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Effect of Iron Concentration in the Growth Medium on the Sensitivity of *Pseudomonas aeruginosa* to Pyocin S2

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The iron concentration in the growth medium was found to affect the susceptibility of *Pseudomonas aeruginosa* PML1550 to pyocin S2, a bacteriocin. The efficiency of killing by pyocin S2 was very low when the indicator cells were grown in an iron-rich medium. The capacity of these cells to adsorb pyocin S2 was reduced. Cultivation under limitation of iron (1 μM or less) was necessary to produce a fully sensitive cell population. The growth under iron limitation was accompanied by the appearance of four protein components in the outer membrane of the cells. Nine mutants resistant to pyocin S2 were isolated and their outer membranes were analyzed. They all lacked one component (Fe-b protein) as well as the adsorption capacity for pyocin S2. These findings suggest a possible role of this protein as the receptor for pyocin S2.

The outer membrane proteins of *Pseudomonas aeruginosa* have recently been analyzed by Mizuno and Kageyama (*1*). They also found that several protein components appeared in the outer membrane of strain PAO1 depending on iron limitation in the growth medium. These proteins are considered to be analogous to the receptor proteins of *Escherichia coli* for iron-chelator complexes (reviewed in *2, 3*) because of their localization, molecular weights (70,000–90,000) and the conditions of their appearance. In *E. coli*, the receptor proteins for iron-chelator complexes also act as receptors for some phages and bacteriocins (*3*). On the other hand, no receptors that are protein in nature have so far been identified on the outer membrane of *P. aeruginosa*.

Pyocin S2 is a bacteriocin produced by *P. aeruginosa* PAO1, and has a molecular weight of approximately 75,000 (*4*). It causes preferential inhibition of the biosynthesis of phospholipids in the sensitive strain *P. aeruginosa* PML1550 (*5*). However, the nature of the receptor for pyocin S2 has not been studied.

In this paper, we demonstrate that the appearance of sensitivity of PML1550 to pyocin S2 depended on iron limitation during growth, which also induced the appearance of four proteins in the outer membrane. A certain component of these proteins was lacking in all 9 clones isolated as resistant to pyocin S2, suggesting a possible role of this protein as the receptor for pyocin S2.

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Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

EXPERIMENTAL PROCEDURE

Bacterial Strains and Media—P. aeruginosa PML1550 was used as a pyocin S2-sensitive strain. It is a spontaneous prototrophic revertant of PML1516d, formerly referred to as P15-16d (*5, 6*). *P. aeruginosa* PAO3047 (formerly M47) was used as a producer of pyocin S2.

The glutamate medium for the cultivation of the indicator strain and its derivatives consisted of 20 g of Na glutamate, 5 g of glucose, 5.6 g of $Na_2HPO_4 \cdot 12H_2O$, 0.25 g of KH_2PO_4, 0.15 g of nitrilotriacetic acid, 0.14 g of KOH, 0.3 g of $MgSO_4 \cdot 7H_2O$, 0.06 g of $CaCl_2 \cdot 2H_2O$, and an indicated amount of $FeSO_4$ per liter. To examine the effects of other metal ions, a mixture of Zn^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, borate, and molybdate was used, as described by Hoshino and Kageyama (*7*).

The medium employed for the cultivation of PAO3047 was the same, except that casamino acids were added at 1 g/liter and $FeSO_4 \cdot 7H_2O$ at 2 mg/liter. Nutrient agar was purchased from Kyokuto Seiyaku Kogyo Co. (Tokyo). The dilution buffer for pyocin S2 (DBS) was 10 mM Tris-HCl (pH 7.7) containing 0.2 M NaCl and 0.1% gelatin. The activity of pyocin S2 diluted with DBS did not decrease during several days at 4°C. The dilution buffer for cells (DB) was 10 mM Tris-HCl (pH 7.6) containing 85 mM NaCl and 1 mM $MgCl_2$. Glass-distilled water and chemicals of guaranteed reagent grade were used throughout this study.

Culture Conditions—The indicator strain was cultured at 37°C with shaking as follows. An overnight preculture of PML1550 grown with 7.2 μM $FeSO_4$ was inoculated into 50 volumes of fresh medium with (usually 36 μM, iron-rich culture) or without $FeSO_4$ (iron-limited culture). After about 3 h (about 3 generations), cells at the middle log phase were harvested for experiments. No marked difference in growth was observed between iron-rich and iron-limited cultures for at least 3–4 h under the above conditions.

Preparation of Pyocin S2—The purification procedure described by Ohkawa *et al.* (*4*) was modified as follows, improving the yield of pure pyocin S2 5-fold. PAO3047 was cultivated in 1 liter of the medium at 37°C with good aeration. At the middle logarithmic phase of growth (about 5×10^8 cells/ml), 1.5 mg of mitomycin C was added. The cells lysed 3 h later, and the lysate, which contained 9×10^3 units of pyocin S2 per ml, was left to stand for 1 h at 37°C with a few drops of chloroform and 1 mg of bovine pancreatic DNase to reduce the viscosity. All subsequent procedures were carried out at 4°C. After centrifugation at $9,000 \times g$ for 30 min, 490 g of $(NH_4)_2SO_4$ (70% saturation) was added to the supernatant and it was left overnight. The precipitate (wet weight 5.8 g) was collected by centrifugation ($9,000 \times g$, 30 min), and dissolved in 4 volumes of 10 mM sodium phosphate buffer (pH 6.8)–10 mM Na_3 EDTA. The insoluble material was centrifuged off ($15,000 \times g$, 10 min). The solution (25 ml) was dialyzed against 10 liters of 8 mM sodium phosphate, 0.5 mM EDTA buffer (pH 6.8) for 10 h. Crude pyocin S2 tended to lose its activity. This inactivation was probably due to coexisting proteolytic enzymes, and EDTA seemed to slow down the inactivation. Pyocin S2 is also unstable at low concentrations of salt even after complete purification (*4*). Therefore, prolonged dialysis should be avoided. The conductivity of the dialysate was adjusted, if necessary, by dilution with water to give approximately the same value as that of 10 mM sodium phosphate buffer, pH 6.8. After removal of the precipitate ($15,000 \times g$, 10 min), the dialysate was immediately applied to a column (0.76 cm × 20 cm) of CM-Sepharose CL-6B (Pharmacia Fine Chemicals) prepared with 10 mM sodium phosphate buffer, pH 6.8. More than 99% of the 280 nm-adsorbing material (including 193 mg of protein) passed through the column. After washing with 20 ml of the same buffer, pyocin S2 was eluted with an NaCl gradient, 0–0.15 M, 100 ml. The flow rate was 16 ml/h. Small volumes of 2.0 M NaCl were added to the fractionation tubes to make the NaCl concentration of the elute 0.2 M or more. The elution profile is shown in Fig. 1a. Peak fractions with pyocin S2 activity were pooled and subjected to gel filtration on a Sephadex G-100 column (1.3 cm × 33 cm) prepared with 10 mM sodium phosphate buffer (pH 6.8) containing 0.2 M NaCl. The flow rate was 27 ml/h. The elution profile is shown in Fig. 1b.

SDS polyacrylamide-slab gel electrophoresis (PAGE) showed that the pyocin fraction eluted from the CM-Sepharose CL-6B column consisted mainly of pyocin S2 (about 1.5 mg) with minor contamination which was completely eliminated by

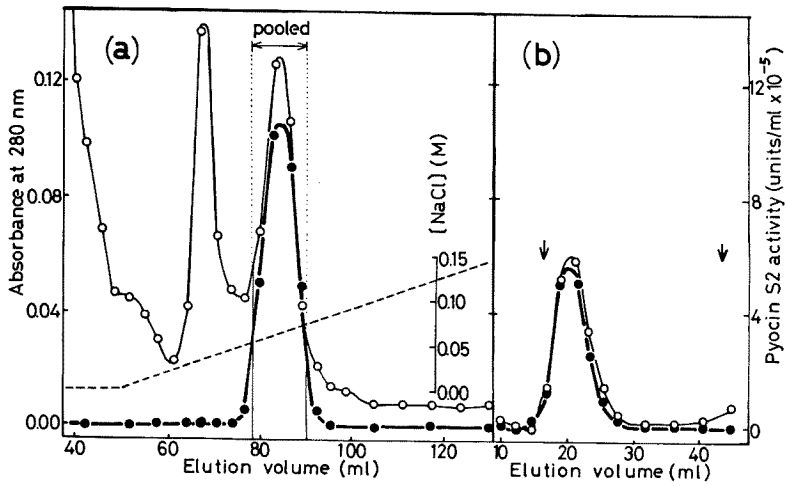

Fig. 1. Purification of pyocin S2. (a) CM-Sepharose CL-6B chromatography. A crude pyocin S2 preparation was applied to the column as described in " EXPERIMENTAL PROCEDURE." Only the significant part of the elution profile is shown. (b) Sephadex G-100 gel filtration. A half (6 ml) of the pooled fractions of Fig. 1a was applied to the column. Details are given in " EXPERIMENTAL PROCEDURE." Arrows indicate the void volume and the bed volume. Vertical scales are common to (a) and (b). ○, Absorbance at 280 nm; ●, pyocin S2 activity; ----, NaCl concentration.

the gel filtration. The overall yield was 65%. The specific activity of 6×10^6 units/mg and the mobility on SDS-PAGE (corresponding to a molecular weight of 72,000) were the same as reported previously (*4*).

Assay of Pyocin S2—The spot test method for the assay of pyocin S2 activity (pyocin unit) was essentially as described previously (*4, 8*). PML1550 cells grown in the iron-limited glutamate medium were used as an indicator.

Assay of Sensitivity to Pyocin S2—The sensitivity of a culture to pyocin S2 was determined quantitatively by measuring the killing of bacteria in liquid media. Colony-formers that survived treatment with pyocin were counted as follows. PML1550 cells were washed and resuspended in DB or indicated media. The cell density was adjusted to $1\text{–}2\times10^8$ per ml. The cell suspension was warmed to 37°C and 0.1 volume of DBS containing an appropriate amount of pyocin S2 (at a multiplicity of 10–15 unless otherwise noted) was added. Portions of 0.05 ml were withdrawn after indicated periods at 37°C, diluted with DB and plated on nutrient agar. Colonies were counted after 24 h at 37°C. The survival ratios were calculated in comparison with the control without pyocin. Multiplicity is an expression of the amount of pyocin S2: pyocin at a multiplicity of 1 gives a survival of 37% or e^{-1} of a sensitive cell suspension (*4*), which corresponds to about 5×10^{-8} unit of pyocin S2 per bacterium in the spot test method.

Adsorption of Pyocin S2 by Intact Cells—Pyocin S2 was added to about 5×10^8 washed cells in 2 ml of DBS at a multiplicity of 5 and incubated at 37°C for 30 min. The cells were removed by centrifugation, the supernatant was treated with a drop of chloroform, and the remaining activity of pyocin S2 was assayed by the spot test.

Analysis of Outer Membrane Proteins—About 7×10^{10} cells were harvested from 100 ml of culture, then washed with and resuspended in 10 mM sodium phosphate buffer, pH 7.2, and disrupted (subsequently handled at 4°C) with a sonicator. After centrifugation of the supernatant at $5{,}000\times g$ for 10 min, the cell envelopes were collected ($60{,}000\times g$, 30 min) and washed once with the same buffer. They were then washed with 10 mM Tris-HCl (pH 7.2) containing 2 mM $MgCl_2$, suspended in 2 ml of 2% Triton-X-100–10 mM Tris-HCl (pH 7.2) and

incubated at 40°C for 30 min. The insoluble membrane fraction was collected (100,000×*g*, 60 min), and washed once with 10 mM sodium phosphate, pH 7.2. The protein composition of the sample was analyzed by SDS-PAGE with a Laemmli buffer system (*9*) followed by staining with Coomassie Brilliant blue R250. The conditions of electrophoresis were as described previously (*1*). Triton X-100 treatment in the presence of $MgCl_2$ solubilized most of the inner membrane proteins, as reported by Schnaitman for *E. coli* (*10*), so that the remaining membrane fraction gave a protein profile similar to that of the separated outer membranes of *P. aeruginosa*, described by Mizuno and Kageyama (*1*). Apparent molecular weights of proteins were determined from their mobilities on SDS-PAGE compared with those of RNA polymerase (165,000, 155,000, 39,000), bovine serum albumin (68,000), trypsin inhibitor (21,500), β-galactosidase (128,000), and catalase (57,000).

Isolation of Mutants Resistant to Pyocin S2—PML1550 cells were treated with 0.5% ethylmethane sulfonate in glutamate medium ($FeSO_4$ 18 μM) at 37°C for 30 min with shaking. The survival ratio was 1.7%. Cells were resuspended in a fresh medium ($FeSO_4$ 7.2 μM), divided into ten tubes, and grown for 7 h. Pyocin S2 (multiplicity≧1,500) was added to 1 ml portions of these cultures, and cultivation was continued. After the addition of pyocin S2, the number of colony formers rapidly decreased to a level of about 5%. The cells, plated after another 7 h, were checked for their sensitivity to pyocin S2.

RESULTS

Appearance of Sensitivity to Pyosin S2 upon Iron Limitation in the Growth Medium—The effect of iron supply in the growth medium on the sensitivity to pyocin S2 is shown in Fig. 2. The survival ratio of PML1550 cells grown under iron limitation was usually about 1% after treatment with a sufficient amount of pyocin S2. However, when cells grown in an iron-rich (36 M $FeSO_4$) medium were treated similarly, most of the population (often up to 70%) survived in spite of prolonged incubation with pyocin S2 in DB. The effect of other minerals (a mixture of Zn^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, $B_4O_7^{2-}$, $(NH_4)_6Mo_7O_{24}$) in the growth medium was negligible. Thus, the concentration of iron ions in the growth medium was exclusively responsible for the change of the sensitivity to pyocin S2.

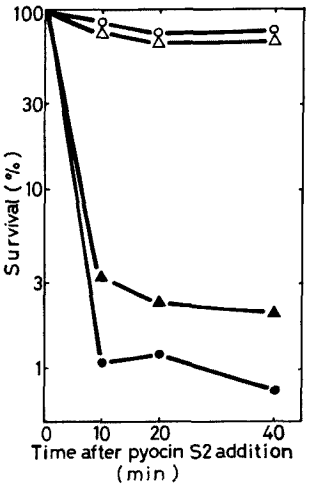

Fig. 2. Effects of metal ions in the culture medium on the susceptibility of the cells to pyocin S2. PML1550 was grown in glutamate medium with various metal supplements. Washed cells were treated with pyocin S2 in DB. Colony formants after indicated periods of incubation with pyocin S2 (multiplicity=7) are shown. △, Glutamate medium with 36 μM $FeSO_4$; ○, with 36 μM $FeSO_4$ and a mixture of other minerals; ▲, without iron, but other minerals were added; ●, without iron and other minerals. The mixture of other minerals consisted of Zn^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, borate, and molybdate.

Growth under iron-rich conditions caused the insensitivity to pyocin S2; addition of iron to the incubation mixture for pyocin S2 treatment had no effect. The sensitive culture (iron-limited) was killed equally well in the presence of $FeSO_4$ (36 M) or its absence in the incubation medium. In other words, iron ions do not inhibit pyocin action.

The relationship between the amount of pyocin added and the extent of killing of cultures grown under the two conditions is shown in Fig. 3. The iron-limited culture showed a high survival which decreased only gradually with larger amounts of pyocin S2. On the other hand, the survival of the iron-limited culture decreased exponentially down to 1% or less with increasing amounts of pyocin S2. However, a small portion of the culture survived even in the presence of a large excess of pyocin. In other words, even the iron-depleted

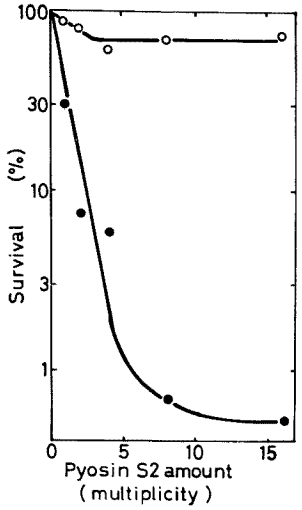

Fig. 3. Relationship of cell survival to the amount of pyocin S2 added. Cells were treated with the indicated amounts of pyocin S2. Survivals after 40 min are shown. ●, Cells grown under iron limitation; ○, grown in the medium containing 36 μM $FeSO_4$.

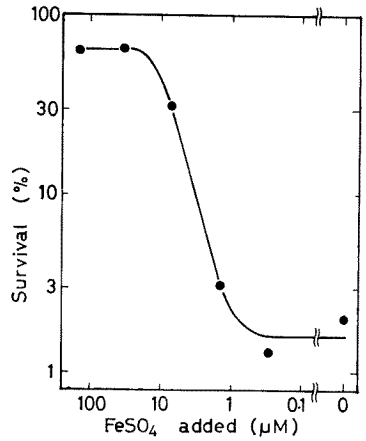

Fig. 4. The relation of iron concentration in the growth medium to the appearance of pyocin S2 sensitivity. Cells were cultivated in media containing $FeSO_4$ at the indicated concentrations. Survivals after incubation with pyocin S2 for 40 min are shown.

culture contained a few tenths percent of insensitive cells. Most of the surviving population in either the iron-rich or the iron-limited culture were not genetically resistant to pyocin S2. The surviving colonies showed pyocin sensitivity under appropriate conditions (see below).

The relation between the iron concentration in the growth medium and the sensitivity of the culture to pyocin S2 is shown in Fig. 4. Sensitivity of PML1550 cells to pyocin S2 appeared when they were cultivated in a medium containing 1 μM or less iron, under our experimental conditions. Thus, sensitivity to pyocin S2 can be modified by the concentration of iron in the growth medium.

Time courses of the change of sensitivity after shifts of iron concentration in the medium are shown in Fig. 5. When cells cultivated under iron-rich conditions were resuspended to grow in a fresh medium without iron, the population sensitive to pyocin S2 immediately began to increase and reached the maximum level within 40 min (Fig. 5a). On the other hand, when 36 μM $FeSO_4$ was added to the culture grown without iron, the sensitive population decreased with time (Fig. 5b). In this case, the change occurred rather slowly. It took 3 h or more (3 generations) to reach the final, maximally insensitive level.

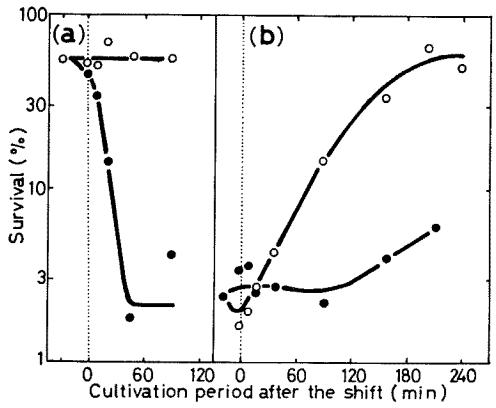

Fig. 5. Time courses of the change of pyocin S2 sensitivity after shifts of iron concentration in the medium. (a) Two portions of a culture grown with 36 μM $FeSO_4$ were taken at the middle logarithmic phase. Cells were washed with and resuspended in 4 volumes of fresh medium with (36 μM) or without $FeSO_4$ and growth was continued at 37°C. At the indicated times, aliquots were withdrawn, diluted with the same medium to give a cell density of $1-2\times10^8$/ml and immediately treated with pyocin S2 for 5 min, then survivals were determined. ○, 36 μM $FeSO_4$ added; ●, without iron supplement. (b) A culture without iron supplement was divided into two portions, diluted 3-fold with the same (fresh) medium; 36 μM $FeSO_4$ was added to one of them, and they were cultivated. Pyocin sensitivity was determined at the indicated times. The symbols are the same as in (a).

TABLE I. Characteristics of PML1550 and mutants resistant to pyocin S2.

Strain		[$FeSO_4$] in the growth medium (μM)	Survival after pyocin S2 treatment (%)[a]	Pyocin S2 unadsorbed (units/ml)[a]	Missing outer membrane proteins[b]
Parent	PML1550	0	4.3	1	—
	PML1550	36	108	32	(Fe-a,-b,-c,-d)[c]
Mutant	PML1567	0	100	50	Fe-b
	PML1566	0	99	50	Fe-b,-d
	PML1563	0	108	32	Fe-b,-d
	PML1564	0	71	32	Fe-b,-d
	PML1565	0	90	50	Fe-b,-d
	PML1568	0	110	ND	Fe-a,-b,-c,-d
	PML1560	0	91	50	Fe-b,D
	PML1561	0	138	64	Fe-b,D
	PML1562	0	87	50	Fe-b,D

[a] Survival after pyocin treatment and the capacity of intact cells to adsorb pyocin S2 were determined as described in "EXPERIMENTAL PROCEDURE." [b] Analyzed by SDS-PAGE. [c] Synthesis of these proteins is repressed. ND, Not determined.

Capacity of Cells to Adsorb Pyocin S2—The adsorption of pyocin S2 by intact cells was tested. As shown in the top two lines of Table I, cells grown in the iron-rich medium scarcely adsorbed pyocin S2, whereas iron-limited cells adsorbed most of pyocin S2 at a multiplicity of 5 (63 units/ml).

Appearance of Certain Outer Membrane Proteins under Conditions of Iron Deficiency—The above findings suggest a possible change in the composition of the outer membrane. The proteins of the outer membrane were analyzed as described in "EXPERIMENTAL PROCEDURE." Densitograms of the stained gels after SDS-PAGE are shown in Fig. 6. When cultivation was carried out under iron-rich conditions, the pattern of outer membrane proteins of PML1550 (Fig. 6b) was the same as that of PML15 (formerly designated as P15), the ancestor of PML1550, reported by Mizuno and Kageyama (*1*). Following their designation, major protein bands are labeled D, E, F, G, and H in Fig. 6. Cultivation under conditions of iron limitation caused a reduction of the major protein band G and the appearance of at least 4 new protein bands referred to here as Fe-a, Fe-b, Fe-c, and Fe-d, as indicated in Fig. 6a. Fe-b was minor, whereas the amounts of Fe-a, Fe-c, and Fe-d were comparable to those of other major proteins of the outer membrane. These 4 proteins were not detected at all in the sample from iron-rich culture (Fig. 6b). In SDS-PAGE co-electrophoresis, these protein exhibited mobilities identical with those of analogous proteins of PAO1 (Mizuno, T., unpublished data). Their apparent molecular weights were estimated to be in the range of 70,000–90,000. When the cells grown with iron were shifted to an iron-depleted medium, these proteins appeared again.

Isolation and Characterization of Pyocin S2-Resistant Mutants—Mutants resistant to pyocin S2 were isolated to investigate the compositions of their outer membranes. Nine fully resistant mutants were selected. They are listed in Table I. They had all lost not only susceptibility to the killing action of pyocin S2 but also the capacity to adsorb it (Table I). Analyses of outer membrane proteins revealed that one mutant, PML1567, shown in Fig. 6c, lacked Fe-b protein; 4 mutants, PML1563–1566, giving essentially the same densitogram as that shown in Fig. 6d, lacked two proteins, Fe-b and Fe-d; 3 mutants, PML1560–1562, gave the densitogram shown in Fig. 6e, lacking Fe-b protein and protein D. One mutant,

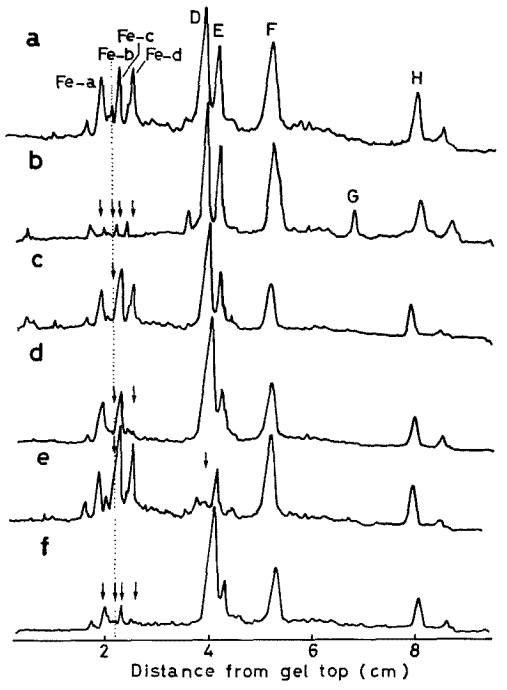

Fig. 6. Densitograms of the outer membrane proteins analyzed by SDS-PAGE. a, Parent PML1550 grown under iron limitation; b, PML1550 grown with iron; c, d, e, f, mutant PML1567, PML1564, PML1560, and PML1568, respectively, all grown under iron limitation.

PML1568, contained reduced amounts of all four proteins (Fig. 6f). Thus all nine mutants lacked Fe-b in their outer membranes.

Protein G was detected in PML1550 when cultured under iron-rich conditions (Fig. 6b), but was missing in the iron-limited culture (Fig. 6a). However, the appearance of this protein in the outer membrane was influenced by various cultural conditions, and was not directly linked to the concentration of iron in the medium (Mizuno, T., personal communication). Other mutants shown in Fig. 6 (c–f) were not defective in protein G: they showed this band under appropriate conditions. All nine mutants retained sensitivity to R-type pyocins.

DISCUSSION

PML1550 cells grown in nutrient broth have been used as an indicator for the assay of pyocin S2 on nutrient agar plates (*11*). Although they were sensitive to pyocin S2 by the spot test method on nutrient agar, we often observed that such cultures showed a considerable survival ratio when treated with pyocin S2 in liquid media. For biochemical studies of the action mechanism of pyocin S2, a cell suspension fully sensitive to pyocin in the liquid is required. In the previous studies, we used indicator cells grown in glutamate medium without addition of heavy metal ions. However, it was difficult to obtain a fully sensitive culture reproducibly, probably because of iron contamination of the water or chemicals. As described in this paper, we found that the sensitivity of a culture to pyocin S2 depended upon the iron concentration in the growth medium, and conditions required to grow a fully sensitive culture were determined. Iron concentrations of 1 μM or less in the glutamate medium are required to produce sensitivity; cultures with 10 μM or more $FeSO_4$ are mostly insensitive to the pyocin (Fig. 4). Insensitive cultures could be made sensitive by growing them for one generation under iron-deprivation (Fig. 5).

However, iron-rich cultures are not totally insensitive to pyocin S2: about 30% of the cells were killed under the conditions employed (Fig. 3), and even more on prolonged incubation with an excess of pyocin. On the other hand, iron-deficient cultures contained an insensitive population amounting to about 0.1–1%, which was not genetically resistant, under our experimental conditions. These findings suggest that some physiological factors of cells (*e.g.*, growth phase) might also be responsible for the sensitivity or insensitivity to the pyocin. The sensitivity to pyocin S2 on agar plates was not greatly influenced by the growth conditions of indicator cells.

The cells grown under iron-rich conditions showed reduced capacity to adsorb pyocin as compared with the iron-limited cells (Table I). These data suggest that the amount of receptor on the cell surface for adsorption of pyocin depends on the growth conditions: failure to synthesize the receptor substance is the reason for the apparent insensitivity.

The receptor substance for R-type pyocins and some bacteriophages of *P. aeruginosa* has been identified as a lipopolysaccharide (*12–14*). On the other hand, the nature of receptors for S-type pyocins has not been investigated. We found that

at least four protein bands appeared in response to iron limitation in the Triton-insoluble fraction, using Schnaitman's procedure (*10*). The localization of these proteins in the outer membranes is clear, since the same bands appeared in the separated outer membrane of strain PAO under similar conditions, and the profile of protein bands of that fraction was essentially the same as those of the outer membranes of strains PAO and PML15 (*1*). The present study suggests that among the four proteins responding to iron limitation, one, designated here as Fe-b, is the most likely candidate for the pyocin S2 receptor. The bases for this inference are as follows. The presence of protein Fe-b in the outer membrane corresponded to the appearance of sensitivity to pyocin S2. All of the 9 mutants isolated as resistant to pyocin S2 were deficient in Fe-b protein (Fig. 6). These mutants had lost the capacity to adsorb pyocin S2 (Table I).

In addition to Fe-b, a few other protein bands were found in the outer membrane of *P. aeruginosa* in response to iron limitation during growth (Fig. 6). These proteins might function in iron transport.

With regard to iron uptake, three high-affinity systems are known in *E. coli*; enterochelin-mediated (*feu* B), ferrichrome-mediated (*ton* A), and citrate-mediated (*cit*) systems (*2, 3*). The product of *feu* B gene is an outer membrane protein which acts as a receptor for either iron-enterochelin complex or colicins B and D (*15, 16*). The *ton* A product is also a protein in the outer membrane and is a receptor for either ferrichrome or colicin M or phages such as T1 or T5 (*2*). The amounts of *feu* B (81K=O-2b) and the two other proteins in the outer membrane are regulated by the amounts of iron associated with the cell (*17*) or present in the growth medium (*18*). Pugsley and Reeves reported (*19*) an increased receptor activity for colicin B in cells grown under iron limitation, although the cells grown with iron possessed sufficient receptors to be killed by colicin B. Competition for the receptor between colicins and ferri-enterochelin has been demonstrated by Guterman with colicin B (*20*) and by Pugsley and Reeves with colicin D (*21*).

Fe-b protein in *P. aeruginosa* seems to be analogous to *feu* B in *E. coli*, since the syntheses of both proteins are controlled by iron in the growth media, and both serve as receptors for certain bacteriocins, although the nature and action mechanism of pyocin S2 are quite different from those of colicins B (*20*), D (*22*), or M (*23*). Incidentally, a phage which seems to have a common receptor specificity with pyocin S2 has been found. The 9 mutants which were resistant to pyocin S2 and lacked Fe-b were all resistant to phage ϕSLF7, which is one of the typing phages selected by Sjöberg and Lindberg (*24*) (data not shown).

However, it is not yet clear whether Fe-b serves as a receptor for iron transport. The supernatant of overnight culture of PML1550 in an iron-limited medium contained a dye with yellow-green fluorescence, while that of iron-rich cultures showed no fluorescence. This dye may be a chelator for iron, as reported by Cox and Graham (*25*). We investigated whether there was any competition between pyocin S2 and iron ions or iron-chelator complex. In our preliminary experiments, no inhibition of the killing action of pyocin S2 was observed by either ferric or ferrous ions with or without the fluorescent supernatant. Therefore, Fe-b protein appears not to be the receptor for the fluorescent dye-iron complex. It might be a receptor for some other kind of iron complex. More detailed studies are needed in this connection. The nature of other proteins which respond to iron deficiency is of interest, but remains to be investigated.

Some pyocin S2-resistant mutants lacked Fe-d protein, and others lacked protein D (Fig. 6), which is one of the major proteins of the outer membrane (its synthesis is regulated by glucose) (*1*). It is not yet clear what relationships exist between these mutations and the pyocin sensitivity.

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