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Zinc affects siderophore-mediated high affinity iron uptake systems in the rhizosphere *Pseudomonas aeruginosa* 7NSK2

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Zinc concentrations ranging between 0.1 and 1 mM only slightly reduced maximal growth of wild-type *Pseudomonas aeruginosa* 7NSK2 in iron-limiting casamino acid medium, but had a clear negative effect on the growth of mutant MPFM1 (pyoverdin negative) and especially mutant KMPCH (pyoverdin and pyochelin negative). Production of pyoverdin by wild-type strain 7NSK2 was significantly increased in the presence of 0.5 mM zinc and could not be repressed by iron even at a concentration of 100 μM. Siderophore detection via isoelectrofocusing revealed that mutant KMPCH did not produce any siderophores, while mutant MPFM1 overproduced a siderophore with an acidic isoelectric point, most likely pyochelin. Pyochelin production by MPFM1 was stimulated by the presence of zinc in a similar way as pyoverdin for the wild-type. Analysis of outer membrane proteins revealed that three iron regulated outer membrane proteins (IROMPs) (90, 85 and 75 kDa) were induced by iron deficiency in the wild-type, while mutants were found to have altered IROMP profiles. Zinc specifically enhanced the production of a 85 kDa IROMP in 7NSK2, a 75 kDa IROMP in MPFM1 and a 90 kDa IROMP in KMPCH.

Keywords: iron deficiency, *Pseudomonas aeruginosa*, pyochelin, pyoverdin, zinc

Introduction

Under iron-limiting conditions most aerobic microorganisms produce siderophores, virtually ferric-ion-specific ligands and cognate outer membrane receptor proteins called IROMPs (iron regulated outer membrane proteins) (Neilands 1981). *Pseudomonas aeruginosa* produces two siderophores, pyochelin, a thiazoline derivative (Cox *et al.* 1981), and the yellow–green fluorescent pyoverdin (Wendenbaum *et al.* 1983). In Enterobacteriaceae expression of the siderophore-mediated high affinity iron uptake systems is negatively regulated by Fur, a repressor protein which uses Fe(II) as a cofactor (Bagg & Neilands 1987). Although a *fur*-like repressor from a fluorescent *Pseudomonas* was cloned (O'Sullivan & O'Gara 1990), the regulation of the high affinity iron uptake systems in fluorescent pseudomonads appears to involve other mechanisms as well since two positive regulatory genes have been identified which can independently activate transcription of genes coding for the biosynthesis of pseudobactin 358, a pyoverdin-type siderophore in *P. putida* WCS 358 (Leong *et al.* 1991). Furthermore, two exogenous siderophores, deferrioxamine b and enterobactin, are able to induce new IROMPs in the outer membrane of *P. aeruginosa*, implying that the siderophore itself can regulate the expression of its cognate receptor (Cornelis *et al.* 1987, Poole *et al.* 1990). It was noticed that *P. aeruginosa* 7NSK2, a plant growth promoting bacterium (Höfte *et al.* 1991) produced pyoverdin on Luria Bertani (LB) medium, provided the medium was supplemented with Zn^{2+}, Cd^{2+} or Ni^{2+} (unpublished results). Similar observations have been reported in the case of *P. fluorescens* and *P. aeruginosa* where zinc was found to increase the production of a green fluorescent pigment (Baghdiantz 1952, Labeyrie & Neuzil 1977). Cu^{2+} or Ni^{2+} were also found to promote the production of a yellow pigment in *P. fluorescens-putida* (Chakrabarty & Roy 1964). More recently, it was observed that addition of Zn^{2+} to cultures of

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Azotobacter vinelandii grown with low levels of iron increased siderophore production (Huyer & Page 1988). We report here that Zn^{2+} at concentrations above 500 μM specifically induces the high affinity pyoverdin system in *P. aeruginosa* 7NSK2, the pyochelin system in a pyoverdin-negative mutant and a third system in a double pyoverdin–pyochelin mutant.

Material and methods

Bacterial strains and growth conditions

P. aeruginosa 7NSK2 is a plant growth promoting bacterium isolated from barley roots (Iswandi *et al.* 1987). Mutant MPFM1 is a Tn*5* pyoverdin mutant (Pvd^-) (Höfte *et al.* 1991) obtained by mutagenesis with the suicide vector pJB4JI (pPH1JI::Mu::Tn*5*; Gen^R, Spe^R, Str^R, Kan^R) in the *Escherichia coli* strain J53 Nal (pro$^-$, met$^-$, Nal^R) (Beringer *et al.* 1978). KMPCH is a pyoverdin and pyochelin (Pvd^-, Pch^-) deficient mutant obtained after ethylmethane-sulfonate (EMS) mutagenesis of MPFM1 (Seong 1991 and this study).

Growth media used were LB medium (Maniatis *et al.* 1982), modified King's medium B (MKB: proteose pepton 5 gl^{-1}, $MgSO_4 \cdot 7H_2O$ 1.5 gl^{-1}, K_2HPO_4 1.2 gl^{-1}, glycerol 2 mll^{-1}, iron content about 6 μM), casamino acid medium (CAA, casamino acids 5 gl^{-1}, K_2HPO_4 0.9 gl^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 gl^{-1}, iron content about 2μM) and M9 mineral medium (Maniatis *et al.* 1982) containing 5 gl^{-1} succinate. For solid media, 1.5% agar was used. Filter sterilized $ZnSO_4$ was added after autoclaving. $FeCl_3$ was added after autoclaving from a 0.1 M stock maintained in 0.1N HCl.

Growth experiments were carried out in 50 ml glass tubes containing 5 ml medium. Overnight cultures in LB broth were centrifuged, pellets were resuspended in distilled water and 1/500 dilutions from these suspensions were used to inoculate the culture medium. Cultures were incubated at 26 °C with continuous shaking at 160 r.p.m. All treatments were carried out in triplicate and experiments were repeated at least once. All glassware used was acid washed.

Measurement of growth and pyoverdin production

Bacterial growth was measured turbidimetrically at 600 nm. Samples were taken periodically between late exponential and stationary growth phase to determine maximal biomass production.

Pyoverdin production was measured by fluorescence or by the spectrophometric method. For fluorescence measurements, the supernatant of the bacterial culture was diluted 1/100 in 0.1 M Tris–buffer (pH 7.4). The relative intensity of fluorescence was measured at 460 nm while exciting at 405 nm with a Sequoia-Turner spectrofluorimeter. A quinine sulfate solution in H_2SO_4 (pH 2) was used as an internal standard. For spectrophotometric measurements, the concentration of pyoverdin in the culture supernatant was measured directly at 400 nm. This method was preferred for pyoverdin measurements in media containing Zn^{2+} and iron. The molar extinction coefficient of 2×10^4 M^{-1} cm^{-1} was used to determine the quantity of pyoverdin present (Meyer & Abdallah 1978). Appropriately diluted uninoculated culture media served as a blank.

Determination of pyochelin

Pyochelin was partially purified by ethyl-acetate extraction followed by paper chromatography as described by Cox & Graham (1979). Chromatograms were developed ascending in water:acetic acid:acetone (90:10:1). After drying, chromatograms were sprayed with an iron reagent (0.1 M $FeCl_3$ in 0.1N HCl) or a phenolate spray reagent (one volume iron spray reagent and one volume of 0.1 M potassium ferricyanide). On the chromatograms, pyochelin turned red when sprayed with iron reagent and turned blue when sprayed with the phenolate reagent.

Isoelectrofocusing and detection of siderophores

Samples (80 μl) from culture supernatants were applied at the center of a polyacrylamide-ampholine (pH 3–10) gel in a LKB multiphor apparatus. The electrophoresis was run at a constant power of 10 W and a voltage increasing from 200 to 1000 V. After the separation was completed, the gel was first observed under UV to visualize the fluorescent pyoverdin bands before an agarose overlay of chromazurol-S medium (CAS medium) was layered on top to detect siderophores by decoloration of the dye (Schwyn & Neilands 1987).

Outer membrane proteins

Outer membranes were prepared by the sarkosyl differential solubilization technique (Filip *et al.* 1973) as described previously (Cornelis *et al.* 1989). Electrophoresis in 10% polyacrylamide gels was performed according to the method of Laemmli (1970). Gels were stained with 0.2% Coomassie blue.

Chemical mutagenesis

For chemical mutagenesis, MPFM1 cells grown until late exponential phase in M9 succinate medium supplied with 10 μM Fe^{3+} and 200 mgl^{-1} kanamycine, were centrifuged and washed with 10 mM $MgCl_2$ and 10 mM HEPES. After the addition of 125 μl EMS, the suspension was shaken for 2 h at 37 °C in the dark followed by centrifugation. Cell pellets were washed twice with M9 succinate medium and cells were plated out on MKB medium, CAA medium and CAS solid medium in order to detect mutants unable to produce pyochelin.

Results

Mutagenesis of P. aeruginosa *7NSK2*

In order to study the effect of zinc on both pyoverdin-mediated and pyochelin-mediated high affinity iron uptake, attempts were made to obtain a mutant deficient in pyoverdin and pyochelin production. The pyoverdin-negative mutant MPFM1 still produced pyochelin on iron-limiting media as evidenced by the CAS assay and ethyl-acetate extraction of the media (Seong 1991). After mutagenesis of the pyoverdin-negative mutant MPFM1 with EMS and testing of 1837 colonies, one colony was non-fluorescent on MKB medium under a long wave UV lamp (366 nm) and did not show a yellow halo on CAS solid medium. The growth of this latter mutant was completely inhibited by 5 $\mu g\,ml^{-1}$ EDDHA. In this mutant, called KMPCH, no pyochelin production could be detected after ethyl-acetate extraction of the medium (Seong 1991).

Growth of P. aeruginosa *7NSK2 and mutants in different conditions*

Figure 1 shows the maximal growth of 7NSK2 (Pvd^+, Pch^+), MPFM1 (Pvd^-, Pch^+) and KMPCH (Pvd^-, Pch^-) in the presence of concentrations of $ZnSO_4$ ranging from 0.1 to 1 mM. In the absence of added iron (Figure 1A), the highest concentration of zinc used (1 mM) caused a 25% reduction of growth in the wild-type strain while the same concentration of zinc reduced the growth by 54 and 62% in MPFM1 and KMPCH, respectively. The presence of 50 μM iron in the medium (Figure 1B) with 1 mM zinc enhanced the growth of both wild-type and Pvd mutant MPFM1 and, to a much lesser extent, the growth of the double mutant KMPCH. Indeed a growth reduction of 46% compared with the control without zinc was observed in the case of KMPCH, with reductions of 22 and 19% for the strains MPFM1 and 7NSK2, respectively. Since similar growth reductions could already be observed for 0.5 mM zinc, this concentration was selected for further work since higher amounts of $ZnSO_4$ caused precipitation in the medium. Table 1 shows that the wild-type 7NSK2 produced between 121 and 133 μmol of Pvd per OD_{600} nm in CAA medium when the zinc concentration was below 0.5 mM. At 0.5 mM zinc, 196 μmol Pvd/OD_{600} were produced and at 1 mM zinc, 213 μmol Pvd/OD_{600}. In the presence of 50 μM iron, a concentration which is sufficient to completely repress pyoverdin production in CAA medium, 34 μmol of pyoverdin were still produced in the presence of 0.5 mM $ZnSO_4$ and

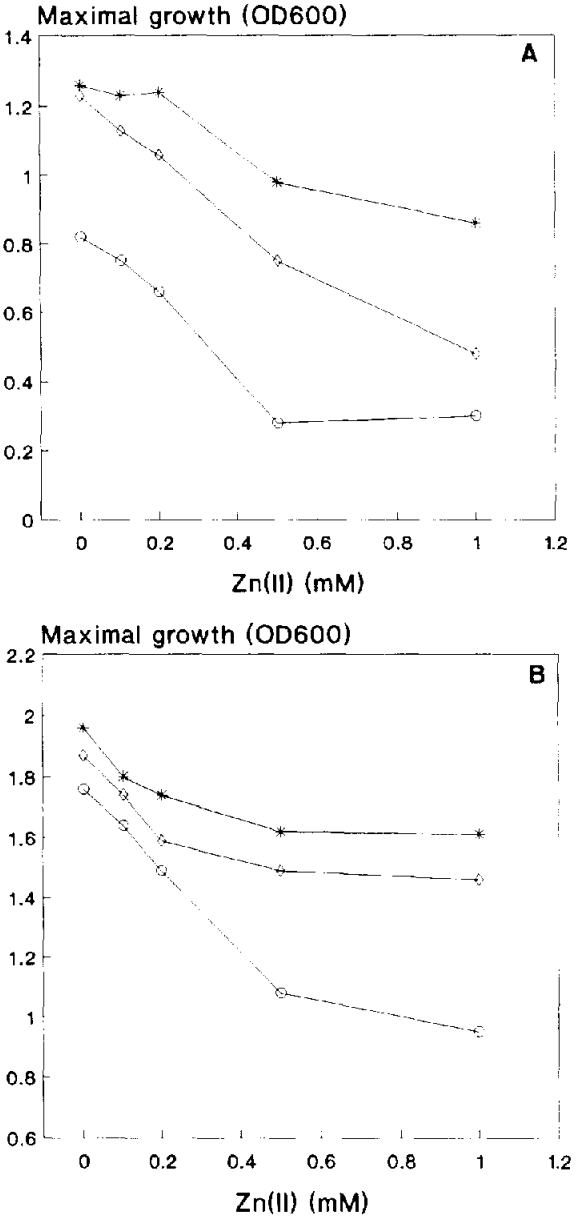

Figure 1. Maximal growth of *P. aeruginosa* 7NSK2 (*), the Pvd^- mutant MPFM1 (◇), and the Pvd^- and Pch^- mutant KMPCH (○) in CAA broth with increasing concentrations of Zn(II) without (**A**) and with (**B**) added Fe(III) (50 μM).

40 μmol in the presence of 1 mM $ZnSO_4$ (Table 1). Figure 2 shows that even in the presence of iron concentrations as high as 100 μM, pyoverdin production by 7NSK2 was not stopped if CAA medium was supplemented with 0.5 mM $ZnSO_4$.

Visualization of siderophore production by isoelectric focusing

After isoelectric focusing several fluorescent bands were visible in the case of the supernatant of 7NSK2

Table 1. Pyoverdin production (μM Pvd/OD_{600}) by *P. aeruginosa* 7NSK2 in CAA medium with increasing concentrations of Zn(II) with and without added Fe(III) (50 μM)

Zn(II)	−Fe(III)		+Fe(III)	
(mM)	mean	SD	mean	SD
0	127[a]	4	7.7[a]	0.3
0.1	133[a]	19	9.1[a]	1.4
0.2	121[a]	27	13.9[a]	0.5
0.5	196[b]	18	34.1[b]	8.6
1	213[b]	20	40.5[b]	0.6
Least significant difference ($P = 0.05$)	52		15.6	

Values followed by the same letter are not significantly different for $P = 0.05$. Values of a representative experiment are shown and are the means of three repetitions.

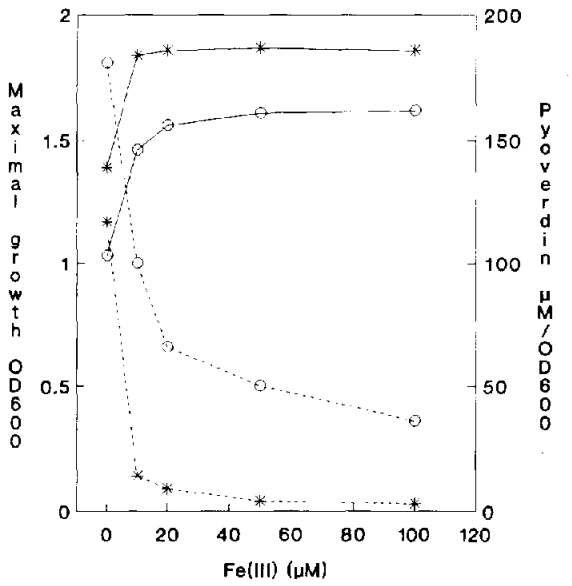

Figure 2. Maximal growth (full lines) and maximal pyoverdin production (dashed lines) of *P. aeruginosa* 7NSK2 in CAA broth containing various concentrations of Fe(III) with added Zn(II) (0.5 mM) (○) and without added Zn(II) (*).

cultures grown in CAA (Figure 3A, lane 1) and in CAA plus zinc (0.5 mM) (Figure 3A, lane 2). All bands were found to possess a siderophore activity as judged by the discoloration in the CAS assay, except for the first band at the bottom of the gel with a basic p*I* (Figure 3B, lanes 1–4). A second, non-fluorescent siderophore was also visible in lane 2 corresponding to the culture in the presence of zinc. This non-fluorescent siderophore had an acidic p*I* and was sometimes difficult to detect since it migrated very close to the gel electrode zone which

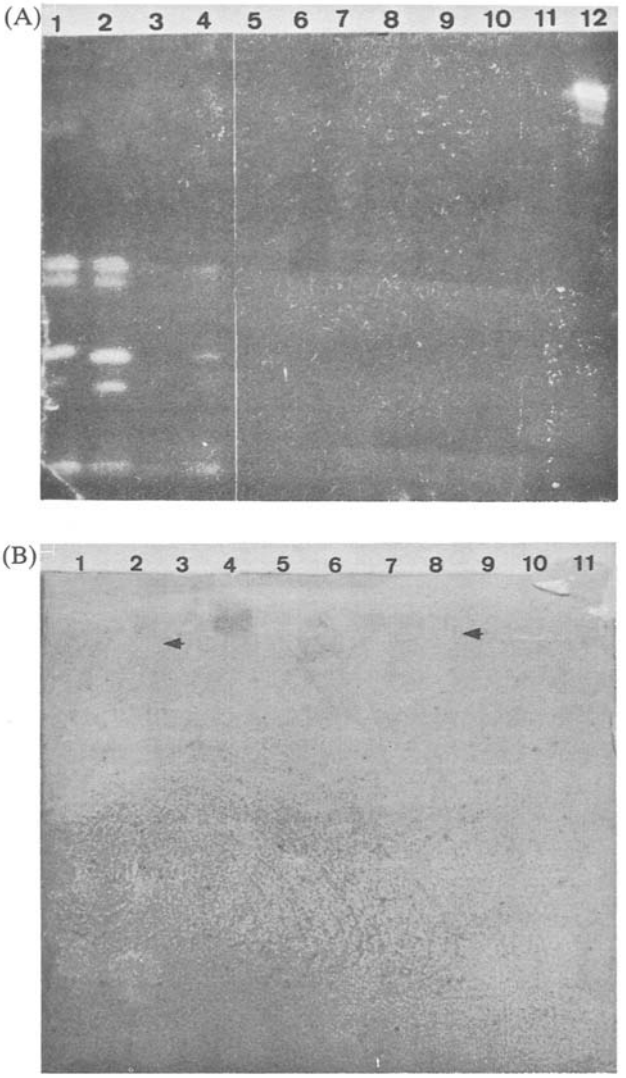

Figure 3. Siderophore production in CAA (lanes 1, 5 and 9), CAA plus 0.5 mM Zn(II) (lanes 2, 6 and 10), CAA plus Fe(III) (50 μM) (lanes 3, 7 and 11) and CAA plus 0.5 mM Zn(II) plus 50 μM Fe(III) (lanes 4 and 8) visualized via isoelectrofocusing. (**A**) Gel viewed under UV light to show fluorescent bands. (**B**) CAS overlay to show bands with siderophore activity. Lanes 1–4: 7NSK2; lanes 5–8: MPFM1; lanes 9–11: KMPCH. In (**A**), lane 12 contains protein markers. Only phycocyanin with a p*I* of 4.65 is visible under UV light.

was soaked with phosphoric acid and also discolorized. The acidic p*I*, the fact that 7NSK2 is a *P. aeruginosa* strain and the presence of an identical spot in *P. aeruginosa* PAO1 (results not shown) led us to the conclusion that this second siderophore was pyochelin. No fluorescent band or siderophore activity was detectable in the supernatants from cultures grown in the presence of iron (lane 3). In lane 4 (cultures with iron plus zinc) faint fluorescent

bands were visible but these bands were hardly detectable by the CAS assay. In the case of mutant MPFM1 (Figure 3, lanes 5–8) no fluorescent band could be seen while the second, non-fluorescent siderophore was detected by the CAS assay in CAA medium (Figure 3B, lane 5), in CAA plus zinc (lane 6) and in CAA plus iron plus zinc (lane 8). This second siderophore was clearly overproduced by MPFM1 as compared with the wild-type. However, the siderophore of MPFM1 was not visible in the lane corresponding to the iron supplemented culture supernatant (lane 7). As expected, and confirming our previous observations, no siderophore could be visualized in the case of mutant KMPCH (Figure 3, lanes 9–11), which was previously shown to be a pyochelin-negative mutant of MPFM1 (Seong 1991).

Analysis of outer membrane proteins

Figure 4(A) shows the separation of outer membrane proteins from *P. aeruginosa* 7NSK2 and the two mutants MPFM1 (Pvd^-, Pch^+) and KMPCH (Pvd^-, Pch^-) grown in CAA (lanes 2–4) and in CAA plus iron (lanes 5–7). The wild-type expressed three iron-repressible proteins of about 90, 85 and 75 kDa (lane 2). The mutant MPFM1 expressed the same proteins although the amount of the 85 kDa protein was lower (lane 3). The mutant KMPCH produced a very small amount, if any, of the 75 kDa protein but expressed both the 85 and the 90 kDa IROMPs (lane 4). A band of about 82 kDa was lacking in the outer membrane of KMPCH grown in the presence of 50 μM iron (lane 7). Previous experiments indicated that this particular band had no relation with the three IROMPs described above (results not shown).

The effect of the addition of zinc to the medium on the IROMP composition is shown in Figure 4(B). For the wild-type we could see an obvious increase in the production of the 90 kDa protein and, especially, the 85 kDa protein (lane 2), while the amount of the 75 kDa IROMP was decreased. On the contrary, the 75 kDa band was increased in the case of MPFM1 grown in the presence of 0.5 mM $ZnSO_4$ (lane 3), while the expression of the two other IROMPs was strongly reduced. In the case of KMPCH and as seen before for the cells grown in CAA medium, the 75 kDa protein was almost undetectable while the two other iron repressible outer membrane proteins were present (lane 4). Additionally, the expression of the 90 kDa protein, was strongly enhanced by the presence of zinc in the medium.

In media containing both zinc (0.5 mM) and iron (50 μM) (lanes 5–7), the synthesis of IROMPs was not repressed and the pattern observed was not much different from the one described for cultures in the presence of zinc alone, except for the wild-type where an equal amount of the 85 and 75 kDa proteins could be observed.

Finally, we observed that the addition of zinc to the medium had a negative effect on the production of a protein of about 60 kDa which was present in outer membranes of all three strains grown in CAA (Figure 4A) but absent in membranes from cells grown in CAA plus 0.5 mM $ZnSO_4$ (Figure 4B). A protein of about 50 kDa was strongly induced by the

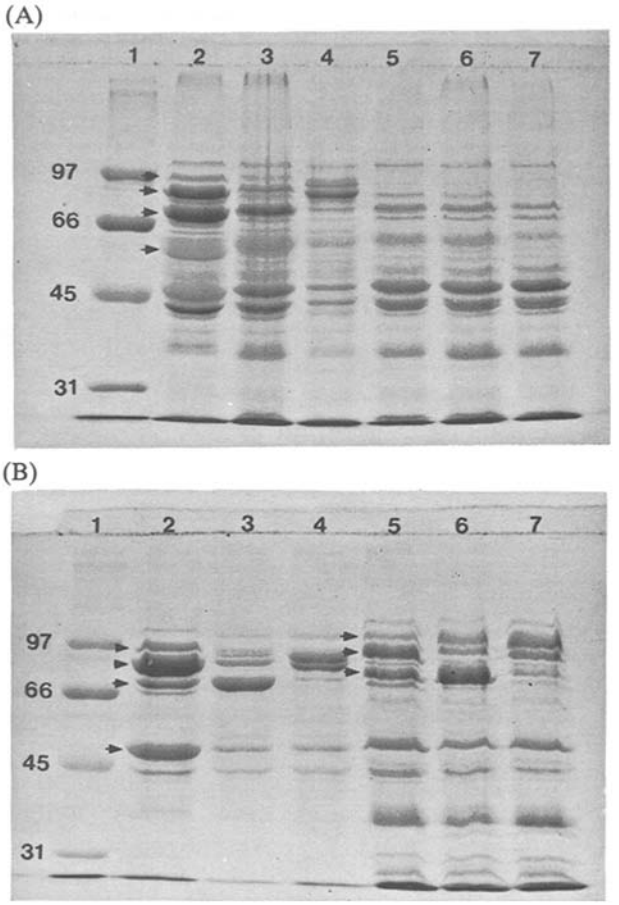

Figure 4. (**A**) SDS–PAGE of outer membranes from *P. aeruginosa* 7NSK2 (lanes 2 and 5), mutant MPFM1 (lanes 3 and 6) and mutant KMPCH (lanes 4 and 7) in CAA medium without (lanes 2–4) and with added Fe(III) (50 μM) (lanes 5–7). Arrowheads indicate IROMPs and the 60 kDa protein. Lane 1 contains protein standards. (**B**) SDS–PAGE of outer membranes from *P. aeruginosa* 7NSK2 (lanes 2 and 5), mutant MPFM1 (lanes 3 and 6) and mutant KMPCH (lanes 4 and 7) in CAA medium with 0.5 mM Zn(II) without (lanes 2–4) and with added Fe(III) (50 μM) (lanes 5–7). Arrowheads indicate IROMPs and the 50 kDa protein. Lane 1 contains protein standards.

presence of zinc in the medium, but only in the membranes from the wild-type strain 7NSK2. At the present time the significance of this observation is unknown.

Discussion

From our results it can be concluded that zinc at concentrations above 0.5 mM in an iron restricted CAA medium exerts a negative effect on the iron uptake by *P. aeruginosa* 7NSK2. The addition of zinc to the medium, even when iron is present, causes an increase in pyoverdin production and in the expression of an 85 kDa protein. A protein of similar size has already been identified as being the ferripyoverdin receptor by three different groups (Meyer *et al.* 1990, Poole *et al.* 1991, Smith *et al.* 1992).

Pyoverdin seems to be important for optimal growth in the presence of zinc, since both the pyoverdin mutant MPFM1 and the pyoverdin plus pyochelin mutant KMPCH are more sensitive to zinc than the wild-type strain 7NSK2. Pyochelin also appears to play a role in lessening the growth restriction caused by zinc, since mutant MPFM1 (Pvd^-, Pch^+) was less affected by zinc than mutant KMPCH (Pvd^-, Pch^-). Pyoverdin production by 7NSK2 and pyochelin production by MPFM1 are not completely repressed in the presence of iron in CAA medium supplemented with zinc. Moreover, growth of mutant KMPCH in CAA medium supplemented with more than 0.2 mM zinc cannot be restored to wild-type levels by addition of iron. However, growth of KMPCH is restored to wild-type levels when iron is supplied under the form of ferripyoverdin (data not shown). These observations indicate that zinc interferes with low affinity iron uptake and therefore causes a state of iron deficiency in the cell. In *A. vinelandii*, zinc was found to increase the production of azotobactin, a fluorescent siderophore with a structure similar to pyoverdin, and to inhibit the ferric reductase activity in the cell (Huyer & Page 1988, 1989). In the pyoverdin mutant MPFM1 the production of an acidic siderophore was increased compared to the wild-type. The production of this siderophore which we consider to be pyochelin is further increased when zinc is present in the medium. This increase is accompanied by a larger amount of the 75 kDa IROMP in the outer membrane. A protein of identical size was proved to be the ferripyochelin receptor and its gene was recently cloned (Heinrichs *et al.* 1991, Ankenbauer 1992). In mutant MPFM1 there is apparently an overproduction of the ferripyochelin receptor together with pyochelin. Pyochelin was shown recently to be able to bind transition metals such as Mo(VI) and Cu(II), but very little binding could be demonstrated for Zn(II) (Visca *et al.* 1992). This rules out the possibility that pyochelin detoxifies the growth medium by chelating zinc.

A third IROMP is expressed in the outer membrane of the three strains. This IROMP does not seem to correspond to any siderophore produced by 7NSK2 since no iron chelating compound could be detected in culture supernatants of KMPCH. However, this IROMP might be a receptor for an exogenous siderophore. Such receptors have already been identified in *P. aeruginosa*, namely a receptor for enterobactin, an *E. coli* siderophore (Poole *et al.* 1990) and an IROMP induced by the presence of deferrioxamine B (Desferal) in the medium (Cornelis *et al.* 1987). The 90 kDa IROMP is overproduced in the mutant KMPCH grown in the presence of zinc while the 75 kDa IROMP (assumed to be the pyochelin receptor) becomes undetectable. The genes for pyochelin synthesis and uptake could be organized in an operonic structure and the mutation induced by EMS could, by a polar effect, affect the expression of the genes involved in the uptake of the siderophore, including the receptor.

In conclusion, our results suggest that, by hindering low affinity iron uptake, zinc causes iron deficiency which can be relieved by pyoverdin and, to a lower extent, pyochelin. The presence of zinc seems to turn on preferentially the genes for the high affinity pyoverdin uptake system. When pyoverdin is absent, the genes for pyochelin uptake are turned on, while a receptor for a putative exogenous siderophore is overproduced in the absence of any siderophore as it is the case for KMPCH. The fact that all three IROMPs are always present, although in different proportions, suggests a two-level regulation of the iron uptake systems. The first, a general regulation, would involve a Fur-like repressor protein like in *E. coli* (Bagg & Neilands 1987). Such a Fur-like regulation also appears to exist in a fluorescent *Pseudomonas* (O'Sullivan & O'Gara 1990). The second regulation level would be specific for each siderophore uptake system, possibly involving siderophore-dependent activation of its own biosynthesis and uptake genes. More experiments, however, will be needed to verify this hypothesis.

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