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Genetic Organization and Transcriptional Analysis of a Major Gene Cluster Involved in Siderophore Biosynthesis in *Pseudomonas putida* WCS358

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In iron-limited environments, the plant-growth-stimulating *Pseudomonas putida* WCS358 produces a yellow-green fluorescent siderophore called pseudobactin 358. The transcriptional organization and the iron-regulated expression of a major gene cluster involved in the biosynthesis and transport of pseudobactin 358 were analyzed in detail. The cluster comprises a region with a minimum length of 33.5 kilobases and contains at least five transcriptional units, of which some are relatively large. The directions of transcription of four transcriptional units were determined by RNA-RNA hybridization and by analysis in *Escherichia coli* minicells. The latter also demonstrated that large polypeptides were encoded by these transcriptional units. The results allowed us to localize several promoter regions on the DNA. The iron-dependent expression of at least two genes within this cluster appears to be regulated at the transcriptional level.

The availability of iron in aerobic environments at neutral pH (e.g., soil) is limited because much of it is present as various polymers of ferric hydroxides with low solubility. Since iron is an essential component of many important enzyme systems in all living cells, most microorganisms have evolved mechanisms for acquiring iron to sustain their growth. They synthesize and excrete siderophores [i.e., low-molecular-weight high-affinity iron(III)-chelating agents] that form complexes with iron(III), which then are taken up specifically via high-affinity transport systems (32).

When grown under iron-limited conditions, fluorescent *Pseudomonas* spp. produce yellow-green fluorescent siderophores, which are called pyoverdines or pseudobactins (12, 14, 35). All the characterized siderophores have a common structure consisting of a specific oligopeptide linked to a fluorescent chromophore, a quinoline derivative. We are investigating the biosynthesis of pseudobactin 358, the siderophore of the rhizosphere-colonizing *Pseudomonas putida* WCS358 (29). Recently, its chemical composition and structure have been determined. In the nonapeptide, lysine, threonine, alanine, serine, aspartic acid, β-hydroxyaspartic acid, and N^{δ}-hydroxyornithine are present in a 2:2:1:1:1:1:1 ratio. The last two amino acids, together with the dihydroxyquinoline moiety, probably deliver the three bidentate iron(III)-chelating groups (G. A. J. M. van der Hofstad et al., manuscript in preparation). Pseudobactin 358 has a relatively high affinity for iron(III), as demonstrated by its affinity constant for this cation, which is 2.10^{25} M^{-1} at pH 7.0.

P. putida WCS358 can reduce crop yield losses caused by deleterious microorganisms (both bacteria and fungi) present in the root environment (18). Such yield losses occur especially under conditions of increased cropping frequencies (33). The protective activity is thought to be caused by the production and excretion of siderophores by the cells of WCS358 and other pseudomonads, which efficiently chelate the iron(III) in the root environment. The deleterious microorganisms are deprived of iron(III) and prevented from growing efficiently (18, 23).

This hypothesis has recently been tested with mutants disturbed in siderophore biosynthesis; siderophore-defective Tn*5* insertion mutants of the root-colonizing *P. putida* WCS358 were unable to stimulate potato plant growth or increase tuber yield, whereas wild-type WCS358 was very effective in both aspects (6, 7).

For a better understanding of the relationship between growth stimulation and the production of siderophores, more knowledge of the organization and regulation of siderophore biosynthesis is needed. We have previously described the isolation of mutants defective in the biosynthesis of pseudobactin 358 after mutagenesis with transposon Tn*5* (29). Complementation of these mutants with the cosmid clones of a genomic library of WCS358 resulted in the identification of five separate gene clusters involved in siderophore biosynthesis. The major gene cluster A, which measures 33.5 kilobases (kb) and comprises six cosmid clones complementing Flu^- Sid^+ and Flu^- Sid^- mutants, was analyzed in more detail. In this report, the transcriptional organization and the iron-regulated expression of this gene cluster were studied.

MATERIALS AND METHODS

Bacterial strains and plasmids. Wild-type *P. putida* WCS358 and its siderophore-defective Flu^- Sid^- and Flu^- Sid^+ mutants have been described previously (29). *Escherichia coli* strains and plasmids used in this study are listed in Table 1. The pMA1- and pMA3-derived subclones in pKT240 and pSPT18 are given in Fig. 1C.

Media and growth conditions. *E. coli* and *Pseudomonas* strains were grown as described previously (29). Concentrations of antibiotics (in micrograms per milliliter) were: ampicillin, 100 (for *E. coli*) and 500 (for *Pseudomonas* strains); kanamycin, 25; nalidixic acid, 25; tetracycline, 25 (all from Boehringer Mannheim Biochemicals). For growth under iron-rich or iron-limited conditions, King B (KB) medium (29) was supplemented with 100 μM $FeCl_3$ or 400 μM

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TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Relevant properties	Reference or source
Strains		
HB101	*recA hsdR hsdM* Str^r Pro Leu Thi	10
JA221	*recA hsdA* derivative of K-12 C600	11
P678-54	Minicell producing	1
Plasmids		
pKT240	Ap^r Km^r Mob$^+$ IncP4	5
pRK2013	Km^r Mob$^+$ Tra$^+$	17
pSUP202	Cm^r Ap^r Tc^r Mob$^+$	34
pSUP2021	Cm^r Ap^r Km^r Tc::Tn*5* Mob$^+$	34
pSPT18	Transcription vector with SP6 and T7 promoters	Pharmacia
pMA1	Tc^r Mob$^+$; comprises 25.5-kb left part of cluster A	29
pMA3	Tc^r Mob$^+$; comprises 27.4-kb right part of cluster A	29

2,2-bipyridyl (a nonutilizable iron chelator), respectively (both from BDH).

DNA manipulations. Basic techniques (e.g., plasmid isolation, restriction endonuclease mapping, gel electrophoresis, electroelution, ligation, transformation, and nick translation) were performed as described by Maniatis et al. (28). Isolation of chromosomal DNA and Southern hybridization were carried out as described previously (29). Wild-type *Hin*dIII, *Xho*I, *Bgl*II, and *Eco*RI subclone fragments were obtained from cosmids pMA1 and pMA3 and ligated into plasmids pKT240 and pSPT18. The recombinant plasmids were transfected into *E. coli* JA221. Plasmid pAK25, with a 2.5-kb *Bgl*II-*Eco*RI insert, was constructed by deletion of a 4.9-kb *Eco*RI fragment (i.e., a 2.8-kb *Bgl*II-*Eco*RI fragment from the insert plus a 2.1-kb *Bam*HI-*Eco*RI fragment from the vector) from pAK22, containing a 5.3-kb *Bgl*II insert (Fig. 1C).

Complementation of mutants. Triparental matings were performed with the helper plasmid pRK2013 (16). Cells of donor *E. coli* JA221, harboring pKT240 recombinant plasmids, helper *E. coli* HB101(pRK2013), and each *P. putida* WCS358 siderophore-defective mutant were grown to late log phase (1.10^9 cells per ml), mixed in an Eppendorf tube (ratio, 1:1:1), and concentrated by short centrifugation (30 s). The mating mixture was carefully suspended in 50 to 100 μl of KB medium and spread on a prewarmed KB agar plate. After incubation for 3 to 16 h at 30°C, cells were suspended in 1 ml of KB medium, and appropriate dilutions were spread onto KB agar plates supplemented with ampicillin and nalidixic acid. After incubation for 16 to 24 h at 30°C, transconjugants were screened for complementation by checking fluorescence (29).

RNA isolation, Northern (RNA) transfers, and in vitro transcription. RNA was isolated from *P. putida* WCS358 mid-log-phase (A_{600}, 0.4 to 0.5) cultures (40 ml) by the hot-phenol method (2) and was stored at −20°C as an ethanol precipitate. RNA (20 μg) was analyzed by formaldehyde gel electrophoresis following formamide-formaldehyde denaturation as described elsewhere (28). Size-fractionated RNA was transferred onto nitrocellulose (Schleicher & Schuell, Inc.) as described by Thomas (36). Hybridization to radiolabeled RNA probes followed basically the procedures described by Melton et al. (30), except that the filters were hybridized at 60 to 65°C, with extensive washing at 68 to 70°C. The 7.6-kb *Hin*dIII fragment and the 5.3-kb *Bgl*II fragment from pMA3 were cloned into the multiple cloning site of the transcription vector pSPT18 (Pharmacia, Inc.) at the *Hin*dIII and *Bam*HI sites, respectively. The resulting recombinant DNAs were linearized downstream of the insert at a site in (*Sma*I or *Xba*I) or just behind (*Nae*I) the multiple cloning site before transcription was initiated at the upstream SP6 or T7 promoter to produce RNA runoff transcripts labeled with [^{32}P]UTP (30).

Protein synthesis in *E. coli* minicells. Minicells were isolated and labeled with [^{35}S]methionine (10 μCi) essentially as described by Andreoli et al. (3). Radiolabeled proteins were analyzed by autoradiography after electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (26).

RESULTS

Tn*5* integration sites. The construction of Tn*5* mutants in siderophore biosynthesis has been described elsewhere (29). These mutants were used to distinguish several transcriptional units in cluster A involved in this biosynthetic pathway. A restriction map of cluster A was constructed (Fig. 1B). A first determination of the site of integration of Tn*5* in each mutant was done by digestion of the mutant chromosomal DNA with the restriction enzyme *Eco*RI and separation of the fragments on agarose gels, followed by blotting onto nitrocellulose filters and hybridization with a ^{32}P-labeled cosmid clone of cluster A. Cosmids pMA1 and pMA3 (Fig. 1C) were chosen as probes, since they cover the entire region of cluster A and complement all nine mutants (29). The autoradiogram of the wild-type DNA (Fig. 2A, lane 4) shows a pattern that corresponds with the sizes of the fragments of the probe (i.e., pMA3) insert (13.5-, 4.8-, 3.4-, 3.2-, and 2.5-kb fragments). For the mutant chromosomal digests, however, the fragment which contains the Tn*5* insertion is approximately 5.8 kb larger, because Tn*5* does not have sites for the *Eco*RI restriction enzyme (13). The wild-type fragment was therefore replaced by a fragment with lower mobility on the gel. Hybridizations of chromosomal *Hin*dIII and *Xho*I digests (which have cleavage sites in the inverted repeats of Tn*5* at 1.195 and 0.485 kb, respectively, from the termini [13]) and the known restriction map of group A allow accurate mapping of the Tn*5* insertions relative to the flanking *Hin*dIII and *Xho*I sites. Only the results for the mutants JM101, JM205, and JM209 are given in Fig. 2, but a similar analysis was carried out for all mutants in this region. Mutants JM101, JM205, and JM209 did not have the wild-type *Eco*RI bands of 3.2, 13.5, and 3.4 kb, respectively, but had new bands of 9.0, 19.3, and 9.2 kb, respectively (Fig. 2A, lanes 1 to 3). The three wild-type *Hin*dIII bands of 15.8, 5.0, and 7.6 kb were replaced by new bands with different mobilities and approximate lengths of 5.0 kb (JM101), 4.4 and 3.0 kb (JM205), and 5.8 and 4.2 kb (JM209) (Fig. 2B, lanes 1 to 3). The *Xho*I pattern (Fig. 2C) showed differences from the wild-type bands of 13.5 kb for JM101, 9.3 kb for JM205, and 9.4 kb for JM209. The resulting positions within this cluster for all the Tn*5* insertions are given in Fig. 1A.

A number of other mutants, which did not map in cluster A by complementation, were also tested by hybridization. Two mutants, JM212 and JM217, were found to contain Tn*5* insertions inside the region of cluster A (Fig. 1A).

Hybridization of the *Eco*RI chromosomal digests of DNA from all the mutants with ^{32}P-labeled pSUP2021, the Tn*5*-containing plasmid (34) that was used to construct the mutant strains, as the probe demonstrated that the shifted

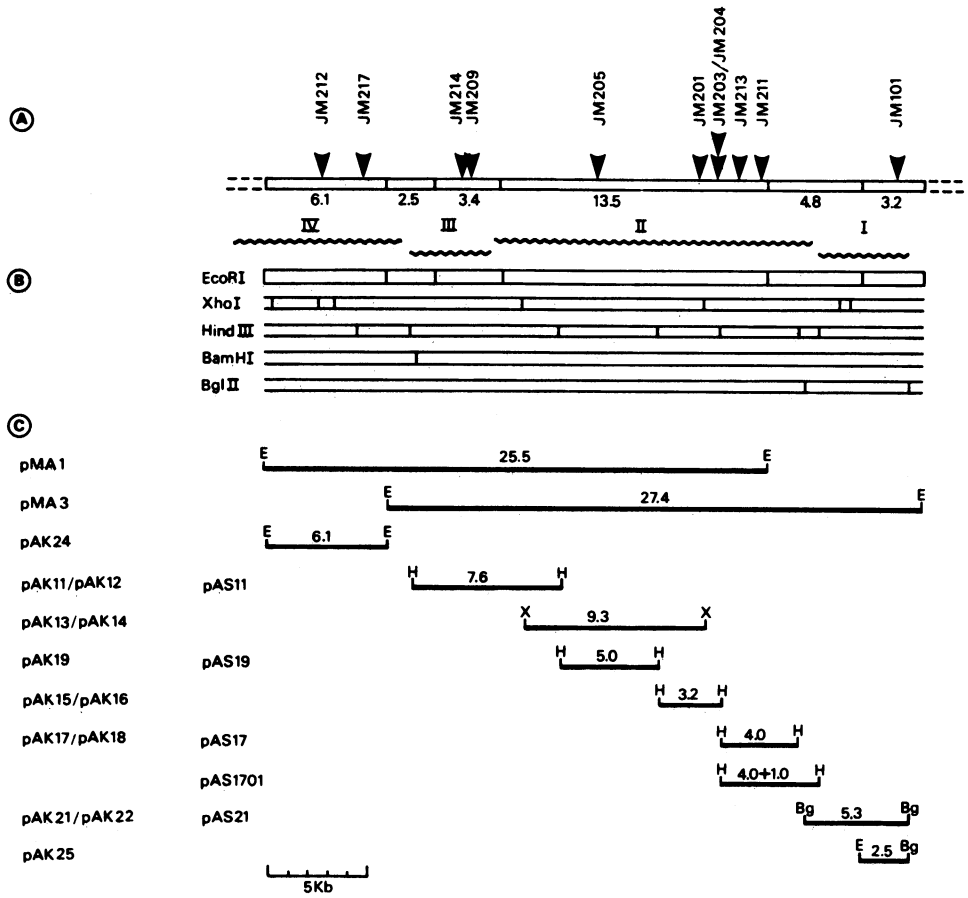

FIG. 1. Physical map of cluster A. The presence of transcriptional units I through IV and their approximate limits (~~~) were deduced from previous complementation data (29) and the mapping of the Tn*5* insertions of 11 siderophore-defective mutants (see text). ▼, Tn*5* integration sites. The sizes of the *Eco*RI fragments on the map are given in kilobases. (B) Restriction map of cluster A. The map was constructed by combining the mapping data of the cosmids pMA1 and pMA3 (29). (C) Schematic overview of the subclones of cluster A that were constructed in this study. The inserts of the subclones and of the cosmids pMA1 and pMA3 are shown. E, *Eco*RI; H, *Hin*dIII; X, *Xho*I; Bg, *Bgl*II. The fragments were cloned into the broad-host-range vector pKT240 (first column), and some of them were also cloned in the transcription vector pSPT18 (second column). The numbers denote the fragment sizes in kilobases.

bands (Fig. 2A, lanes 1 to 3) indeed contained Tn*5*-specific DNA, as shown in Fig. 2D for mutants JM101, JM205, and JM209. Hybridization with a probe that only contained the DNA of the vector and no Tn*5* DNA showed that all mutants tested acquired only the Tn*5* insertion and no measurable lengths of vector DNA (data not shown).

Complementation experiments. Previous complementation experiments showed the presence of at least three transcriptional units in cluster A (29). Transcriptional unit I (Fig. 1A) overlaps part of a 3.2-kb *Eco*RI fragment in which the mutation of JM101 resides and which was shown to be necessary for complementation of JM101. A second, large

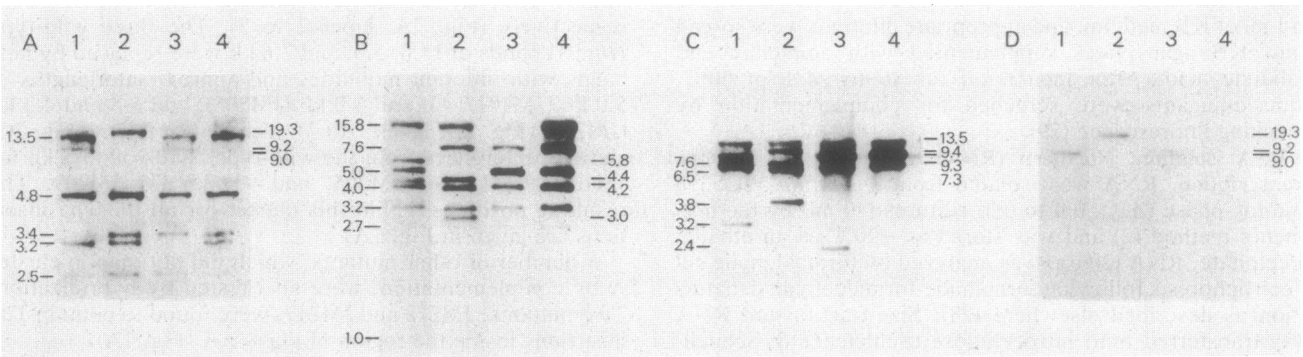

FIG. 2. Southern blot analysis of representative *P. putida* WCS358 siderophore-defective Tn*5* mutants. Total genomic DNA (10 μg) was digested with *Eco*RI (A and D), *Hin*dIII (B), and *Xho*I (C), run on 0.8% agarose gels, transferred to nitrocellulose, and hybridized with the ^{32}P-labeled cosmid pMA3 (A, B, and C) or the ^{32}P-labeled plasmid pSUP2021 (D). Lanes: 1, JM209; 2, JM205; 3, JM101; 4, WCS358 wild type. Fragment sizes are given in kilobases.

transcriptional unit (unit II) stretches out on the *Eco*RI 13.5-kb fragment, in which the mutations of JM201, JM203, JM204, JM205, JM211, and JM213 are located, and on the adjacent 4.8-kb fragment, which is essential for complementation. At the other end, a part of the flanking 3.4-kb fragment contributes to this transcriptional unit (J. D. Marugg et al., manuscript in preparation). Transcriptional unit III is located on the *Eco*RI fragments of 2.5 and 3.4 kb, since the latter fragment carries the Tn*5* insertions of mutants JM209 and JM214, while the first fragment is needed for restoration of the wild-type phenotype. The hybridization results with the mutants JM212 and JM217 show the presence of an additional transcriptional unit. This fourth transcriptional unit (unit IV) covers the *Eco*RI 6.1-kb fragment and extends outside cluster A, as mutants JM212 and JM217, both with insertions in this fragment, were not complemented by any of the cosmids of cluster A. Furthermore, these (and the following) results suggest that it is not very likely that the rescue of the mutants after introduction of cloned wild-type DNA fragments occurs via homologous recombination; rescue has to be the result of true complementation. This implies that conclusions that indicate the possible presence of transcriptional units on such fragments are legitimate. The correlated physical and genetic map, with the approximate limits of the transcriptional units, is shown in Fig. 1A.

To determine more precisely the transcriptional organization of this region, several subclones were constructed that contained (parts of) the transcriptional units of cluster A. The mobilizable, broad-host-range plasmid pKT240 (5) was used as a vector (Fig. 3). *Hin*dIII and *Xho*I fragments, derived from cosmids pMA3 and pMA1, were cloned in the corresponding sites inside the kanamycin resistance gene, and a 5.3-kb *Bgl*II fragment was ligated in the *Bam*HI-site just downstream of the gene. *Eco*RI subfragments were cloned in front of the promoterless streptomycin resistance gene present in pKT240. The cloned fragments, together with their positions on the map, are given in Fig. 1C.

Each of these plasmid clones was mobilized, by use of a pRK2013-containing helper strain (17), to the corresponding siderophore-defective mutant. Transconjugants were screened for restoration of yellow-green fluorescence on iron(III)-limited medium as a measure of siderophore biosynthesis (29).

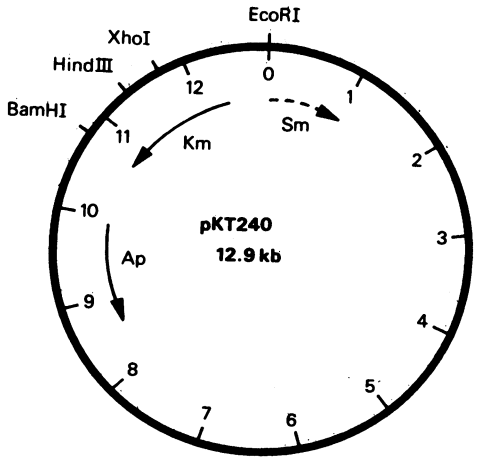

FIG. 3. Broad-host-range vector pKT240 (5). The plasmid encodes genes for kanamycin resistance (Km) and ampicillin resistance (Ap). The streptomycin resistance gene (Sm) lacks a functional promoter. Only the restriction sites used in this study are shown.

Both plasmids pAK21 and pAK22, with the same 5.3-kb *Bgl*II fragment in different orientations, complemented mutant JM101. Based on the polarity of Tn*5* insertions (13), this means that a complete transcriptional unit is present on this cloned fragment. The 3.2-kb *Eco*RI fragment that overlaps the right half of this fragment (Fig. 1C) contains the Tn*5* insertion in JM101 and was needed for proper complementation of JM101. Therefore, a subclone was constructed to check if this region of overlap was sufficient for complementation. The resulting plasmid, pAK25, with the *Bgl*II-*Eco*RI insert of 2.5 kb, indeed restored the mutation of JM101 and limited the position of the transcriptional unit to between the borders of this subclone.

The plasmids pAK11 and pAK12 both contain the *Hin*dIII 7.6-kb fragment but in different orientations. Both complemented the mutants JM209 and JM214. This demonstrates the presence of a complete transcriptional unit within the 7.6-kb insert of the plasmids. The mapping data for JM209 and JM214 in combination with the previous complementation results demonstrate that at least parts of the 3.4- and 2.5-kb *Eco*RI fragments were needed. Therefore, this transcriptional unit covers the border parts of the two fragments.

The *Hin*dIII fragments of 5.0, 3.2, and 4.0 kb and a 9.3-kb *Xho*I fragment are part of the presumed large transcriptional unit in the middle of cluster A (Fig. 1). Each subfragment was cloned in both directions in pKT240 (with the exception of the 5.0-kb *Hin*dIII fragment, which was found in only one orientation) and tested for complementation of mutants JM201, JM203, JM204, JM205, JM211, and JM213. None of these fragments in pAK13 through pAK19 (Fig. 1C) was able to complement any of the six mutants, whereas cosmids with inserts which entirely overlap the four subclone fragments (e.g., pMA3) complemented each of them. This again shows the presence of a large transcriptional unit on the 13.5-kb *Eco*RI fragment that extends within or beyond the 4.0-kb *Hin*dIII fragment at the right end.

The *Eco*RI 6.1-kb fragment in pAK24 did not rescue the mutants JM212 and JM217. This confirms earlier complementation data of pMA1 and shows that this fragment contains only a part of the transcriptional unit that was disturbed in the two mutants.

RNA-RNA hybridization. The direction of transcription and the approximate location of the transcriptional regulation regions (promoters and operators) of transcriptional units I and III (Fig. 1A) were determined by hybridization of total cellular RNA with single-stranded RNA probes with known polarity. The 7.6-kb *Hin*dIII fragment and the 5.3-kb *Bgl*II fragment from cluster A were cloned into the transcription vector pSPT18, resulting in pAS11 and pAS21, respectively. With this vector, which contains both the SP6 and T7 promoters, each strand of a cloned insert can be transcribed into an (radioactive) RNA molecule in vitro. This RNA was used as a probe in a Northern hybridization experiment; only RNA with opposite polarity hybridized to such a probe, and this yielded the direction of transcription of these DNA regions.

Total RNA was isolated from cultures of wild-type WCS358 which were grown under iron(III)-rich and -limited conditions; the RNA was fractionated on a denaturing agarose gel, blotted to nitrocellulose, and subsequently hybridized separately with each of the [^{32}P]UTP-labeled RNA probes derived from both strands of the inserts of pAS11 and pAS21. Only the pAS11-SP6-derived probe (Fig. 4A, lane 2) and the pAS21-SP6-derived probe (lane 3) hybridized spe-

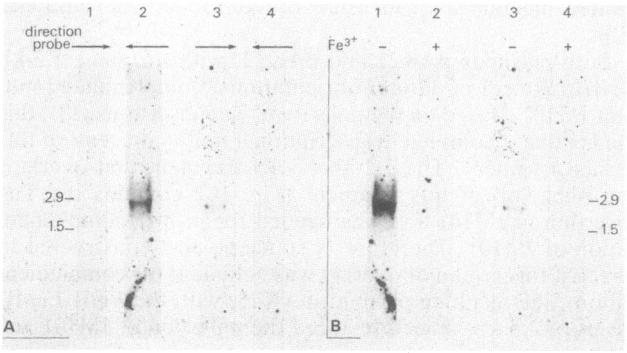

FIG. 4. (A) Northern blot analysis of total RNA (20 μg) from *P. putida* WCS358 grown under iron(III)-limited conditions by using in vitro-synthesized ^{32}P-labeled RNA probes with different polarities. Probes were prepared from the *Hin*dIII 7.6-kb insert of pAS11 by using the T7 promoter (lane 1) and the SP6 promoter (lane 2) and from the *Bgl*II 5.3-kb insert of pAS21 by using the SP6 promoter (lane 3) and the T7 promoter (lane 4). (B) Northern blot analysis of total RNA (20 μg) from *P. putida* WCS358 grown under iron(III)-limited (lanes 1 and 3) and iron(III)-rich (lanes 2 and 4) conditions by using pAS11-SP6-derived (lanes 1 and 2) and pAS21-SP6-derived (lanes 3 and 4) ^{32}P-labeled RNA molecules as probes. The sizes of rRNA markers are given in kilobases.

cifically with the blotted RNA from cells grown under iron-limited conditions. In both cases, the hybridizing bands correspond with mRNAs of about 2.4 to 2.8 kb. (For the directions of transcription deduced from the results for these fragments, see Fig. 6.) As described below, the complementation data and the expression in minicells demonstrate the presence of two independent transcriptional units on the 5.3-kb *Bgl*II fragment. Therefore, the detection of only one band with the pAS21-derived probe was unexpected. It may well be that the hybridized band represents two different transcripts with equal lengths.

To determine whether iron regulation takes place at the transcriptional level, the correct probes were also hybridized with the RNA that was extracted from iron-rich cultures. Hybridization was only observed when RNA was isolated from cultures which were grown under iron(III)-limited conditions (Fig. 4B, lanes 1 and 3) and not when RNA was isolated from cultures grown in iron(III)-rich conditions (lanes 2 and 4). This means that expression of the genes of these transcriptional units is regulated at the transcriptional level.

Since the 7.6-kb *Hin*dIII fragment also overlaps with a part of the large transcriptional unit (unit II), it should be possible to detect the corresponding RNA (and identify its direction of transcription) with the probe. However, no large band could be detected on the autoradiogram, but a faint smear of bands was visible (Fig. 4A, lane 2). The reason for this may be the instability of this large transcript. The faint smear of bands may represent degradation products of this transcript.

Expression in *E. coli* minicells. The translational capacities of the different transcriptional units were analyzed by in vivo expression of the various plasmid subclones in a minicell-producing strain of *E. coli*, P678-54 (1) (Fig. 5).

Both pAK11 and pAK12, with different orientations of the same 7.6-kb *Hin*dIII fragment, caused the appearance of two new large polypeptides with apparent molecular weights of 115,000 (the 115K polypeptide) and 145,000 (145K polypeptide) (Fig. 5A, lanes 2 and 3). The polypeptide product from the kanamycin resistance gene of pKT240 (Fig. 5A, lane 1) disappeared in both clones. Both precursor and mature proteins from the ampicillin resistance gene remained unchanged. Furthermore, pAK11 and pAK12 also produced

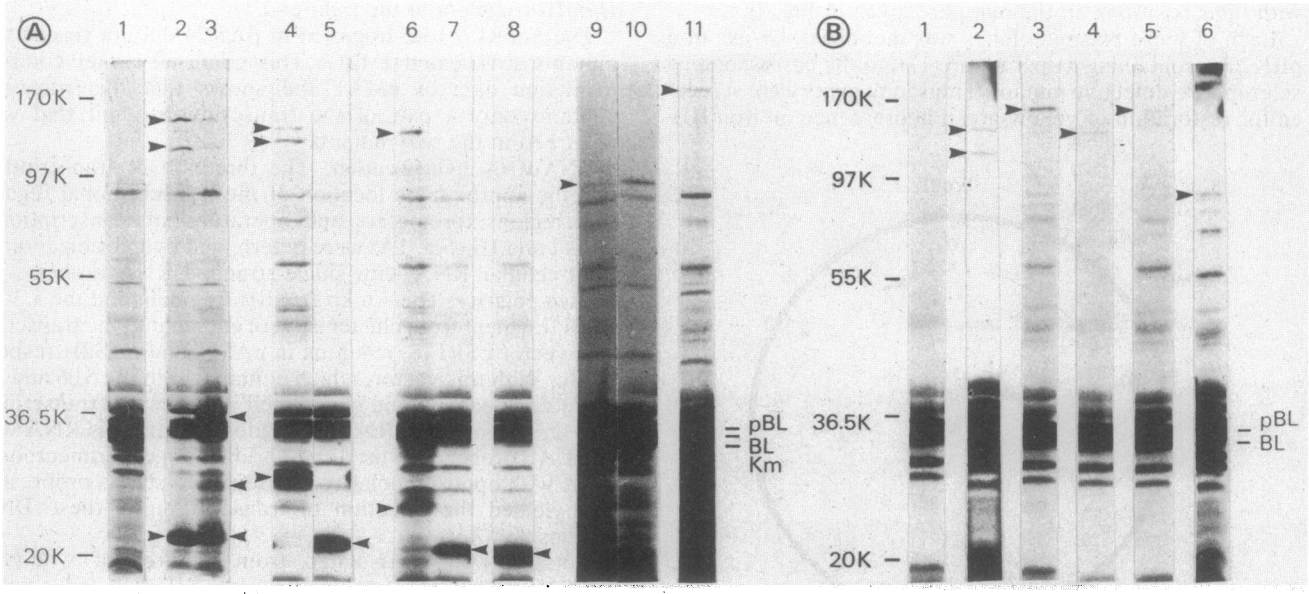

FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ^{35}S-labeled polypeptide products in minicells containing pKT240-derived (A) or pSPT18-derived (B) subclones of cluster A (see also Fig. 1). The polypeptides marked with arrowheads are described in the text. Molecular size marker proteins were macroglobulin (reduced; 170K), phosphorylase *b* (97K), glutamate dehydrogenase (55K), lactate dehydrogenase (36.5K), and trypsin inhibitor (20K). Major polypeptides encoded by the vectors are pre-β-lactamase (pBL), β-lactamase (BL), and the kanamycin resistance gene product (Km). Lanes in panel A: 1, vector control pKT240; 2, pAK11; 3, pAK12; 4, pAK15; 5, pAK16; 6, pAK17; 7, pAK18; 8, pAK19; 9, pAK22; 10, pAK21; 11, pAK24. Lanes in panel B: 1, vector control pSPT18; 2, pAS11; 3, pAS19; 4, pAS17; 5, pAS1701; 6, pAS21.

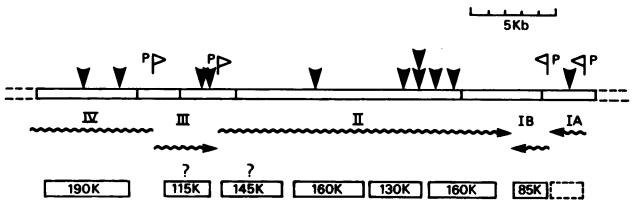

FIG. 6. Genetic organization of cluster A. ⇝, Localization and orientation of the transcriptional units (units IA, IB, II, III, IV); ▼, Tn*5* insertions in the siderophore-defective mutants on the *Eco*RI restriction map; ◁, possible promoter regions; [115K] proposed locations (and sizes) of the polypeptide products encoded by the different subclones (see also Discussion); [---] protein of unknown size.

several low-molecular-weight polypeptides of about 21,000 (pAK11) and 21,000, 25,000, and 35,000 (pAK12), which probably represent artificial proteins or products that are the result of fusion of the kanamycin resistance gene with insert DNA stretches. When the inserts were expressed in another vector (pSPT18), these small polypeptides were absent but the 115K and 145K proteins were present (Fig. 5B, lane 2). Nevertheless, the expression of the 115K and 145K polypeptides from both pAK11 and pAK12 means that at least one promoter must be present on the insert, which is in agreement with the complementation data obtained with these clones. Since the fragment was cloned downstream of the kanamycin resistance gene promoter, expression can still depend to a large extent on the orientation of the insert with respect to this promoter. This may well be the reason why the expression of both polypeptides was much more pronounced in pAK11 than it was in pAK12. In pAK12, the direction of transcription is toward the kanamycin promoter.

Subclones pAK15 through pAK19 contain three *Hin*dIII fragments of 3.2, 4.0, and 5.0 kb (Fig. 1), which are part of the presumed large transcriptional unit, in different orientations. Clone pAK15, with a 3.2-kb *Hin*dIII fragment (Fig. 5A, lane 4), and clone pAK17, with a 4.0-kb *Hin*dIII fragment (Fig. 5A, lane 6), expressed 130K and 140K (pAK15) and 140K (pAK17) polypeptides. Considering the length of the cloned insert of pAK15 (3.2 kb), which is too small to encode both proteins separately, it is likely that this phenomenon is caused by readthrough or by an internal translation initiation event. The other orientations of the 3.2- and 4.0-kb fragments (in pAK16 and pAK18, respectively), as well as pAK19 carrying a 5.0-kb *Hin*dIII fragment, did not produce any of the large extra products (Fig. 5A, lanes 5, 7, and 8). All five clones produced some low-molecular-weight proteins of 21,000 to 27,000, most likely representing fusion or artificial products as described above (Fig. 5A, lanes 4 to 8). Also, the protein of the kanamycin resistance gene was not produced. The 5.0-kb insert of pAK19 was cloned in plasmid pSPT18, giving pAS19, and in minicells it produced a 160K protein (Fig. 5B, lane 3). The expression pattern of the pKT240 derivatives suggests that the fragments contain no promoter and that the observed expression probably is dependent on transcription from the kanamycin resistance gene promoter of the vector. This is in agreement with the complementation data of these fragments, which suggest the existence of a large transcriptional unit. Furthermore, the cloned fragments which gave rise to the polypeptides have the same polarity, and this suggests that the direction of transcription is toward the 4.8-kb *Eco*RI fragment (Fig. 6). As in pAK17, plasmid pAS17 contains the 4.0-kb *Hin*dIII fragment and gave rise to a 140K protein (Fig. 5B, lane 4). However, in minicells containing pAS1701 with a partial *Hin*dIII fragment of the same 4.0 kb and a flanking 1.0-kb fragment, the 140K protein was not produced, but instead, a 160K protein was expressed (Fig. 5B, lane 5). This again indicates the presence of a transcriptional unit which is directed toward the 4.8-kb fragment.

Both orientations of a 5.3-kb *Bgl*II fragment (Fig. 5A, lanes 9 and 10) cloned in pKT240 revealed the synthesis of an 85K polypeptide, demonstrating the presence of a promoter on this fragment, as could have been concluded from the complementation data. The expression pattern displayed the same phenomenon as with pAK11 and pAK12. This suggests a direction of transcription as shown in Fig. 6, which is in agreement with the previously described results. The pSPT18 derivative pAS21 also produced this 85K protein (Fig. 5B, lane 6). The 85K protein was not produced by pAK25, which carries the right 2.5-kb *Eco*RI-*Bgl*II half of the 5.3-kb fragment (data not shown). This means that the gene of the 85K protein is located in the left end of the 5.3-kb *Bgl*II fragment, with a start close to the *Eco*RI site in the middle (Fig. 6, IB). Moreover, this implies that this gene is on a different transcriptional unit than the one that was disturbed in mutant JM101, which is positioned in the right side of the fragment (Fig. 6, IA).

The transcriptional unit overlapping the 6.1-kb *Eco*RI fragment, as represented by pAK24, gave rise to a polypeptide of about 190,000 (Fig. 5A, lane 11). Since we cloned only one orientation of this fragment and expression may be influenced by vector moieties, no conclusions concerning the direction of transcription can be drawn here.

The tentative localization and sizes of the polypeptide products encoded by the different plasmid subclones are shown in Fig. 6.

DISCUSSION

A major gene cluster involved in the biosynthesis of pseudobactin 358, the yellow-green fluorescent siderophore of *P. putida* WCS358, has been analyzed in more detail. The mapping of the Tn*5* integration sites of 11 siderophore-defective mutants, together with the complementation data presented here, demonstrates that a region of at least 33.5 kb is required for synthesis of this compound. The genetic information continues beyond one end of the region, as two mutants, JM212 and JM217, with insertions located near this end could not be complemented by any of the cosmids or subclones. Recently, the other end of the region was extended by 18 kb by the isolation of a cosmid clone from a WCS358 genomic library which overlaps the *Eco*RI fragments of 4.8 and 3.2 kb (van der Hofstad et al., in preparation). Besides the biosynthetic genes, this overlap also contains DNA information for the uptake and utilization of pseudobactin 358. More specifically, the 85K protein (Fig. 6) probably serves as the outer membrane receptor protein for ferric pseudobactin 358 (van der Hofstad et al., in preparation). Altogether, the data presented here demonstrate that several genes for production and transport of pseudobactin 358 are clustered in a limited region of the *P. putida* WCS358 genome. However, our previous work (29) already demonstrated the involvement of five different gene clusters in siderophore biosynthesis. Other investigators have shown that several gene clusters are involved both in the production of a fluorescent compound in a *Pseudomonas syringae* isolate (24) and in the biosynthesis of pseudobactin, the siderophore of *Pseudomonas* sp. strain B10 (31). One of the gene clusters of *Pseudomonas* sp. strain B10 was also found

to contain the gene coding for the outer membrane receptor protein. The gene is on a separate operon and flanked on both sides by biosynthetic genes (27). In *Pseudomonas aeruginosa*, PAO mutants defective in biosynthesis of pyoverdine have been obtained, and their mutations were mapped on the PAO genome. One set of mutations was mapped at 65 to 70 min on the chromosome (4, 21), while a second set had a map position of 35 min (21). In general, it seems that in the different fluorescent *Pseudomonas* species, the biosynthetic genes of these fluorescent compounds are dispersed around the genome, although large regions with clustered genes are present.

The transcriptional organization of cluster A was analyzed. We have shown that at least five transcriptional units are present in this region (Fig. 6, IA, IB, II, III, and IV). From the combined data from the Tn*5* mapping and the complementation experiments, the presence of transcriptional units IA, II, III, and IV was deduced. The minicell analysis demonstrated the presence of a fifth transcriptional unit (unit IB), located near transcriptional unit IA. The direction of transcription of units IA, IB, and III was determined by RNA-RNA hybridization by using in vitro-labeled RNA probes. The direction of transcription of unit II could be deduced from the minicell analysis. Transcriptional units IA and IB have orientations which are opposite to those of transcriptional units II and III. The transcriptional orientation of transcriptional unit IV was not determined. Some of the transcriptional units are remarkably long; transcriptional unit II seems to cover the *Eco*RI fragment of 13.5 kb entirely, while transcriptional unit IV seems to be at least 5 kb. It is not yet clear whether these transcriptional units form large open reading frames or consist of operonlike structures with multiple genes. The proteins which are produced in minicells do not have to be complete products of the biosynthetic pathway, but they may be truncated forms of one or several large proteins. This was concluded from pAS17 and pAS1701 expression; a 140K protein, produced by pAS17, was not produced by pAS1701, which instead produced a 160K protein, suggesting that the 140K protein is a truncated gene product. In a similar way, it is quite possible that proteins produced by the other subclones are incomplete, making it difficult to determine precisely the total number of genes on this cluster.

In *P. putida* WCS358, pseudobactin 358 is produced under iron-limited conditions (29). Under these low-iron conditions, certain outer membrane proteins are also expressed specifically (15). By RNA-RNA hybridization, we have shown that the expression of some of the genes within cluster A was regulated by iron at the transcriptional level. This effect of iron can be mediated via a system with a regulator protein (activator or repressor) which allows initiation of transcription strictly under iron-limited conditions. This implies that the promoter or operator regions of the respective genes contain specific features that are recognized by such a regulator protein in the absence or presence of iron. Another form of regulation may be an effect on the mRNA level by an increased rate of mRNA degradation. Since the positions and directions of transcription of four transcriptional units in cluster A have been determined, we have been able to localize the promoter regions on the DNA (Fig. 6). We are now analyzing these promoter regions in promoter probe vectors by the construction of transcriptional fusions with the structural genes for β-galactosidase (*lacZ*) and catechol-2,3-dioxygenase (*xylE*). These fusions will be used to identify the structure of the promoter regions and to isolate regulation-defective mutants. The isolation of the regulator gene itself will be very useful in the elucidation of the mechanism of this iron regulation. In *P. aeruginosa*, the production of exotoxin A and other extracellular products is also iron regulated (8, 9). It has been demonstrated that exotoxin A expression is regulated by iron at the transcriptional level (19, 25). Whether this occurs via a negative or a positive control mechanism is not completely clear, but some evidence is provided (19, 20) which favors the latter possibility.

In general, the expression of *Pseudomonas* genes in *E. coli* is rather poor, probably because *Pseudomonas* promoters are not recognized well by the *E. coli* transcription machinery (22). Our results confirm this phenomenon; in minicells, the expression of most *Pseudomonas*-specific proteins was poor and depended a great deal on vector moieties, such as the promoter of the kanamycin resistance gene in pKT240. Thus, either there is a lack of recognition of expression signals (transcriptional and/or translational) by *E. coli* or a regulatory factor (activator) is absent in *E. coli*.

With the exception of the 85K protein, we cannot assign (biosynthetic) functions to the genes of this cluster. However, mutant siderophores from JM203, JM217, and JM101 were purified, and their amino acid compositions were determined (G. A. J. M. van der Hofstad, unpublished results). This analysis demonstrated that all contain the complete wild-type peptide. This is an indication that the mutants of cluster A are probably defective in certain steps of the synthesis of the hydroxyquinoline-derived group. The structures of these and other mutant pseudobactins are being analyzed further in order to obtain more insight into the biosynthesis of pseudobactin 358.

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