JOURNAL OF BACTERIOLOGY, Jan. 1984, p. 53–58
0021-9193/84/010053-06$02.00/0

Vol. 157, No. 1

Cloning of Genes Involved in the Biosynthesis of Pseudobactin, a High-Affinity Iron Transport Agent of a Plant Growth-Promoting *Pseudomonas* Strain

JANE C. MOORES,[1]† MARILYN MAGAZIN,[1]‡ GARY S. DITTA,[2] AND JOHN LEONG[1]‡*

Departments of Chemistry[1] and Biology,[2] University of California, San Diego, La Jolla, California 92093

Received 22 August 1983/Accepted 21 October 1983

A gene bank of DNA from plant growth-promoting *Pseudomonas* sp. strain B10 was constructed using the broad host-range conjugative cosmid pLAFR1. The recombinant cosmids contained insert DNA averaging 21.5 kilobase pairs in length. Nonfluorescent mutants of *Pseudomonas* sp. strain B10 were obtained by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, ethyl methanesulfonate, or UV light and were defective in the biosynthesis of its yellow-green, fluorescent siderophore (microbial iron transport agent) pseudobactin. No yellow-green, fluorescent mutants defective in the production of pseudobactin were identified. Nonfluorescent mutants were individually complemented by mating the gene bank en masse and identifying fluorescent transconjugants. Eight recombinant cosmids were sufficient to complement 154 nonfluorescent mutants. The pattern of complementation suggests that a minimum of 12 genes arranged in four gene clusters is required for the biosynthesis of pseudobactin. This minimum number of genes seems reasonable considering the structural complexity of pseudobactin.

Specific rhizosphere-colonizing strains of the *Pseudomonas fluorescens-Pseudomonas putida* group have recently been used as seed inoculants on crop plants to promote growth and increase yields. These yellow-green fluorescent pseudomonads, generically termed plant growth-promoting rhizobacteria, rapidly colonize plant roots of a variety of crops and cause statistically significant yield increases (29). Plant growth-promoting rhizobacteria exert their plant growth-promoting activity in part by depriving certain native microflora of iron(III), thereby reducing microbial root colonization (18). Under iron-limiting conditions, they produce extracellular siderophores [microbial iron(III) transport agents] (26) which efficiently chelate environmental iron, making it less available to certain endemic microorganisms, thus inhibiting their growth. In addition, the plant growth-promoting strain *Pseudomonas* sp. strain B10 is an effective biological control agent of *Fusarium* wilt and take-all diseases, caused by the soil-borne fungi *Fusarium oxysporum* f. sp. *lini* and *Gaeumannomyces graminis* var. *tritici*, respectively (17). Pseudobactin, the yellow-green fluorescent siderophore of *Pseudomonas* sp. strain B10, apparently denies iron(III) to these pathogens, thus inhibiting their growth.

The molecular structure of pseudobactin consists of a linear hexapeptide, L-lysine-D-*threo*-β-hydroxyaspartic acid-L-alanine-D-*allo*-threonine-L-alanine-D-N^{δ}-hydroxyornithine, in which the N^{δ}-OH nitrogen of the ornithine is cyclized with the C-terminal carboxyl group, and the N^{ϵ}-amino group of the lysine is linked via an amide bond to a quinoline derivative, which is responsible for the yellow-green fluorescence of both pseudobactin and *Pseudomonas* sp. strain B10 (33). The iron-chelating groups consist of a hydroxamate group derived from N^{δ}-hydroxyornithine, an α-hydroxy acid from β-hydroxyaspartic acid, and an *o*-dihydroxy aromatic group derived from the quinoline moiety. The combination of metal-chelating ligands and the alternating L- and D-amino acids is unusual.

We have begun a study to understand the genetic organization of the high-affinity pseudobactin-dependent iron(III) assimilation system of *Pseudomonas* sp. strain B10. We describe here the construction of a gene bank of *Pseudomonas* sp. strain B10 DNA and the subsequent determination of the number of complementation groups needed for the biosynthesis of pseudobactin.

MATERIALS AND METHODS

Bacterial strains. Yellow-green, fluorescent *Pseudomonas* sp. strain B10 Nal^r Rif^r, which was isolated as a spontaneous mutant of *Pseudomonas* sp. strain B10 resistant to both 100 μg of nalidixic acid and 100 μg of rifampin per ml, was obtained from J. W. Kloepper. Yellow-green, fluorescent *Pseudomonas* sp. strain Wasco 9, which aggressively colonizes sugar beet roots and subsequently inhibits plant growth (31), was obtained from M. N. Schroth. *Escherichia coli* strains were HB101 *pro leu thi lacY rpsL endA recA hsdR hsdM* and the λ lysogenic strains of Sternberg et al. (30), NS428 (λ *A*am11 *b2 red*3 *c*I857 *S*am7) and NS433 (λ *E*am4 *b2 red*3 *c*I857 *S*am7).

Media. *E. coli* was grown in LB medium or M9 minimal medium (25) at 37°C, whereas *Pseudomonas* strains were cultured in King's medium B (KB) (16) at 30°C. Antibiotics were used at the following concentrations: 50 μg/ml kanamycin sulfate (Kn) and 3 μg/ml tetracycline hydrochloride (Tc) for *E. coli* HB101 and 35 μg/ml rifampin (Rif) and 10 μg/ml tetracycline hydrochloride for *Pseudomonas* sp. strain B10.

Plasmids. The broad host-range cosmid pLAFR1 (11), which is a 21.6-kilobase (kb) derivative of pRK290 (8), contains a single *Eco*RI restriction enzyme site and confers resistance to tetracycline; pRK2013, which is the helper plasmid of pLAFR1, carries the conjugal transfer genes of RK2 (8) cloned into a ColE1 replicon (10) and confers resistance to kanamycin.

Chemicals. Pseudobactin and ferric pseudobactin were

* Corresponding author.
† Present address: Department of Medicine, University of California, San Diego, La Jolla, CA 92093.
‡ Present address: Helicon Foundation, San Diego, CA 92121.

isolated as described previously (33). All chemicals were reagent grade. The *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), ethyl methanesulfonate (EMS), lysozyme, diethyl pyrocarbonate, and kanamycin sulfate were purchased from Sigma Chemical Co., St. Louis, Mo. Pronase, RNase, rifampin, and tetracycline hydrochloride were obtained from Calbiochem-Behring Corp., La Jolla, Calif. Restriction endonucleases and T_4 DNA ligase were obtained from Bethesda Research Laboratories, Rockville, Md., and were used as specified by the manufacturer. Seakem agarose ME was obtained from FMC Corp., Rockland, Maine, and Sarkosyl was from Ciba-Geigy Corp., Summit, N.J.

DNA isolation. Preparative amounts of cosmid DNA were isolated from *E. coli* essentially as described by Currier and Nester (7) with omission of Mg^{2+} and PO_4^{3-} in the precipitation step. Cosmid DNA for clonal analyses was prepared from *E. coli* by a cleared-lysate technique with Triton X-100 (15). Rapid isolation of small amounts of cosmid DNA from *Pseudomonas* sp. strain B10 was performed by modifications of the methods of Currier and Nester (7) and Hansen and Olsen (13). Briefly, after the shearing step, the DNA was precipitated with isopropanol. The pellet was allowed to dissolve for 1 h in TE buffer (50 mM Tris [pH 8.0], 20 mM EDTA) containing 1% sodium dodecyl sulfate. After enough freshly prepared 3 M NaOH was added to the above solution to achieve a final pH of 12.3, the resultant solution was immediately mixed gently for 3 min (at ca. 20 inversions per min). Enough 2 M Tris (pH 7.0) was added with rapid mixing to give a final pH of 8.2. A 1/10 volume of 5 M NaCl was added with rapid mixing. After this, the solution was extracted with phenol, and the DNA was precipitated with isopropanol. *E. coli* HB101 was transformed with this cosmid DNA.

Total DNA from *Pseudomonas* sp. strain B10 was obtained from 500 ml of stationary phase cells according to a modification of the procedure of Clewell and Helinski (4) as described by Takeda et al. (32).

Size fractionation of *Pseudomonas* sp. strain B10 DNA. A partial *Eco*RI digest of total *Pseudomonas* sp. strain B10 DNA was size fractionated with a preparative vertical gel electrophoresis apparatus from Bethesda Research Laboratories (model 1100PG) with 0.6% agarose in Tris-acetate (40 mM Tris, 20 mM acetic acid, 2 mM Na_2EDTA [pH 8.1]) buffer. Fractions were monitored for DNA size on 0.5% agarose gels. Those fractions containing DNA in the size range of 10 to 35 kb were pooled and used for construction of the gene bank.

Construction of *Pseudomonas* sp. strain B10 gene bank. *Eco*RI-digested pLAFR1 (67 μg/ml) was ligated with size-fractionated *Pseudomonas* sp. strain B10 DNA (466 μg/ml) with T_4 DNA ligase (83 U/ml) (11). The λ packaging extracts were prepared from the λ lysogenic strains *E. coli* NS428 and NS433 essentially as described (24) with the following modifications. Cultures of strains NS428 and NS433 were used in the ratio of 1:2. Putrescine was omitted from the CH buffer (24). The packaging of ligated DNA into λ phage heads was performed in vitro (11). The resulting phage particles containing recombinant DNA molecules were used to infect *E. coli* HB101 in LB medium. After expression of tetracycline resistance proceeded for 1 h at 37°C, cells were spread on LB agar plates containing tetracycline.

Isolation of mutants. *Pseudomonas* sp. strain B10 was mutagenized with NTG, EMS, or UV light according to modifications of procedures of Miller (25). About 10^8 cells per ml were treated with NTG (2 μg/ml) in KB medium for 4 h at 30°C. Mutagenesis with EMS (4%) was performed with 2 $\times$ 10^8 cells per ml in M9 minimal medium containing 2 mM $MgSO_4$ for 1 h at 30°C. About 10^8 cells per ml in 0.1 M $MgSO_4$ were irradiated with UV light to give a survival rate of 0.1 to 1%. After each of the above treatments, cells were washed to remove the mutagen, if necessary, and then subcultured overnight in KB medium.

Nonfluorescent mutants defective in the production of pseudobactin were obtained as follows. About 150 to 200 cells from each of the above treatments were spread per KB agar plate. Colonies were scored for nonfluorescence after incubation overnight first at 30°C and then overnight at room temperature.

The possibility of detecting yellow-green, fluorescent mutants defective in the biosynthesis of pseudobactin was explored by the following two methods. Only EMS-treated cells were screened.

About 50 cells were spread per KB agar plate. After these plates were incubated overnight first at 30°C and then overnight at room temperature, they were sprayed with a suspension of nonfluorescent mutant 177 (10^6 cells per ml) (see below) derived from *Pseudomonas* sp. strain B10 by treatment with EMS. These plates were incubated at 30°C overnight. Any yellow-green, fluorescent colonies surrounded by clear zones of growth inhibition of mutant 177 were to be purified by restreaking on KB agar plates.

The above procedure was adapted with the following modifications. About 30 cells were spread per KB plate. These plates were sprayed with a suspension of *Pseudomonas* sp. strain Wasco 9 (10^6 cells per ml). Yellow-green, fluorescent colonies of strain B10 which did not exhibit clear zones of growth inhibition of strain Wasco 9 were first purified by streaking on KB plates containing rifampin and then retested.

Plate bioassay for siderophore utilization. A published procedure (34) was followed with the following modifications. A single colony of each nonfluorescent mutant defective in the production of pseudobactin was inoculated onto KB plates containing 1 mg of ethylenediaminedi-(*o*-hydroxyphenylacetic acid) (EDDA) per ml and seeded with *Pseudomonas* sp. strain B10 (10^3 cells per ml). *Pseudomonas* sp. strain B10 and filter paper disks containing 10 μM pseudobactin or 10 μM ferric pseudobactin served as positive controls. Plates were incubated at room temperature and examined after 24 and 48 h for the presence of halos of single colonies surrounding the mutants.

Complementation of nonfluorescent biosynthetic mutants. Twenty-six nonfluorescent mutants of *Pseudomonas* sp. strain B10 were individually complemented by mating the gene bank en masse by using the triparental conjugation system of Ditta et al. (8). About 4 $\times$ 10^9 cells each of *E. coli* HB101 harboring the gene bank and *E. coli* HB101(pRK2013) and 2 $\times$ 10^8 cells of each recipient mutant were mixed, and the suspension was filtered through 0.45-μm membrane filters (Millipore Corp., Bedford, Mass.). The filters were incubated overnight at 30°C on LB agar plates before the cells were suspended and plated on KB agar plates containing tetracycline and rifampin. These plates were incubated for ca. 2 days at 30°C, and the cells were suspended and pooled. About 100 to 200 cells were then spread per KB plate containing tetracycline; the plates were incubated overnight first at 30°C and then overnight at room temperature. Complemented transconjugants were yellow-green fluorescent.

Recombinant cosmid DNA was individually isolated from between one to five complementing clones per mutant as described above and was used to transform *E. coli* HB101.

Triparental conjugations were performed to check the ability of each recombinant cosmid to complement the mutant from which it was originally isolated (5). Recombinant cosmids which did not complement the original mutant were not studied further.

The 80 recombinant cosmids (see below), which were obtained from complementation of the original 26 mutants, were tested for their ability to complement each of the remaining 156 mutants (see below). Conjugations were performed by mixing with a 49-prong replicator the 80 clones harbored in *E. coli* HB101, which were maintained in 7 by 7 ordered arrays on LB plates containing tetracycline (3 μg/ml), with lawns of *E. coli* HB101(pRK2013) and of each mutant onto LB plates. After these plates were incubated for 2 days at 30°C, transconjugants were obtained by replicating the cells onto KB plates containing tetracycline and rifampin. After 2 days at 30°C, cells were replicated again onto KB plates containing tetracycline and scored for fluorescence after 1 day at 30°C.

RESULTS

Gene bank of *Pseudomonas* sp. strain B10 DNA. A gene bank of *Pseudomonas* sp. strain B10 total DNA, partially digested with *Eco*RI, was constructed using the broad host-range cosmid pLAFR1 and was maintained in *E. coli* HB101. Tetracycline-resistant transductants were obtained at a frequency of 8×10^4/μg of vector DNA. The resultant gene bank contained more than 15,000 independent clones. Fifty-five clones were chosen at random for analysis of their plasmid DNA. Gel electrophoresis of *Eco*RI digests of these plasmids showed that all contained insert DNA, which ranged between 8 and 35 kb and averaged 21.5 kb in length (data not shown). In all cases, a fragment comigrated with *Eco*RI-digested pLAFR1. If the molecular weight of the *Pseudomonas* genome is assumed to be about the same as that of the *E. coli* genome, *i.e.*, 4,200 kb (2), then only about 880 members of the gene bank are needed to guarantee a 99% chance that a given sequence of DNA will be represented (3).

Mutants defective in biosynthesis of pseudobactin. Treatment of yellow-green, fluorescent *Pseudomonas* sp. strain B10 with NTG, EMS, or UV light yielded 177 nonfluorescent mutants defective in the biosynthesis of yellow-green, fluorescent pseudobactin. One hundred fifty-seven of these mutants were stably nonfluorescent and were used in subsequent experiments. The reversion frequency of these latter mutants could not be directly determined but was less than 10^{-4} (data not shown). Seven of these mutants were derived from treatment with NTG, 13 from exposure to UV light, and 137 from treatment with EMS. Although the 157 nonfluorescent mutants did not produce pseudobactin, the possibility that they might produce other molecules which could function as siderophores for *Pseudomonas* sp. strain B10 was tested. None of these mutants was able to reverse iron starvation of *Pseudomonas* sp. strain B10 induced by the synthetic ferric complexing agent EDDA, the iron of which is not utilized by the cells in the plate bioassay. In contrast, a halo of single colonies was observed surrounding the site of inoculation of the positive control, *Pseudomonas* sp. strain B10, and surrounding the disks of pseudobactin or ferric pseudobactin. Therefore, the 157 mutants did not produce siderophores utilizable by *Pseudomonas* sp. strain B10.

In addition to nonfluorescent biosynthetic mutants, other mutants were sought which were still yellow-green fluorescent. Two classes of such mutants could be envisioned: the first class would produce yellow-green, fluorescent derivatives of pseudobactin which still bind iron(III), whereas the second class would produce fluorescent derivatives which do not bind iron(III) at all.

The screening procedure for fluorescent mutants of the first class was based upon the assumption that the outer membrane receptor protein for ferric pseudobactin is specific for this molecule and would not recognize altered or incomplete derivatives. Outer membrane receptor proteins for the siderophores ferric enterobactin and ferrichrome appear to be remarkably specific for the stereochemistry of the ferric siderophore (27, 35). Since pseudobactin and ferric pseudobactin were able to reverse EDDA-induced iron starvation of nonfluorescent mutant 177 (data not shown), this mutant was able to utilize pseudobactin as a siderophore and hence had a functional iron assimilation system for ferric pseudobactin, including its outer membrane receptor protein. Thus, mutant 177 should be susceptible to iron(III) starvation by fluorescent mutants of the first class because this indicator strain would not be able to utilize altered derivatives of pseudobactin to transport iron. About 32,000 yellow-green, fluorescent colonies of EMS-treated *Pseudomonas* sp. strain B10 were screened. None inhibited the growth of mutant 177.

The screening procedure for fluorescent mutants of the second class exploited the inability of their fluorescent products to bind iron(III). *Pseudomonas* sp. strain B10 or pseudobactin inhibits the growth of *Pseudomonas* sp. strain Wasco 9, presumably by iron deprivation (J. Leong, unpublished data). Since these fluorescent products are presumed not to bind iron(III), they would not starve *Pseudomonas* sp. strain Wasco 9 for iron(III) and hence would not inhibit its growth. About 19,000 yellow-green, fluorescent colonies of EMS-treated *Pseudomonas* sp. strain B10 were screened. None failed to inhibit the growth of *Pseudomonas* sp. strain Wasco 9.

In summary, no yellow-green, fluorescent mutants were identified.

Complementation of nonfluorescent mutants. Twenty-six nonfluorescent mutants of *Pseudomonas* sp. strain B10, which were chosen at random, were individually complemented by mating the gene bank en masse by using helper plasmid pRK2013; tetracycline-resistant and rifampin-resistant transconjugants were selected and screened. Tetracycline-resistant transconjugants typically appeared at frequencies of 2×10^{-5} to 2×10^{-3} per recipient cell. Complemented transconjugants were yellow-green fluorescent and appeared at frequencies of 10^{-3} to 10^{-2} per transconjugant.

Cosmid DNAs from several complemented transconjugants from each mutant were isolated and used to transform *E. coli*. We confirmed the ability of the recombinant cosmids to complement the mutant from which each was originally isolated to eliminate the possibility that fluorescence resulted from reversion to wild type. Eighty such *E. coli* transformants were then used in pairwise conjugations with the entire collection of nonfluorescent mutants to deduce the overall pattern of complementation. In this way, eight cosmid DNAs were identified as being sufficient to complement 154 of the 157 original mutants. Three mutants could not be complemented by any of the 80 cosmids or by mating the gene bank en masse. They may contain multiple mutations in genes required for pseudobactin biosynthesis. Alternatively, the gene bank may lack DNA carrying the appropriate wild-type allele.

Table 1 summarizes the complementation data obtained for the eight cosmids. The mutants could be arranged into

TABLE 1. Complementation analysis of pseudobactin biosynthetic genes

Complementing cosmid	No. of mutants complemented[a]		Sizes of *Eco*RI fragments from insert DNA (kb)[b]	Size of total insert DNA (kb)[b]
pJLM1	6		8.7, 8.1, 5.3	22.1
pJLM2	4		17.5, 14.0	31.5
pJLM3A	26	3	13.6, 3.3, 3.0, 2.6	22.5
pJLM3B	27		13.2, 11.2, 3.3, 2.6	30.3
pJLM3C	14	2	11.2, 8.1, 3.6, 1.4	24.3
pJLM4A	62	51	13.5, 4.7, 3.7, 2.7, 2.2	26.8
pJLM4B	66		13.0, 3.7, 3.3, 3.1, 2.7, 2.2, 1.1, 1.0	30.1
pJLM4C	6	1	10.5, 5.3, 4.1, 2.6, 1.9, 1.5, 1.3, 1.0	28.2

[a] The numbers to the right of the brackets indicate the number of mutants complemented in common by any two recombinant cosmids.
[b] These sizes were obtained from agarose gels.

four groups, which probably represent different gene clusters. Recombinant cosmids pJLM1 and pJLM2 complemented all mutants in groups 1 and 2, respectively, whereas three cosmids each, pJLM3A, pJLM3B, and pJLM3C and pJLM4A, pJLM4B, and pJLM4C were needed to complement all of the mutants in groups 3 and 4, respectively. For the latter two groups, some of the mutants were complemented by two different cosmids. In each such case, the cosmids appeared to have one or more *Eco*RI fragments of identical size (Fig. 1 and Table 1). These common fragments probably represent regions of overlap between the cosmids and suggest that the complementing genes for the mutants are located in the regions of overlap.

For the purposes of this study, a complementation group containing one or more genes is defined as a set of mutants which can be complemented by a particular combination of cosmid clones. Thus, the group of six mutants which could only be complemented by pJLM1 is a complementation group. Likewise, the 4 mutants which could only be complemented by pJLM2 and the 23 mutants which could only be complemented by pJLM3A constitute two more groups. The three mutants which could be complemented by both pJLM3A and pJLM3B are yet a fourth group. Figure 2 summarizes the data in Table 1 according to this analysis. As illustrated, a minimum of 12 genes are thus identified as being essential for pseudobactin production.

It is conceivable that some of the genes essential for pseudobactin biosynthesis are also necessary for cellular growth under iron-sufficient conditions. However, only 12 auxotrophic mutants were found that did not grow on M9 minimal medium plates, and these were randomly distributed within the 12 complementation groups (data not shown). These 12 auxotrophs probably have multiple mutations.

DISCUSSION

To date, the genetic organization of only two siderophore-dependent iron transport systems have been studied in any detail. These are the high-affinity enterobactin and aerobactin systems of enteric bacteria.

Enterobactin is a cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine and is produced under iron(III)-limiting conditions (28). Currently, nine genes are known to be involved in the high-affinity, enterobactin-dependent iron transport system of *E. coli* K-12 (21, 22). These genes are clustered within 26 kb of DNA and are organized into at least six transcriptional units (21, 22). Seven *ent* genes appear to be involved in the biosynthesis of enterobactin. The *fep* gene codes for the 81,000 dalton outer membrane receptor protein for ferric enterobactin. The *fes* gene product is involved in the release of iron from ferric enterobactin, but its precise role is unclear (26).

The siderophore aerobactin is a conjugate of 6-(*N*-acetyl-*N*-hydroxyamino)-2-aminohexanoic acid and citric acid (12). The aerobactin-dependent iron transport system has recently been cloned from the ColV-K30 plasmid on a 16.3-kb fragment (1). This fragment codes for proteins necessary for the biosynthesis of aerobactin and the 74,000 dalton outer membrane receptor protein for ferric aerobactin (1, 19), all of which are coordinately regulated by iron (1).

The structural complexity of pseudobactin necessitates that multiple genes be involved in its biosynthesis. Since pseudobactin consists of a short peptide and contains unusu-

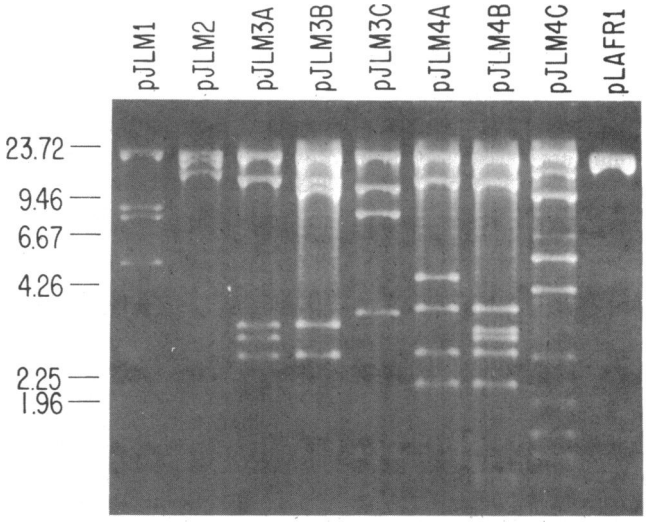

FIG. 1. *Eco*RI digestion of eight recombinant cosmids required to complement 154 nonfluorescent mutants and *Eco*RI digestion of pLAFR1. Fragments were separated by gel electrophoresis through a 0.5% agarose gel. Fragment sizes (in kb) of *Hin*dIII-digested bacteriophage λ DNA are indicated at the left. The 1.0-kb and 1.1-kb fragments of pJLM4B, the 1.0-kb fragment of pJLM4C, and the 1.4-kb fragment of pJLM3C may not be discernible. The 14.8-kb fragment of pJLM4C is a partial digest.

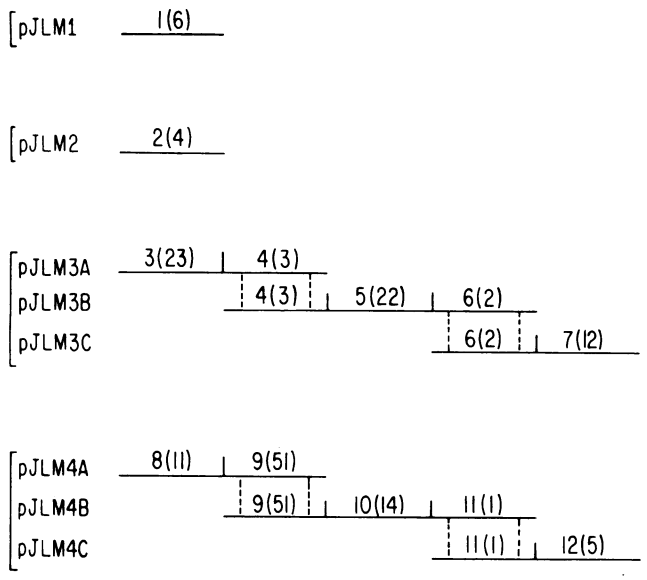

FIG. 2. Schematic showing the proposed arrangement of 12 pseudobactin complementation groups among the eight cosmids. Cosmid DNAs containing the complementation groups are indicated by horizontal lines; the groups are arbitrarily numbered from 1 to 12 and are delineated by hatch marks. Dashes indicate boundaries of regions of DNA overlap. For each of the eight recombinant cosmids shown, the number of mutants used to identify each complementation group is shown in parentheses. The cosmids are arranged into four groups, indicated by brackets, which tentatively represent different gene clusters.

al amino acids, the biosynthesis of the hexapeptide moiety is almost certainly not mediated by ribosomes. Specific enzymes including isomerases must be required for the biosynthesis of the unusual amino acids D-*threo*-β-hydroxyaspartic acid, D-*allo*-threonine, and D-N^{δ}-hydroxyornithine. For example, two enzymes appear to be responsible for the formation of the hydroxamate bond which is present in several siderophores; the first enzyme catalyzes the formation of N^{δ}-hydroxyornithine from ornithine, whereas the second enzyme specifically acylates N^{δ}-hydroxyornithine (9). By analogy with gramicidin S, a cyclic peptide antibiotic produced by *Bacillus brevis* (20, 23) and made up of two identical pentapeptides joined head to tail, as few as two enzymes may be involved in peptide bond formation of pseudobactin. Little is known about the biosynthesis of the yellow-green, fluorescent quinoline derivative.

Utilizing a gene bank of *Pseudomonas* sp. strain B10 DNA and 154 nonfluorescent mutants defective in the production of pseudobactin, we have identified eight different recombinant cosmids carrying pseudobactin biosynthetic genes. Although the total number of genes involved in the biosynthetic pathway is not yet known, the complementation pattern suggests that a minimum of 12 genes is needed. This seems reasonable considering the structural complexity of pseudobactin. The biosynthesis of pseudobactin is expected to require more genes than either enterobactin or aerobactin, which are simpler molecules.

We can tentatively conclude that at least four gene clusters are involved in the biosynthesis of pseudobactin, compared with a single cluster involved in the biosynthesis of either enterobactin or aerobactin. In this regard it should be noted that genes coding for the biosynthesis of tryptophan, histidine, and leucine are also clustered in enteric bacteria but are separate in both *Pseudomonas aeruginosa* and *Pseudomonas putida* (6, 14), even though the biosynthetic pathways are similar.

The functional genes for biosynthesis of pseudobactin carried by the eight cosmids described above are being localized by subcloning and by Tn*5* mutagenesis. Ultimately, it is hoped that these and other genes specifying iron assimilation from ferric pseudobactin can be extended to other root-colonizing microorganisms, thereby conferring upon them plant growth-promoting ability similar to that of *Pseudomonas* sp. strain B10.

ACKNOWLEDGMENTS

We thank Donald R. Helinski for valuable encouragement and providing laboratory facilities during the early stages of this work.

This investigation was supported in part by the National Institutes of Health. M.M. is a National Institutes of Health Postdoctoral Trainee, grant AM07233.

LITERATURE CITED

1. **Bindereif, A., and J. B. Neilands.** 1983. Cloning of the aerobactin-mediated iron assimilation system of plasmid ColV. J. Bacteriol. **153**:1111–1113.
2. **Cairns, J.** 1963. The chromosome of *Escherichia coli*. Cold Spring Harbor Symp. Quant. Biol. **28**:43–46.
3. **Clarke, L., and J. Carbon.** 1976. A colony bank containing synthetic ColE1 hybrid plasmids representative of the entire *E. coli* genome. Cell **9**:91–99.
4. **Clewell, D. B., and D. R. Helinski.** 1972. Effect of growth conditions on the formation of the relaxation complex of supercoiled ColE1 deoxyribonucleic acid and protein in *Escherichia coli*. J. Bacteriol. **110**:1135–1146.
5. **Corbin, D., G. Ditta, and D. R. Helinski.** 1982. Clustering of nitrogen fixation (*nif*) genes in *Rhizobium meliloti*. J. Bacteriol. **149**:221–228.
6. **Crawford, I. P.** 1975. Gene rearrangements in the evolution of the tryptophan synthetic pathway. Bacteriol. Rev. **39**:87–120.
7. **Currier, T. C., and E. W. Nester.** 1976. Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal. Biochem. **76**:431–441.
8. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. U.S.A. **77**:7347–7351.
9. **Emery, T.** 1974. Biosynthesis and mechanism of action of hydroxamate-type siderochromes, p. 110–114. *In* J. B. Neilands (ed.), Microbial iron metabolism: a comprehensive treatise. Academic Press, Inc. New York.
10. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. U.S.A. **76**:1648–1652.
11. **Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene **18**:289–296.
12. **Gibson, F., and D. I. Magrath.** 1969. The isolation and characterization of a hydroxamic acid (aerobactin) formed by *Aerobacter aerogenes* 62-1. Biochim. Biophys. Acta **192**:175–184.
13. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227–238.
14. **Holloway, B. W., V. Krishnapillai, and A. F. Morgan.** 1979. Chromosomal genetics of *Pseudomonas*. Microbiol. Rev. **43**:73–102.
15. **Kahn, M., R. Kolter, C. Thomas, D. Figurski, R. Meyer, E. Remaut, and D. R. Helinski.** 1979. Plasmid cloning vehicles derived from plasmids ColE1, F, R6K and RK2. Methods Enzymol. **68**:268–280.

16. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocanin and fluorescin. J. Lab. Clin. Med. **44**:301–307.
17. **Kloepper, J. W., J. Leong, M. Teintze, and M. N. Schroth.** 1980. *Pseudomonas* siderophores: a mechanism explaining disease-suppressive soils. Curr. Microbiol. **4**:317–320.
18. **Kloepper, J. W., J. Leong, M. Teintze, and M. N. Schroth.** 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature (London) **286**:885–886.
19. **Krone, W. J. A., B. Oudega, F. Stegehuis, and F. K. de Graaf.** 1983. Cloning and expression of the cloacin DF13/aerobactin receptor of *Escherichia coli* (ColV-K30). J. Bacteriol. **153**:716–721.
20. **Kurahashi, K.** 1974. Biosynthesis of small peptides. Annu. Rev. Biochem. **43**:445–459.
21. **Laird, A. J., D. W. Ribbons, G. C. Woodrow, and I. G. Young.** 1980. Bacteriophage Mu-mediated gene transposition and in vitro cloning of the enterochelin gene cluster of *Escherichia coli*. Gene **11**:347–357.
22. **Laird, A. J., and I. G. Young.** 1980. Tn*5* mutagenesis of the enterochelin gene cluster of *Escherichia coli*. Gene **11**:359–366.
23. **Lipmann, F.** 1971. Attempts to map a process evolution of peptide biosynthesis. Science **173**:875–884.
24. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Neilands, J. B.** 1982. Microbial envelope proteins related to iron. Annu. Rev. Microbiol. **36**:285–309.
27. **Neilands, J. B., T. J. Erickson, and W. H. Rastetter.** 1981. Stereospecificity of the ferric enterobactin receptor of *Escherichia coli* K-12. J. Biol. Chem. **256**:3831–3832.
28. **Pollack, J. R., and J. B. Neilands.** 1970. Enterobactin, an iron transport compound from *Salmonella typhimurium*. Biochem. Biophys. Res. Commun. **38**:989–993.
29. **Schroth, M. N., and J. G. Hancock.** 1982. Disease-suppressive soil and root-colonizing bacteria. Science **216**:1376–1381.
30. **Sternberg, N., D. Tiemeier, and L. Enquist.** 1977. In vitro packaging of λ*D*am vector containing *Eco*RI DNA fragments of *Escherichia coli* and phage P1. Gene **1**:255–280.
31. **Suslow, T. V., and M. N. Schroth.** 1982. Role of deleterious rhizobacteria as minor pathogens in reducing crop growth. Phytopathology **72**:111–115.
32. **Takeda, Y., N. E. Harding, D. W. Smith, and J. W. Zyskind.** 1982. The chromosomal origin of replication (*ori*C) of *Erwinia carotovora*. Nucleic Acids Res. **10**:2639–2650.
33. **Teintze, M., M. B. Hossain, C. L. Barnes, J. Leong, and D. van der Helm.** 1981. Structure of ferric pseudobactin, a siderophore from a plant growth promoting *Pseudomonas*. Biochemistry **20**:6446–6457.
34. **Teintze, M., and J. Leong.** 1981. Structure of pseudobactin A, a second siderophore from plant growth promoting *Pseudomonas* B10. Biochemistry **20**:6457–6462.
35. **Winkelmann, G.** 1979. Evidence for stereospecific uptake of iron chelates in fungi. FEBS Lett. **97**:43–46.