118.72; *T. koningii*, CBS 401.73; *T. longibrachiatum*, CBS 488.78; *T. pseudokoningii*, CBS 931.69; *T. viride*, CBS 815.68. All strains were maintained on YMG agar composed of (g/l): yeast extract 4, malt extract 10, glucose 4, and agar 20. For the production of siderophores, asparagine medium was used (Anke and Diekmann 1971). Incubations were carried out in 5-l conical flasks containing 2 l medium on a rotary shaker (120 rpm) at 27° C.

Antimicrobial and cytotoxic activities. Antimicrobial assays were carried out on complex and synthetic media as described before (Anke 1977). Cytotoxic activity was evaluated according to Leonhardt et al. 1987).

Measurement of the concentration of siderophores. After removal of the mycelia, to 1 ml culture filtrate was added 35 µl $FeCl_3$ (20 mg/ml); after centrifugation, the absorbance at 440 nm of the supernatant was measured. The absorption coefficient of coprogen B, the main siderophore, was used to calculate the total amount (Konetschny-Rapp et al. 1988a). Culture filtrates containing high amounts of siderophores were diluted before addition of $FeCl_3$.

Isolation and characerization of the siderophores

From the culture filtrate. After addition of excess ferric chloride and centrifugation at 3500 *g*, the pH was brought to 6.5 and the filtrate was passed through a column filled with Diaion HP 21 (Mitsubishi) in H_2O. After washing with H_2O, the siderophores were eluted with acetone H_2O (1:1). After evaporation of the acetone, the aqueous solution was lyophilized. Separation of ferricrocin, coprogen, coprogen B, fusigen and fusigen B was achieved by chromatography on carboxymethyl cellulose (CM52) in ammonium acetate buffer pH 5.0: coprogen and ferricrocin were eluted with 0.01 M buffer, coprogen B and fusigen B with 0.1 M buffer, fusigen with 0.2 M buffer. The final purification steps were chromatography on Sephadex LH 20 in methanol or reversed-phase chromatography on RP 18 in water/methanol (9:1).

From the mycelia. Mycelia were washed with water and extracted with methanol for 4 h. The extracts were concentrated to dryness. After complexing with ferric chloride, the siderophores were identified by TLC or electrophoresis in 0.1 M ammonium acetate buffer pH 5.0 (Diekmann and Zähner 1967).

Isolation of the iron-free form of palmitoylcoprogen. The methanolic extract from the mycelia was evaporated to dryness. The oily residue was suspended in water and extracted twice with ethyl acetate. The aqueous phase contained deferri-ferricrocin and deferri-coprogen B. The combined organic phases were evaporated, and the oily residue was, after washing with *n*-heptane, dissolved in methanol and subjected to chromatography on Sephadex LH 20 in methanol. Fractions containing the siderophore were detected with $FeCl_3$ and combined. Final purification was achived by HPLC on Lichrogel PS 1 in 2-propanol.

Identification of the siderophores. TLC plates coated with silica gel (Merck) or cellulose F (Merck) were used with the following solvent systems: L1, chloroform/methanol/H_2O (70:25:4); L2, dichloromethane/methanol/H_2O (70:25:4); L3, 1-propoanol/acetic acid/H_2O (4:1:1); L4, 1-butanol/1-propanol/H_2O (6:3:5); L5, 1-butanol/acetic acid/H_2O (4:1:5); L6, 1-propanol/25% ammonium hydroxide (100:24).

Identification of 5-hydroxy-3-methyl-2-pentenoic acids, amino acids and plamitic acid. The removal of the iron from the siderophores and the hydrolysis of the desferri compounds were carried out as described before (Anke and Diekmann 1971). For palmitoylcoprogen, this procedure did not work; therefore the iron-free compound was isolated and subjected to hydrolysis (6 M HCl, 20 h, 100° C). The hydrolysate was extracted with ethyl acetate and the organic phase was concentrated and applied onto silica gel TLC plates. 3-Methyl-2-penteno-5-lactone was identified as described before (Anke 1977). Amino acids were identified by HPLC according to Arnoldi (1990). In order to identify the fatty acid part of the compound, the hydrolysate was subjected to GC/MS analysis. Considerable amounts of palmitic acid (M^+ 256) were indicated; comparison of the spectrum with that of an authentic sample confirmed this. A search for $m/z=60$ (which is typical for fatty acids) in the GC/MS chromatogram showed that no other fatty acids were present in the hydrolysate in comparable amounts.

Spectroscopic measurements. NMR experiments were performed with a Varian XL-300 spectrometer operating at 300 MHz for proton and at 75 MHz for ^{13}C. Palmitoylcoprogen (10 mg) was dissolved in 0.75 ml CD_3OD or $(CD_3)_2SO$ and all spectra were run at 25° C. Positive FAB spectra were performed with a Jeol MS-SX 102 spectrometer calibrated with poly(ethyleneglycol).

Table 1. Effect of $ZnSO_4$ on the production of siderophores by three strains of the genus *Trichoderma* in asparagine medium after 18 days

$ZnSO_4$ (μM)	Siderophore production (g/l) by strain		
	HA 35-84	HA 113-84	HA 34-88
0	0.106	0.090	0.041
20	0.881	1.209	0.260
40	1.189	1.689	0.302
60	1.115	1.564	0.300
80	1.150	1.663	0.268

Table 2. Growth of nine strains of the genus *Trichoderma* and excretion of siderophores into the culture broth

Strain	Incubation time (days)	Mycelia (g/l)	Siderophores (mg/g mycelia)	Antagonistic properties
HA 35-84	28	3.9	270	+++
HA 34-88	13	5.6	48	+++
HA 113-84	30	3.8	550	+++
T. hamatum	19	7.0	93	+++
T. harzianum	30	3.2	140	+
T. viride	30	1.8	330	++
T. koningii	14	5.4	83	++
T. longibrachiatum	30	4.0	160	+++
T. pseudokoningii	18	4.3	245	+

Antagonistic properties were measured towards *Pythium ultimum* on agar plates

Results and discussion

Production of siderophores

When grown under iron limitation, all strains excreted siderophores into the culture medium. Addition of $ZnSO_4$ drastically increased the production; the highest yields were obtained at 40 μM $ZnSO_4$ (Table 1). Similar effects have been reported for *Azotobacter vinelandii* and other bacteria (Chakrabarty and Roy 1964; Huyer and Page 1988).

Depending on the strain, the production of siderophores, the mycelial mass and the fermentation time varied considerably. The cultures were harvested when the carbon source (glucose) was used up. At the same time, the concentration of siderophores in the culture filtrates had reached its highest level. The results are summarized in Table 2. The mycelial mass varied over 1.8–7.0 g/l and the siderophore production ranged over 270–2080 mg/l culture. The ability to overgrow and parasitize *Pythium ultimum* in agar cultures did not correlate with the production of siderophores. Compared to other fungi, the yields of siderophores from all *Trichoderma* species were rather high. *Botrytis cinerea*, a phytopathogen, was reported to excrete 30 mg siderophores/l (Konetschny-Rapp et al. 1988b). In a survey of 14 species of the *Aspergillus fumigatus* group, six species produced less than 100 mg/l, none produced more than 1000 mg/l (Diekmann and Krezdorn 1975).

Table 3. Composition of the siderophore mixture excreted into the culture broth

Strain	Siderophore (g/l)	Coprogen %	Coprogen B %	Ferricrocin %	Fusigen %
HA 35-84	1.05	≤10	≥90	≤10	0
HA 34-88	0.27	≤10	≥90	≤10	0
HA 113-84	2.08	≤10	≥90	≤10	0
T. hamatum	0.65	≤ 5	≥95	≤ 5	0
T. harzianum	0.45	≤ 5	≥95	≤ 5	0
T. viride	0.75	≤10	≥90	≤10	0
T. koningii	0.45	≤ 5	≥95	≤ 5	0
T. longibrachiatum	0.65	≤10	45	≤10	45
T. pseudokoningii	1.05	≤10	30	≤10	55

Isolation and characterization of the siderophores from the culture filtrate

The composition of the siderophore mixture from the culture medium of nine different strains is given in Table 3. Coprogen B was the main component of all strains with ferricrocin and coprogen being minor components. *T. longibrachiatum* and *T. pseudokoningii* produced siderophores of the fusigen type, in addition to ferricrocin and coprogen B. Simultanous production of siderophores of three different types as in these latter two species has not been observed before. Usually only one or two types can be found: *Neurospora crassa* produces chelators of the ferrichrome and coprogen type, *Gibberella fujikuroi* excretes only fusigen (Winkelmann 1986; Winkelmann and Huschka 1987) and *Aspergillus deflectus* triacetylfusigen (Anke 1977). According to their morphological features, *T. longibrachiatum* and *T. pseudokoningii* were assigned to a new section in the genus *Trichoderma* by Bissett (1984). This segregation is underlined by the differences in the production of siderophores.

Isolation and characterization of the siderophores from the mycelia

In the dark-yellow-coloured methanolic extracts from *T. longibrachiatum* and *T. pseudokoningii* no siderophores could be detected. The production of yellow pigments by these two species is another feature used by Bissett (1984) to separate them from the other species defined by Rifai (1969). In the extracts of *T. harzianum, T. hamatum, T. koningii, T. viride, Trichoderma* sp. HA 35-84, HA 113-84, and HA 34-88 coprogen B, ferricrocin and a new lipophilic siderophore were detected. *Trichoderma* sp. HA 35-84 was the best producer of this new compound. The isolation scheme of the new siderophore from strains HA 35-84 and HA 34-88 is depicted in Fig. 1. Palmitoylcoprogen is the first fungal siderophore which is not excreted into the culture broth but is found solely in the cells. With the exception of mycobactins and nocobactins from bacteria (Snow 1970; Ratledge and Patel 1976), all siderophores identified so far are easily soluble in water.

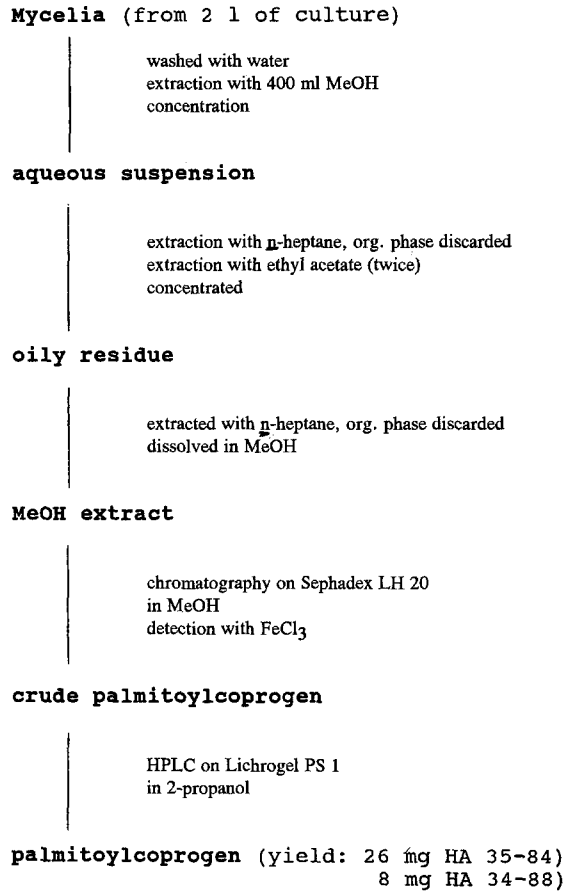

Fig. 1. Isolation of palmitoylcoprogen

In addition to the chemical degradation which yielded 5-hydroxy-3-methyl-2-pentenoic acids, *N*-$^{\delta}$hydroxyornithine and palmitic acid, the structure of palmitoylcoprogen was also determined by extensive NMR and mass-spectroscopic investigations. The relative molecular mass of the iron-free compound is 964, and weak signals for both M+H (965) and M+Na (987) could be detected by positive-ion FAB mass spectroscopy with glycerol as a matrix. ^{1}H-NMR spectra of coprogen in the same solvents were used for comparison, all comparable proton chemical shifts and multiplicities (given below for palmitoylcoprogen) were in agreement, and the presence of acidic protons in both samples was confirmed by spectra run in $(CD_3)_2SO$. The palmitic acid moiety was identified through proton couplings, outlined by two-dimensional correlation spectroscopy (COSY). The structure of the vinylic part of the molecule was confirmed by selective decoupling of the vinylic protons and by a two-dimensional nuclear Overhauser experiment (NOESY). The identical ^{1}H-NMR chemical shift for H-2″ in both coprogen which has an acetyl group on N(3″) and palmitoylcoprogen indicates that the fatty acid is positioned on N(3″). Chemical shifts for ^{13}C (given below for palmitoylcoprogen) could be obtained from one-dimensional ^{13}C spectra combined with heteronuclear two-dimensional correlation spectroscopic experiments (HET-

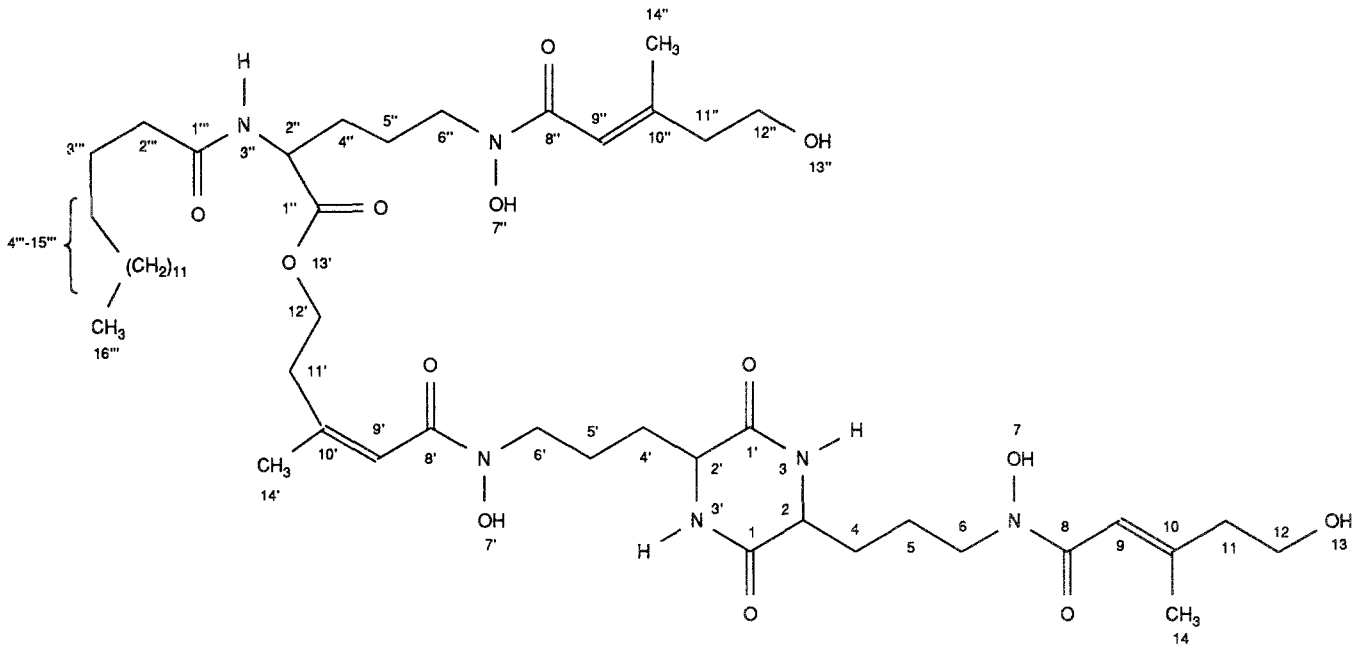

Fig. 2. Structure of desferripalmitoylcoprogen

COR) for assignment of shifts. The structure is shown in Fig. 2.

Analysis of palmitoylcoprogen ($C_{49}H_{84}O_{13}N_6$)

NMR (300 MHz, CD_3OD, 25° C, δ relative to tetramethylsilane): ^1H δ: 6.3 (br.s, 3 H, H-9, H-9′, H-9″), 4.37 (br.m, 1 H, H-2″), 4.30 (br.m, 2 H, H-12′), 4.02 (br.m, 2-H, H-2, H-2′), 3.71 (t, $J=6.6$ Hz, 4 H, H-12, H-12″), 3.66 (br.m, 6-H, H-6, H-6′, H-6″), 2.50 (t, $J=6.3$ Hz, 2-H, H-11′), 2.37 (t, $J=6.4$ Hz, 4 H, H-11, H-11″), 2.24 (t, $J=7.3$ Hz, 2 H, H-2‴), 2.08 (br.s, 9 H, H-14, H-14′, H-14″), 1.9–1.8 (br.m, H-4, H-4′), 1.8–1.7 (br.m, H-4″), 1.75–1.65 (br.m, H-5, H-5′, H-5″), 1.65–1.55 (br.m, H-3‴), 1.32 (br.s, 24 H, H-4‴ to H-15‴) and 0.90 (t, $J=6.4$ Hz, 3 H, H-16‴). ^{13}C δ: 176.6, 173.5, 170.4, 169.6, 169.3 (s, carbonyl-C), 152.7 (s, C-10, C-10″), 151.2 (s, C-10′), 118.5 (d C-9′), 117.9 (d, C-9, C-9″), 63.7 (t, C-12′), 60.9 (t, C-12, C-12″), 55.7 (d, C-2, C-2′), 53.7 (d, C-2″), 49.3 (t, C-6, C-6′, C-6″), 44.8 (t, C-11, C-11″), 40.6 (t, C-11′), 36.7 (t, C-2‴), 33.1 (t, C-14‴), 32.4 (t, C-4, C-4′), 32.2, 30.8, 30.7, 30.5, 30.4, 30.3 (C-4‴ to C-13‴), 29.5 (t, C-4″), 27.0 (C-3‴), 24.6 (t, C-5″), 23.7 (t, C-15‴), 23.5 (t, C-5, C-5′), 18.9, 18.8 (q, C-14, C-14′, C-14″) and 14.5 (q, C-16‴).

Ultraviolet spectrum (EtOH): $\lambda_{max}=215$ nm, $\log\varepsilon=4.51$, very weak shoulder at 250 nm, the iron complex has a broad maximum at 440 nm.

The deferri compound is almost insoluble in water. It is easily soluble in MeOH, acetone or ethyl acetate.

Biological properties of palmitoylcoprogen

Unlike desferritriacetylfusigen, desferripalmitoylcoprogen did not exhibit antibacterial, antifungal or algicidal activity, indicating that iron in the form of palmitoylcoprogen is available to all organisms tested. When assayed according to Anke (1977) the growth of bacteria (*Bacillus brevis, B. subtilis, Staphylococcus aureus,* and *Proteus vulgaris*) and fungi (*Paecilomyces varioti, Curvularia lunata, Candida albicans* and *Saccharomyces cerevisiae*) was inhibited by desferritriacetylfusigen, but not by desferripalmitoylcoprogen (100–200 mg/l). At concentrations up to 50 µg/ml, no cytotoxic activity towards HeLa S3 or Ehrlich ascitic carcinoma cells was observed. Concentrations of desferripalmitoylcoprogen ≥ 100 µg/ml caused lysis of HeLa S3 cells. Addition of palmitoylcoprogen to peas infected with *Pythium ultimum* had no effect on the pathogen, whereas the addition of *Trichoderma* spp. HA 34-88, HA 113-84, HA 35-84, *T. hamatum* and *T. longibrachiatum* into the soil protected the seedlings. Thus the strains with the highest and lowest production rate of siderophores as well as a nonproducer of palmitoylcoprogen are equally effective in the biological control of damping-off, indicating that the antagonistic properties of *Trichoderma* strains are not correlated to the type or the amount of siderophores produced. The beneficial effects of fungal siderophores on plants and the role of palmitoylcoprogen in the transport of iron into fungal cells remain to be investigated.

The localization and the structure of this chelator make it a likely candidate for the taxi model for iron transport (Winkelmann 1986) or as iron acceptor and storage compound within the cells.

Acknowledgements. We thank Drs D. Mangold and E.-H. Pommer (BASF AG, Ludwigshafen) for financial support. The help of Dr R. Böker with the HPLC is gratefully acknowledged. We thank A. Helfer for expert technical assistance.

References

Anke H (1977) Desferritriacetylfusigen, and antibiotic from *Aspergillus deflectus*. J Antibiot 30:125–128

Anke H, Diekmann H (1971) Biosynthesis of sideramines in fungi: mevalonate as a precursor of *cis*- and *trans*-5-hydroxy-3-methyl-2-pentenoic acids. FEBS Lett 17:115–117

Arnoldi E (1990) HPLC-Analytik und Fermentationsanalyse des Pilzes *Cyathus striatus*. Dissertation thesis, University of Kaiserslautern

Bissett J (1984) A revision of the genus *Trichoderma*. I. Section *longibrachiatum* sect. nov. Can J Bot 62:924–931

Chakrabarty AM, Roy SC (1964) Effect of trace elements on the production of pigments by a pseudomonad. Biochem J 93:229–231

Chet I (1987) *Trichoderma* – application, mode of action, and potential as a biocontrol agent of soil-borne plant pathogenic fungi. In: Chet I (ed) Innovative approaches of plant disease control. John Wiley, New York, pp 137–160

Cook RS, Baker KF (1983) The nature and practice of biological control of plant pathogens. American Phytopathological Society, St. Paul MN, 539 pp

Diekmann H, Krezdorn E (1975) Ferricrocin, Triacetylfusigen und andere Sideramine aus Pilzen der Gattung *Aspergillus*, Gruppe *Fumigatus*. Arch Microbiol 105:191–194

Diekmann H, Zähner H (1967) Konstitution von Fusigen und dessen Abbau zu Δ^2-Anhydromevalonsäure. Eur J Biochem 3:213–218

Huyer M, Page WJ (1988) Zn^{2+} increases siderophore production in *Azotobacter vinelandii*. Appl Environ Microbiol 54:2625–2631

Kloepper JW, Leong J, Teintze M, Schroth MN (1980a) Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286:885–886

Kloepper JW, Leong J, Teintze M, Schroth MN (1980b) *Pseudomonas* siderophores: a mechanism explaining disease-suppressive soils. Curr Microbiol 4:317–320

Konetschny-Rapp S, Huschka HG, Winkelmann G, Jung G (1988a) High-performance liquid chromatography of siderophores from fungi. Biol Metals 1:9–17

Konetschny-Rapp S, Jung G, Huschka HG, Winkelmann G (1988b) Isolation and identification of the principal siderophore of the plant pathogenic fungus *Botrytis cinerea*. Biol Metals 1:90–98

Leonhardt K, Ankie T, Hillen-Maske E, Steglich W (1987) 6-Methyl-9-β-D-ribofuranosylpurine and 6-hydroxymethyl-9-β-D-ribofuranosylpurine as antiviral metabolites of *Collybia maculata* (Basidiomycetes). Z Naturforsch 42c:420–424

Neilands JB (1981) Microbial iron compounds. Annu Rev Biochem 50:715–731

Neilands JB (1984) Siderophores of bacteria and fungi. Microbiol Sci 1:9–14

Neilands JB, Leong SA (1986) Siderophores in relation to plant growth and disease. Annu Rev Plant Physiol 37:187–208

Ratledge C, Patel PV (1976) The isolation, properties and taxonomic relevance of lipid-soluble, iron-binding compounds (the nocobactins) from nocardia. J Gen Microbiol 93:141–152

Rifai MA (1969) A revision of the *Trichoderma*. Mycol Papers 116:1–56

Schippers B, Lugtenberg B, Weisbeck PJ (1987) Plant growth control by fluorescent pseudomonads. In: Chet I (ed) Innovative approaches to plant disease control. John Wiley, New York, pp 19–39

Sneh B, Dupler M, Elad Y, Baker R (1984) Chlamydospore germination of *Fusarium oxysporum* f. sp. *cucumerinum* as affected by fluorescent and lytic bacteria from *Fusarium*-suppressive soil. Phytopathology 74:1115–1124

Snow GA (1970) Mycobactins: iron-chelating growth factors from *Mycobacteria*. Bacteriol Rev 34:99–125

Winkelmann G (1986) Iron complex products (siderophores). In: Pape H, Rehm HJ (eds) Biotechnology, vol. 4, VCH, Weinheim, pp 215–243

Winkelmann G, Huschka HG (1987) Molecular recognition and transport of siderophores. In: Winkelmann G, van der Helm D, Neilands JB (eds) Iron transport in microbes, plants and animals. VCH, Weinheim, pp 3–33