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Ornibactin production and transport properties in strains of *Burkholderia vietnamiensis* and *Burkholderia cepacia* (formerly *Pseudomonas cepacia*)

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Several strains of *Burkholderia vietnamiensis*, isolated from the rhizosphere of rice plants, and four strains formerly known as *Pseudomonas cepacia* including two collection strains and two clinical isolates were compared for siderophore production and iron uptake. The *B. vietnamiensis* (TVV strains) as well as the *B. cepacia* strains (ATCC 25416 and ATCC 17759) and the clinical isolates K132 and LMG 6999 were all found to produce ornibactins under iron starvation. The two ATCC strains of *B. cepacia* additionally produced the previously described siderophores, pyochelin and cepabactin. Analysis of the ratio of isolated ornibactins (C4, C6 and C8) by HPLC revealed nearly identical profiles. Supplementation of the production medium with ornithine (20 mM) resulted in a 2.5-fold increase in ornibactin synthesis. Ornibactin-mediated iron uptake was independent of the length of the acyl side chain and was observed with all strains of *B. vietnamiensis* and *B. cepacia*, but was absent with strains of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Pseudomonas stutzeri*, known to produce pyoverdines or desferriferrioxamines as siderophores. These results suggest that ornibactin production is a common feature of all *Burkholderia* strains and that these strains develop an ornibactin-specific iron transport system which is distinct from the pyoverdine-specific transport in *Pseudomonas* strains.

Keywords: *Burkholderia*, *Pseudomonas*, ornibactin, siderophore, iron metabolism

Introduction

Ornibactins are iron-chelating compounds synthesized by the bacterial strain TVV69, a nitrogen-fixing bacterium isolated from rice rhizosphere and first identified as *Pseudomonas cepacia* (Trân Van 1994). The chemical structures of the ornibactins have been recently defined as hydroxamate/hydroxycarboxylate peptides (Stephan *et al.* 1983a,b) and are composed of the conserved tetrapeptide $^{\delta}$N-OH-Orn-βOH-Asp-Ser-$^{\delta}$N-OH-Orn harboring a 1–4 diaminobutane (putrescine) residue and an acyl chain of 3-hydroxybutanoic acid, 3-hydroxyhexanoic acid or 3-hydroxyoctanoic acid. This microheterogeneity in the acyl chain results in three different molecular species, the C4-, C6- and C8-ornibactins, which are together excreted by strain TVV69 grown under iron deficiency. Although having some relationship with the pyoverdines of the fluorescent *Pseudomonas* in their peptidic nature, ornibactins by their lack of a chromophore and their ornithine content form a new family of microbial iron-chelating compounds (see Winkelmann 1991 for a compilation of siderophores).

In this paper we investigated the expected siderophore function of ornibactins for the producing strain. Moreover, we checked for ornibactin production by some related strains, among them a collection of natural isolates of identical origin as TVV69 (TVV strains). The TVV strains, together with strain TVV69, have demonstrated interesting biological properties such as antagonistic activity against several fungi such as *Rhizoctonia*, *Helminthosporium*, *Fusarium* and *Pythium* (Trân Van 1994), or, as also demonstrated for at least one strain, plant growth promotion activity (Tabacchioni *et al.* 1993, Trân Van 1994). Because all these strains appeared to be closely related to the *P. cepacia* group, our search for ornibactins was extended to some *P. cepacia* strains, among them two plant- or soil-related strains and two clinical isolates.

According to a new classification (Yabuuchi *et al.* 1992) most of the pseudomonads belonging to the DNA:DNA,

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DNA:RNA homology group II (Palleroni *et al.* 1973) were renamed '*Burkholderia*', i.e. *Bulkholderia cepacia* instead of *Pseudomonas cepacia*. This new terminology will be used throughout this work. Furthermore, the TVV strains as well as one clinical isolate have recently been proposed to constitute a new species among the *Burkholderia* genus, named *Burkholderia vietnamiensis* (Gillis *et al.* 1995). The comparisons developed in the present study between siderophore production and siderophore-mediated iron uptake capabilities of *B. cepacia* and *B. vietnamiensis* reinforce the distinction of these bacteria into two closely related but different species.

Materials and methods

Bacterial strains

TVV strains (TVV69, 70, 71, 72, 74, 75, 115, 116, 127, 128, 131 and 135) were isolated as nitrogen-fixing bacteria associated with the rhizosphere of rice growing in an acidic soil in the region of Binh Thanh (Vietnam). The API (Appareil et Procédés d'Identification, bioMérieux, Marcy-l'Etoile, France) microtube systems API50CH (49 carbohydrates), API50AA (49 amino acids and related compounds) and API50OA (49 organic acids) were used according to the manufacturer's instructions for bacterial strain distinction.

For comparison purposes, the two *B. cepacia* strains ATCC 25416 (type strain) isolated from onion and ATCC 17759 isolated from forest soil were included in this study, as well as two clinical isolates, strain K132, kindly provided by K. Poole and identified as a *B. cepacia* strain by API20NE, and strain LMG 6999, which was isolated from a child's neck abscess in Sweden. Recently, a polyphasic approach including DNA–DNA hybridization, DNA–rRNA hybridization and auxanographic analysis was used by Gillis *et al.* (1995) to more precisely define the internal structure of the former [*Pseudomonas*] rRNA group II (Palleroni *et al.* 1973) containing the *Burkholderia* genus. The data clearly showed that the TVV strains and the clinical, N_2-fixing isolate LMG 6999 were related to *B. cepacia* species but differed from it by a very low DNA–DNA homology ranging from 28 to 48%. A new species was thus created, containing all the N_2-fixing strains of this rRNA group and named *B. vietnamiensis*. The precise taxonomic position of the other clinical isolate (strain K132) remains unknown as its identification was performed with the API20NE microtube system which does not allow a distinction between the *B. cepacia* and the *B. vietnamiensis* species. *Pseudomonas aeruginosa* ATCC 15692, *P. aeruginosa* ATCC 27853, *Pseudomonas fluorescens* ATCC 13525, *P. fluorescens* CCM 2798 and *Pseudomonas stutzeri* ATCC 17588 were also included in iron uptake studies.

Bacterial growth and siderophore detection

Bacterial growth in liquid medium was turbidimetrically followed by directly measuring the OD at 650 nm on a Lumetron colorimeter (Photovolt, New York, NY) for cultures prepared in 180 × 18 mm capped tubes, or by measuring the OD at 650 nm of 1 ml portions of growth medium using an Uvikon-930 spectrophotometer (Kontron Instruments, Montigny-le Bretonneux, France) for cultures done in 1 l Erlenmeyer flasks containing 500 ml medium.

Siderophores were detected by growing the bacteria on the Chrome-Axurol-S (CAS)-agar medium according to Schwyn & Neilands (1987) or directly from liquid growth cultures (succinate medium, Meyer & Abdallah 1978) by mixing 5 ml of growth medium with 5 µl $FeCl_3$ (2 M). After 30 min the assays were centrifuged (10 min, 24 000 *g*) and the absorbance of the supernatants read at 400 nm. Quantification of ferric ornibactins was based on a molar absorption coefficient of 1267 $mM^{-1}\,cm^{-1}$ at 392 nm, as determined from a HPLC-purified sample of C8-ornibactin dissolved in distilled water.

Where indicated, succinate medium was supplemented with sterile $FeCl_3$ (20 mM) solution or with filter-sterilized ornithine (1 mM), pH 7.0, after autoclaving. Ethylenediamine di(hydroxyphenylacetic acid) (EDDHA, 100 or 1000 $mg\,l^{-1}$, final concentration) was added to the succinate medium solidified with agar (20 $g\,l^{-1}$) as previously described (Hohnadel & Meyer 1988).

Siderophore production and purification

Siderophores were produced by growing the bacteria at 30 C on a rotary shaker (200 r.p.m.) in succinate medium (Meyer & Abdallah 1978) supplemented with ornithine (10 mM). When growth had reached the stationary phase (40 h culture) the cells were removed by centrifugation and siderophores isolated from the supernatants. Two procedures were used: (1) a chloroform (or ethyl acetate) extraction of the acidified (pH 2–3) culture supernatant followed by a chromatography on Sephadex LH-20 of the extract dissolved in methanol as described previously for the purification of pyochelin and cepabactin of *B. cepacia* ATCC 25416 (Meyer *et al.* 1989), and (2) the procedure successfully used for the purification of ornibactins of strain TVV69 (Stephan *et al.* 1993a), based on a chloroform–phenol extraction of the siderophore–iron complexes from the culture supernatants. After supplementation with an excess of iron (1 ml $FeCl_3$ (2 M) per liter of supernatant], the culture medium was concentrated under vacuo, saturated with NaCl and treated with one third in volume of chloroform–phenol (1/1, v/w). The ferrisiderophores were then solubilized in the aqueous phase after adding two volumes of diethyl ether and water (100 ml) to the chloroform–phenol solution.

Iron-free ornibactins were obtained from the purified ferric ornibactins by the 8-hydroxyquinoline method according to Wiebe & Winkelmann (1975). Ornibactins were also isolated directly from the spent medium by a repeated methanol extraction: the spent medium was concentrated to dryness under vacuum and the dry residue was repeatedly extracted with 10 ml methanol portions until no more $FeCl_3$-reacting material was detectable in the methanolic extract. The pooled methanolic portions were treated with anhydrous sodium sulfate and concentrated to dryness after filtration.

The dry residue was suspended in methanol and further purified on a Sephadex LH-20 column (35 × 1.5 cm) with methanol as an eluting solvent. The iron-reacting material eluted in a single peak containing the three ornibactins (C4, C6 and C8), as analyzed by HPLC. Pyoverdine from *P. aeruginosa* ATCC 15692, desferriferrioxamine E from *P. stutzeri* ATCC 17588, cepabactin and pyochelin from *B. cepacia* ATCC 25416, were purified according to published procedures (Meyer & Abdallah 1978, 1980, Meyer *et al.* 1989). Desferriferrioxamine B was used as the commercially available form, Desferal® (Ciba-Geigy, Switzerland).

HPLC separation of ornibactins

Ferric ornibactins were separated by HPLC (Shimadzu, Duisburg, Germany) using a system described previously (Stephan *et al.* 1993a). Isolated ornibactin mixtures were separated on a reversed-phase column (Nucleosil C18; 5 μm, 4.6 × 250 mm; Grom, Herrenberg, Germany) using a gradient (6–40% within 20 min) of acetonitrile (containing 0.08% trifluoroacetic acid) in water (containing 0.12% trifluoroacetic acid) at a flow rate of 1.3 ml min^{-1}. Detector wavelength was 210 nm.

Siderophore-mediated ^{59}Fe uptake studies

Cells harvested from 24 h cultures in succinate medium at 30 C (25 C for *P. fluorescens* strains) were washed twice with distilled water and resuspended at $OD_{600} = 0.3$ in succinate medium without the nitrogen source (incubation medium). An aliquot of this bacterial suspension (9 ml) was incubated in an Erlenmeyer flask at 30 C in a shaking (100 r.p.m.) water bath for 10 min. Then a ^{59}Fe-labeled siderophore solution was added at zero time, containing: 5 μl ^{59}Fe chloride (0.1 mCi ml^{-1}, specific activity 25 mCi mg^{-1}; Amersham, UK), 50 μl siderophore solution (1 mM) in distilled water containing ornibactins, pyoverdines or desferriferrioxamines, or 50 μl of a methanolic solution (3 mM) of cepabactin or pyochelin, and 945 μl of incubation medium added 15 min after mixing of the two solutions. Samples (1 ml) were taken at intervals during the 15 min incubation period and filtered through cellulose nitrate membrane filters (Millipore; 0.45 μm porosity). The filters were washed twice with incubation medium (2 ml) and the radioactivity counted in a Gammamatic 4000 counter (Beckman, Palo Alto, CA). Control experiments without bacteria verified that the labeled iron was fully solubilized under the conditions used.

Results

Ornibactin production as a function of iron concentration in the growth medium

Cultures of strain TVV69 reached the stationary phase of growth in succinate medium after about 35 h under the conditions used. Supplementation of the medium with iron ($FeCl_3$, 100 μM final concentration) resulted in increased growth rate and cell yield: the stationary phase was reached after 24 h of growth and the cell yield, as measured by the OD at 650 nm, was two to three times higher compared with the one in succinate medium, demonstrating that cells grown in succinate medium remained iron deficient. The high sensitivity of TVV69 to iron deprivation was also suggested by growth experiments in the presence of the iron chelator EDDHA. The bacterial growth on a succinate–agar medium supplemented with EDDHA (100 μg ml^{-1}) was strongly inhibited as very small colonies appeared with a 2 day delay compared with growth on unsupplemented medium. Full inhibition was observed at a concentration of 1000 μg ml^{-1} and EDDHA.

Production of ornibactins in growth medium was followed colorimetrically as described in Materials and methods. The orange color which developed immediately upon addition of an excess of iron to the culture medium was quantified by measuring the absorbance of the supernatant at 400 nm after centrifugation. This absorbance increased progressively during the exponential growth in succinate medium and reached a maximal value when the culture entered the stationary phase. Since ornithine supplementation of the growth medium increased the production of ornibactins (see below), the production of ornibactins in the growth medium was followed in ornithine-supplemented (10 mM) succinate medium as a function of the iron concentration. As shown in Figure 1, the amount of ornibactins produced during growth of strain TVV69 was maximal at 4 μM added-iron concentration, whereas no production of ornibactins was detectable when the concentration of iron in the growth medium reached 15 μM. At that concentration iron was no longer a growth limiting factor, as indicated by the curve in Figure 1 showing the maximal cell yield versus iron concentration in the growth medium. The amount of ornibactins produced per unit (OD) of cells decreased with increasing iron concentration of the growth medium. This clearly demonstrates that the ornibactin biosynthesis is iron-regulated and characteristic of iron-starved cells.

Ornibactin production as a function of ornithine concentration in the growth medium

From the ornibactin structures it is evident (Stephan *et al.* 1993a,b) that these compounds are particularly rich in ornithine and ornithine-derived residues (Figure 2). Therefore, the influence of ornithine supplementation of the growth medium on the production of ornibactins was checked. As shown in Figure 3, ornithine supplementation at different concentrations (0–20 mM) resulted in significant stimulation of ornibactin production by strain TVV69, which reached a maximum level (2.6-fold) at 10 mM ornithine supplementation. Bacterial growth was also slightly increased with this supplementation, but not in a sufficient manner to explain the increase in ornibactin production as shown by the curve on Figure 3 representing the amount of ornibactin produced per unit (OD) of cells. Other amino acids, at a 10 mM final concentration in the growth medium,

were checked for a potential effect on ornibactin production. Among them, proline and arginine well stimulated the siderophore biosynthesis, although to a slightly lesser extent than ornithine (2.2- and 2.0-fold, respectively, instead of 2.6-fold for ornithine). The other amino acids tested (alanine, serine, valine, aspartic acid, lysine and its decarboxylated derivative cadaverine) had a slight stimulating effect (average of 1.3-fold), whereas putrescine, one of the components of ornibactins, had no apparent effect.

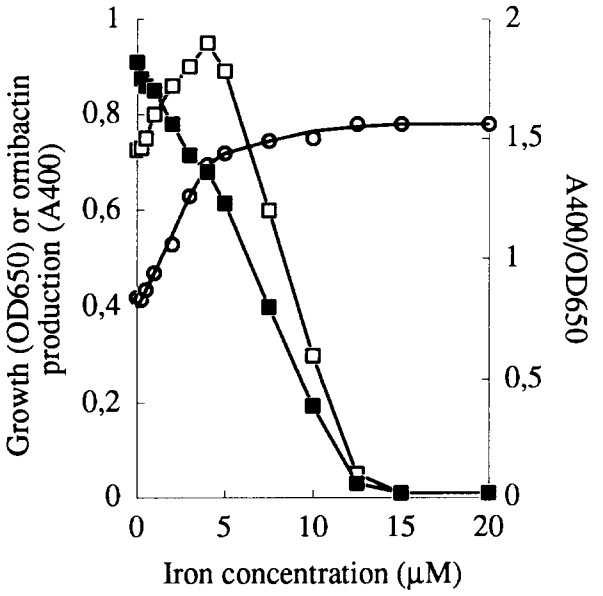

Figure 1. Ornibactin production and growth of *B. vietnamiensis* TVV69 as a function of iron concentration in succinate medium supplemented with ornithine (10 mM). Growth (○) was measured turbidimetrically (OD_{650}) at the plateau. Culture supernatants (7.5 ml) were supplemented with iron (5 μl, $FeCl_3$ 2 M) and the absorbance of the supernatants read after 5 h at 400 nm (□). Specific ornibactin biosynthesis activity expressed by the ratio A_{400}/OD_{650} is also shown (■).

Ornibactin production by other B. vietnamiensis *TVV strains*

Beside TVV69, 11 other nitrogen-fixing bacterial strains were isolated from a rice rhizosphere, all belonging to the *B. vietnamiensis* species (Gillis *et al.* 1995). They differed, however, one from another by some morphological and biochemical variations. For instance, strain TVV71 appeared as very motile, rod-shaped bacteria, usually associated in short chains of two to 10 cells, whereas the other strains were single, motile rods. Some differences also appeared when using the extended auxanographic tests API 50CH, 50 OA and 50 AA. As shown in Table 1, each TVV strain was distinct by usually more than one auxanographic character.

When tested for the production of siderophores, all the

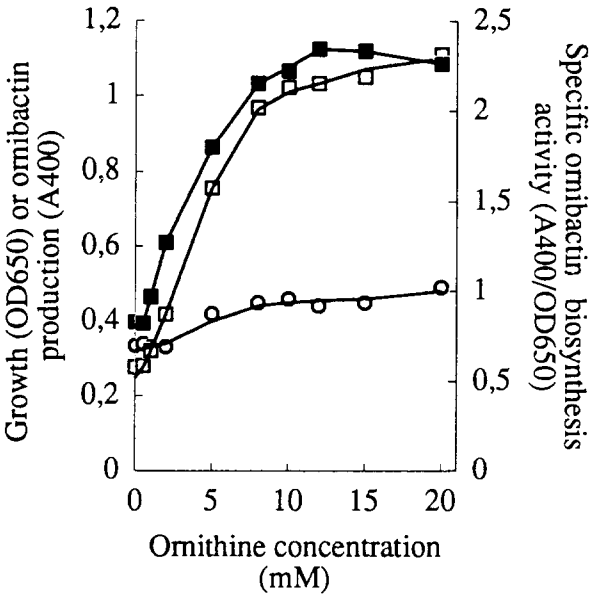

Figure 3. Growth of *B. vietnamiensis* TVV69 and ornibactin production as a function of ornithine concentration in succinate medium. Experimental protocol and symbols are the same as in Figure 1.

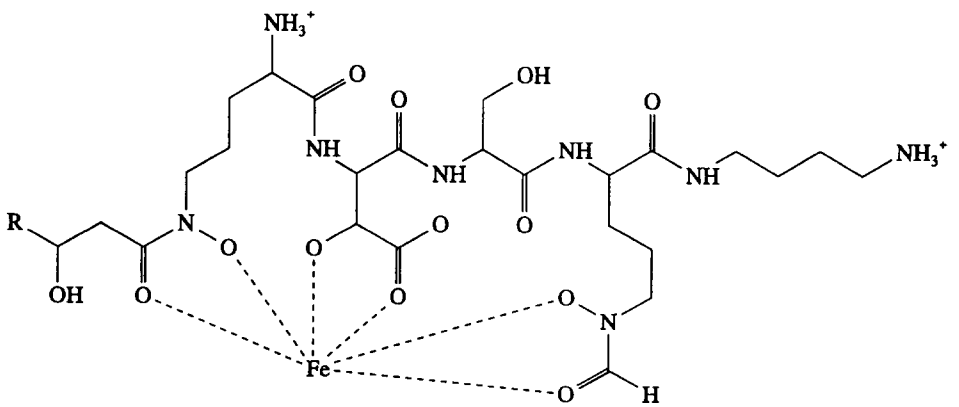

Figure 2. Structure of the iron–ornibactin complex. Ornibactin C4: R = methyl. Ornibactin C6: R = propyl. Ornibactin C8: R = pentyl.

Table 1. Biochemical characteristics of *B. vietnamiensis* and *B. cepacia*

Substrate	*B. vietnamiensis*												*B. cepacia*		
	TVV												LMG	ATCC	
	69	70	71	72	74	75	115	116	127	128	131	135	6999	25416	17759
API50CH															
D-ribose	−	+	+	±	+	−	+	+	+	+	+	+	−	+	+
lactose		±	±	−	−	−	+	−	−	−	±	−	−	±	−
maltose	−	−	−	−	−	−	+	−	±	−	−	−	−	−	−
D-raffinose	±	−	±	±	±	±	+	±	±	±	+	+	−	+	±
D-arabinose	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
rhamnose	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
α-Me-D-man	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
α-Me-D-gluc				−	−	−	+	−	−	−	−	−	−	−	−
D-melibiose	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+
starch	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
D-turanose	−	−	−	−	−	−	+	−	−	−	±	−	−	−	−
amygdaline	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+
arbutin	+	+	±	+	+	+	+	+	+	±	+	+	−	+	+
salicin	±	±	±	+	+	+	+	±	+	±	±	+	−	+	+
API50OA															
malonate	−	±	−	−	−	−	−	±	−	±	±	±	±	+	+
mesotartrate	−	±	±	−	−	−	±	−	−	−	±	−	−	+	±
2-ketoglutarate	−	+	±	+	±	±	±	±	±	±	+	±	−	−	+
suberate	−	±	±	±	±	−	−	±	±	±	+	±	±	+	+
pimelate	−	−	−	−	−	−	−	−	−	±	±	−	−	+	+
itaconate	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
API50AA															
creatine	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
spermine	−	−	+	−	−	−	−	+	−	+	−	−	−	−	−
amylamine	−	+	+	+	+	+	+	+	+	+	+	+	±	+	+
DL-norvaline	−	±	±	−	−	−	+	±	±	±	±	−	±	−	+
L-citrulline	−	±	±	±	−	±	±	±	−	−	±	−	−	+	+
DL-3-am-but	−	+	−	−	−	±	±	−	−	−	±	−	−	+	+
DL-2-am-but	−	±	−	±	+	±	+	±	+	±	±	−	+	+	+

Symbols: +, good growth; ±, slight growth; −, no growth. Only discriminating substrates are listed.

TVV strains reacted positively on CAS-agar and showed an identical orange color when the supernatant was supplemented with iron, as was shown for the ornibactin-producer strain TVV69. No siderophores were extractable by chloroform or ethyl acetate from pH 3-acidified growth supernatants, including TVV69, whereas the chloroform phenol extraction procedure resulted in iron-containing, orange material. HPLC analysis of siderophores resulted in an identical ornibactin profile as shown for TVV69 (Figure 4), demonstrating that all these strains were ornibactin producers. Differences in the total amount of ornibactins produced by the different TVV strains were observed, as estimated by the ferric ornibactins absorbance in the respective growth supernatants (Table 2). HPLC profiles also revealed some differences in the ratio of the C4-, C6- and C8-ornibactins produced. In all cases, C8-ornibactin was the predominant siderophore, representing about 40–70% of the total amount of ornibactins, followed by the C6 compound (26–43%). Except for few strains (TVV74, TVV115 and TVV135), the amount of C4-ornibactin represented usually less than 10% of the total ornibactins production (Table 2).

All the TVV strains behaved the same as TVV69, i.e. ornibactin production was a function of the iron content of the growth medium (data not shown) and in all cases a strong stimulation of the ornibactin biosynthesis by ornithine was observed. As shown in Table 2, the stimulation factor of ornibactin production at 20 mM ornithine supplementation varied from 1.9 (strain TVV135) to 3.3 (strain TVV74), with an average value for all the TVV strains of 2.5. The percentage of ornibactins remained unchanged when comparing the production in succinate medium and in ornithine-supplemented succinate medium (data not shown).

Ornibactin production by B. cepacia *strains and by the clinical isolates K132 and LMG 6999*

B. cepacia ATCC 25416, *B. cepacia* ATCC 17759 and the clinical isolates K132 and LMG 6999 were analyzed for their

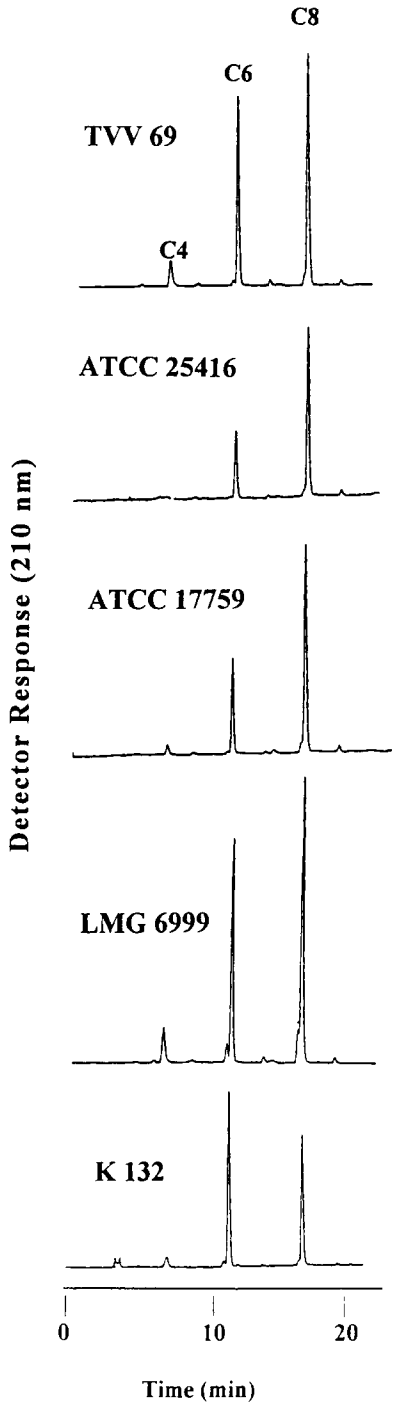

Figure 4. HPLC profiles of isolated ornibactins (C4, C6 and C8) from *B. vietnamiensis* and *B. cepacia* strains.

production of siderophores after growth in succinate medium. The two methods described in Materials and methods were systematically used for comparison with the *B. vietnamiensis* TVV strains.

Chloroform extraction of the pH 3-acidified culture supernatant of *B. cepacia* ATCC 25416, followed by a chromatographic separation in methanol on Sephadex LH-20, led to the isolation of the two already described siderophores cepabactin and pyochelin (Meyer *et al.* 1989). Culture supernatants of *B. cepacia* ATCC 17759 revealed identical results, demonstrating that this strain produced cepabactin and pyochelin as well. From the two clinical strains K132 and LMG 6999, no detectable iron-chelating compounds were extracted by chloroform or ethyl acetate, suggesting that these two strains behaved the same as the TVV strains. The method used for the purification of ornibactins was also successfully applied for strains K132 and LMG 6999. The orange color which formed upon addition of iron to the culture supernatant of these two strains was extractable by chloroform–phenol and was fully released in water after mixing the chloroform–phenol solution with diethyl ether and water. HPLC analysis of the orange material after gel filtration on Sephadex LH-20 revealed a profile which was identical to the one obtained with the TVV strains (Figure 4), demonstrating that the clinical isolates were ornibactin-producing strains. Moreover, culture supernatants of strains *B. cepacia* ATCC 25416 and *B. cepacia* ATCC 17759 extracted by the chloroform–phenol procedure rather surprisingly yielded an iron-chelating material which was identified as ornibactins by HPLC analysis (Figure 4). However, release into water of the chloroform–phenol extracted orange material was never complete, despite repeated attempts. Controls performed with pure cepabactin and pure pyochelin subjected to the same protocol demonstrated that the iron complexes of these two siderophores also could be solubilized in chloroform–phenol but were not subsequently released into water on diethyl ether addition. The amount of ornibactins extracted by the chloroform–phenol procedure from 1 l of culture medium of strain *B. cepacia* ATCC 25416 was 109 mg (dry weight). It thus represents the most important siderophore produced by the bacteria since, for the same volume of culture supernatant treated by the chloroform extraction procedure, only 46 mg of cepabactin and 11 mg of pyochelin were obtained. From these experiments it can be concluded that the two ATCC strains of *B. cepacia* grown under iron starvation produced three siderophores of different chemical structures: cepabactin and pyochelin (already previously recognized by Sokol (1986, Meyer *et al.* 1989) and ornibactins, whereas the two clinical isolates, strain K132 and *B. vietnamiensis* LMG 6999, like the TVV strains, produced ornibactins only.

Ornibactin-mediated iron uptake

HPLC-purified C4-ornibactin, C6-ornibactin and C8-ornibactin were tested for their capacity to facilitate iron transport into iron-starved cells of their producing strain TVV69. As shown in Figure 5, the three compounds mediated iron uptake into the cells with similar efficiency. Other siderophores were tested, among them cepabactin, pyochelin, pyoverdine and desferriferrioxamines. Except for cepabactin, which showed a much less efficient iron uptake as compared to the ornibactins (see Figure 5), all other siderophores tested, including pyochelin, gave negative results.

Table 2. Effect of ornithine supplementation on ornibactin production in strains of *B. vietnamiensis* and *B. cepacia*

Strain	Ornibactin production (mM) in succinate medium supplemented with ornithine at[a]			Percentage of ornibactin[b] produced as		
	0 mM	20 mM	ratio	C4-ornibactin	C6-ornibactin	C8-ornibactin
B. vietnamiensis						
TVV69	0.27	0.66	2.4	2.6	43.2	54.1
TVV70	0.34	0.98	2.8	<1	26.3	72.6
TVV71	0.37	0.84	2.3	<1	27.5	71.3
TVV72	0.32	0.91	2.8	7.6	38.4	53.9
TVV74	0.29	0.98	3.3	17.1	40.6	42.2
TVV75	0.31	0.85	2.7	9.6	40.8	49.5
TVV115	0.43	1.06	2.5	16.4	38.2	45.3
TVV116	0.41	0.85	2.1	6.2	33.9	59.8
TVV127	0.4	0.91	2.3	6.8	34.3	58.8
TVV128	0.36	0.87	2.4	8.1	37.7	54
TVV131	0.3	0.83	2.7	1.2	39.2	59.6
TVV135	0.45	0.84	1.9	19.6	38.5	41.7
LMG6999	0.65	1.14	1.7	8.8	36.8	54.4
B. cepacia						
ATCC 17759	0.30[c]	0.61[c]	2	4.2	28.9	66.8
ATCC 25416	0.45[c]	0.75[c]	1.6	4.5	29.2	66.3
K132	0.83	1.05	1.3	7.2	36.6	56.1

[a] As determined from the absorption at 400 nm of the iron-supplemented culture supernatant using a molar extinction coefficient of 1267 $M^{-1} l^{-1}$ as determined for HPLC-purified C8-ornibactin.
[b] As determined from the HPLC profiles.
[c] Values include cepabactin and pyochelin produced together with ornibactins.

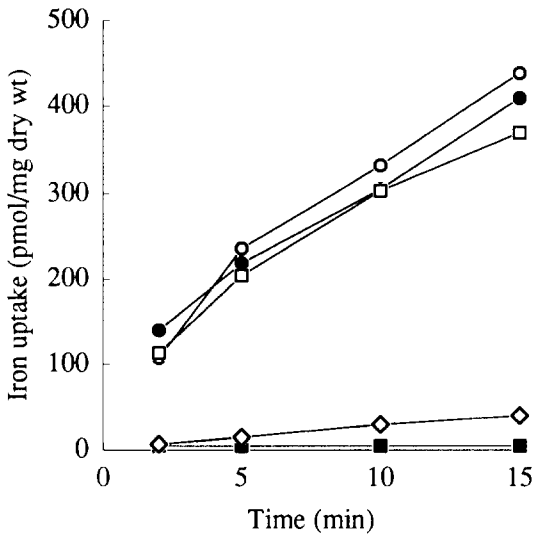

Figure 5. ^{59}Fe incorporation in *B. vietnamiensis* TVV69 mediated by C4-ornibactin (○), C6-ornibactin (●), C8-ornibactin (□), cepabactin (◇) from *B. cepacia* ATCC 25416, pyochelin from *B. cepacia* ATCC 25416 or pyoverdine from *P. aeruginosa* ATCC 15692 or desferriferrioxamine E from *P. stutzeri* ATCC 17588 or desferriferrioxamine B (Desferal®, Ciba-Geigy) (■). Ornibactins from *B. vietnamiens* TVV69 were purified by HPLC.

Ornibactin-mediated iron uptake in iron-starved TVV69 cells was considerably reduced (90–95%) when the cells were incubated at 0°C or when incubation was done in an uptake medium where the energy source (succinate) was omitted. No uptake at all was observed for TVV69 cells harvested from an iron-supplemented (100 μM) succinate growth medium (data not shown).

Ornibactin-mediated iron uptake was also performed with other ornibactin-producing strains (*B. vietnamiensis* TVV and LMG 6999 strains, *B. cepacia* ATCC 25416, *B. cepacia* ATCC 17759 and strain K132), and in the ornibactin non-producer strains *P. aeruginosa* ATCC 15692, *P. aeruginosa* ATCC 27853, *P. fluorescens* ATCC 13525, *P. fluorescens* CCM 2798 and *P. stutzeri* ATCC 17588. These experiments were done by using the purified mixture of C4-, C6- and C8-ornibactins obtained from TVV69 culture supernatants by repeated methanol extraction of the dry residue and LH-20 Sephadex chromatography (see Materials and methods). These results are presented in Table 3 as the percentage of the labeled iron incorporation observed for strain TVV69 at 15 min incubation time. All the ornibactin-producing strains incorporated the iron chelated via the TVV69 ornibactins, with an efficiency which was strain dependent, whereas none of the ornibactin non-producing strains incorporated the labeled ferric ornibactins.

Table 3. Relative ornibactin-mediated iron uptake rates in strains of *Burkholderia* and *Pseudomonas*

Strains		^{59}Fe uptake mediated by ornibactin[a]
B. vietnamiensis	TVV69	100
	TVV70	153
	TVV71	83
	TVV72	86
	TVV74	88
	TVV75	91
	TVV115	144
	TVV116	115
	TVV127	117
	TVV128	144
	TVV131	98
	TVV135	122
	LMG 6999	109
B. cepacia	ATCC 25416	68
	ATCC 17759	70
	K132	90
P. aeruginosa	ATCC 15692	0
	ATCC 27853	0
P. fluorescens	ATCC13525	0
	CCM 2798	0
P. stutzeri	ATCC 17588	0

[a] Expressed as the percentage of iron incorporation obtained after 15 min incubation with strain TVV69. Uptakes were done with the mixtures of C4-, C6- and C8-ornibactins isolated from TVV69.

Discussion

As evidenced by growth and uptake experiments, ornibactins acted as siderophores for their producing strain *B. vietnamiensis* TVV69. Thus ornibactins were specifically synthesized by iron-starved cells and excreted in large amounts in the growth medium (about 1 mM in optimized conditions) and, due to their Fe(III)-chelating properties, they facilitated iron uptake through an active transport, as demonstrated by iron uptake inhibition during energy deprivation experiments.

It has previously been shown that three structurally related ornibactins, differing from each other by the length of the acyl chain, were found in the culture supernatants of iron-deficient TVV69 cells (Stephan *et al.* 1993a). This minor difference in structure between the three compounds apparently did not affect cellular iron uptake since they each showed a similar efficiency. These data suggest that the acyl part of the molecule can vary to some extent and that the bacterial recognition of ferric ornibactins rather relies on the peptidic chain which remains identical for the three compounds.

Of the four amino acids of the peptide chain in ornibactin, two are derived from ornithine, the two others being serine and β-hydroxy-aspartic acid (Figure 2). Taking into account the putrescine (decarboxylated ornithine) residue present in the ornibactin structure, it can be calculated that ornithine, as a precursor, accounts for half of the molecular mass of ornibactin (51% for the C4-ornibactin). Apparently, the internal pool of ornithine in cells growing in succinate medium is the limiting factor for ornibactin biosynthesis since a supplementation of the succinate growth medium with ornithine or with arginine and proline, two amino acids known to be related with ornithine metabolism, resulted in a 2.5-fold (average) increase of ornibactin production for the TVV strains. Such an effect was not observed for ornithine-unrelated amino acids or for putrescine itself. An ornithine-dependent stimulation of ornibactin production by strains K132 and LMG 6999 was also observed, although to a lesser extent compared to TVV strains. This could be explained by the higher level of ornibactin production in the ornithine-unsupplemented succinate medium observed for these strains (Table 2). The possibility of increasing siderophore yield by feeding iron-starved growing bacteria with a potential siderophore precursor, as shown in the present study, has already been described for the production of desferriferrioxamine E by *Streptomyces olivaceus* (Meiwes *et al.* 1990).

The siderophore function of the ornibactins was also demonstrated for the strains which have been shown in this study to synthesize the same ornibactins as strain TVV69. All these strains belong to the *Burkholderia* genus. Among the 12 *B. vietnamiensis* strains investigated together with strain TVV69, 11 were natural isolates from the same Vietnam rice rhizosphere. The differences in the biochemical properties (Table 1) justified their recognition as separate strains. The clinical isolate originated from Sweden, strain LMG 6999, presented strong similarities (nitrogen fixation DNA–DNA, DNA–RNA hybridizations) with the TVV strains and, therefore, was classified among the *B. vietnamiensis* species (Gillis *et al.* 1995). All TVV isolates and LMG 6999 when analyzed for their siderophore production, demonstrated a common feature: they apparently only produced ornibactins as siderophores. Attempts to detect other chloroform (or ethyl acetate)-extractible iron-chelating compounds were unsuccessful. Contrary to *B. cepacia* strains, the *B. vietnamiensis* strains did not synthesize cepabactin (Meyer *et al.* 1989), pyochelin (Sokol 1986, Meyer *et al.* 1989, Visca *et al.* 1993) or salicyclic acid (azurechelin) (Sokol *et al.* 1992, Visca *et al.* 1993). Strain K132, another clinical strain analyzed in this study, behaved the same as *B. vietnamiensis* LMG 6999 or TVV strains, since only ornibactins were detectable in its iron-deficient growth supernatant. A more detailed taxonomic recognition of this strain will be of interest in order to determine whether K132 belongs to the *B. cepacia* species or, as suggested by its siderophore production, to the *B. vietnamiensis* group.

The well defined *B. cepacia* strain ATCC 25416 (type strain) and strain ATCC 17759, which were easily distinguished from the *B. vietnamiensis* and from the K132 strain by their characteristic yellow-pigmented colonies on LB-agar medium, synthesized cepabactin, a hydroxy-pyridinone derivative (Meyer *et al.* 1989), and pyochelin, a salicyclic-substituted cysteinyl peptide (Cox *et al.* 1981). Both compounds have already been described as siderophores from *B. cepacia* ATCC 25416 (Meyer *et al.* 1989), whereas pyochelin was described for *P. aeruginosa* (Liu &Shokrani 1978, Cox & Graham 1979, Cox 1980) and for various clinical *B. cepacia* isolates (Sokol 1986, Visca *et al.* 1993). In all these

studies, the search for siderophores had been undertaken by a unique procedure consisting of the extraction of the iron-chelating compounds from the acidified (pH 2–3) bacterial growth supernatants by chloroform or ethyl acetate. In no case was the classical method for hydroxamate-siderophore purification used. As shown in the present work, this method, based on the extraction of the iron–siderophore complexes by chloroform–phenol, allowed the detection of ornibactins in the two *B. cepacia* culture supernatants. Quantitatively, ornibactins represented the major iron-chelating compounds synthesized by the ATCC 25416 strain, twice as much (in dry weight) as cepabactin and 10 times more than pyochelin. Thus, these two *B. cepacia* strains present a unique feature among siderophore-producing microorganisms, they produce three structurally unrelated siderophores under iron starvation.

It can be concluded from the present results that the previously published statement concerning the discrimination between rhizosphere and clinical isolates of *B. cepacia* strains based on their siderophore production (Bevivino *et al.* 1994) is not valid. These authors stated that the clinical isolates could be easily identified from the rhizosphere isolates by their production of pyochelin. As demonstrated in the present work and already described in a previous publication (Meyer *et al.* 1989), plant- or soil-originated strains, i.e. the type strain *B. cepacia* ATCC 25416 and *B. cepacia* ATCC 17759, produced pyochelin together with the other siderophores cepabactin and ornibactins, whereas a clinical isolate like strain K132 produced ornibactins only, as the closely related *B. vietnamiensis* from rhizosphere (TVV strains) or from clinical (LMG 6999) origin. Moreover, the taxonomical assignment of the isolates described in previous studies as *B. cepacia* strains (Sokol 1986, Visca *et al.* 1993, Bevivino *et al.* 1994) remains doubtful, with strains very likely belonging to different species, such as the *B. vietnamiensis* TVV75 described in Bevivino *et al.* (1994), as a *B. cepacia* strain. As suggested by the present work, analysis of siderophores produced by such strains may be an easy way to discriminate between the *B. cepacia* and *B. vietnamiensis* species.

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