JOURNAL OF BACTERIOLOGY, Jan. 1995, p. 423–431
0021-9193/95/$04.00+0

Vol. 177, No. 2

Genetic and Physical Mapping of Genes Involved in Pyoverdin Production in *Pseudomonas aeruginosa* PAO

MASATAKA TSUDA,* HIROAKI MIYAZAKI, AND TERUKO NAKAZAWA

Department of Microbiology, Yamaguchi University School of Medicine, Ube 755, Japan

Received 15 August 1994/Accepted 4 November 1994

Pseudomonas aeruginosa **PAO was mutagenized with Tn*1737*KH, a type I transcription probe transposon containing a promoterless *lacZ* (β-galactosidase) gene, and 24 insertion mutants that did not grow under iron-deficient conditions were isolated. None of the culture supernatants from any mutants contained pyoverdin, a low-molecular-weight siderophore able to sequester ferric iron at very high affinity, and the growth defects of the mutants were all phenotypically recovered by the addition of the culture supernatant from the wild-type strain. These phenotypes led to the inference that all the mutants had defects in the genes (*pvd* genes) for production of pyoverdin. In some *pvd*::Tn*1737*KH mutants, high levels of β-galactosidase activities were observed, and such activities were drastically reduced by the addition of ferric ion in the culture media, indicating that the expression of at least some *pvd* genes is regulated at the transcriptional level. Molecular cloning and physical analysis of the chromosomal fragments with Tn*1737*KH insertions allowed us to allocate all the mutations within a 103-kb region, referred to as the *pvd* region, that was found to locate at 47 min on the genetic map of PAO. Further physical mapping and Southern analysis showed that there is a 10-kb overlap between the *pvd* region and the 125-kb *catA* region described by Zhang and Holloway (C. Zhang and B. W. Holloway, J. Gen. Microbiol. 138:1097–1107, 1992). We could hence illustrate the physical map of the *P. aeruginosa* chromosome with a size of 218 kb.**

Iron is an essential element for the growth of almost all bacterial species. The solubility of ferric ion is very low in nature, and bacteria have evolved various kinds of efficient mechanisms to acquire iron under limiting conditions (32). The common and well-studied iron acquisition systems in bacteria involve secretion of low-molecular-weight, high-affinity iron chelators, termed siderophores, and uptake of the ferric siderophore complexes into the cell by specific receptors located at the cell surfaces (15). For various pathogenic bacteria that have to acquire iron from severely restricted environments in the hosts, the siderophore-mediated iron uptake systems are considered important pathogenic factors (25).

Pseudomonas aeruginosa, an important opportunistic pathogen of humans, is a representative of fluorescent pseudomonads (19). Under iron limitation, this microorganism, including strain PAO, secretes two siderophores, pyochelin (12) and pyoverdin (13), and some outer membrane proteins specifically bind and transport ferripyochelin or ferripyoverdin into the cell (5, 38, 45). Pyoverdin has a chelating affinity for Fe^{3+} more than 25 orders of magnitude higher than that of pyochelin and promotes cellular growth in the presence of human serum and transferrin (4, 14). Pyoverdin has been therefore considered to play a crucial role in vivo in the mobilization of ferric ion (4).

Pyoverdin secreted by *P. aeruginosa* is a water-soluble, yellow-green fluorescent compound that is composed of a dihydroxyquinoline-containing fluorescent chromophore joined to one terminus of a partly cyclic octapeptide (D-Ser–L-Arg–D-Ser–L-N^5-OH-Orn–L-Lys–L-N^5-OH-Orn–L-Thr–L-Thr) (9). Other fluorescent *Pseudomonas* species of rhizosphere origin also secrete pyoverdin-like siderophores, pseudobactins (35). While the chromophore parts are well conserved among these siderophores, the peptide compositions show some difference (1). The resemblance of the chemical compositions among these siderophores has been expanded to a high degree of sequence homology of the genes responsible for biosynthesis of pyoverdins and pseudobactins among different *Pseudomonas* spp., and these genes are suggested to have evolved from a common ancestral set of genes (41). To date, genetic analyses of pseudobactin-mediated ferric ion acquisition systems have been extensively carried out with a *Pseudomonas putida* strain, WCS358 (27), and a *Pseudomonas* sp. strain, M114 (35). These analyses have unravelled (i) the requirement of a number of genes and a long range of DNA regions for the iron acquisition (e.g., more than 35 genes and 100 kb, respectively, in *P. putida* WCS358 [26]) and (ii) the involvement of both negative and positive regulatory systems for expression of these genes (23, 33, 34, 54).

Regarding the pyoverdin-mediated iron uptake system of *P. aeruginosa* PAO, a ferripyoverdin-binding receptor protein and its gene have been analyzed in detail (37, 38). In contrast, progress in studies of pyoverdin synthesis has been made at low rate. The pyoverdin-deficient (Pvd^-) mutations isolated mainly by chemical mutagens have been mapped at two distinct regions on the chromosomal map (3, 18, 41, 56). An early part of the biosynthetic pathway of pyoverdin has been clarified by Visca et al. (56), and three DNA fragments containing some *pvd* genes have been isolated by (i) selecting a recombinant cosmid that could complement *pvd* mutations (55), (ii) transposon insertion mutagenesis of the *pvd* genes followed by molecular cloning of the transposon-tagged chromosomal fragment (41), and (iii) selecting DNA fragments hybridized with the pseudobactin synthesis genes of *P. putida* WCS358 (28). It has been, however, unknown whether these cloned fragments are physically separated or overlapped with one another and whether they cover all the genes necessary for pyoverdin production.

We are interested in (i) the organization of genes involved in the pyoverdin-mediated ferric ion uptake system on the chro-

* Corresponding author. Mailing address: Department of Microbiology, Yamaguchi University School of Medicine, Ube 755, Japan. Phone: 81-836-22-2227. Fax: 81-836-22-2227.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli		
DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44* Δ(*lac*)*U169* (ϕ80d*lac*ΔM15)	6
S17-1	*pro thi recA hsdR* Tp^r Sm^r; chromosomally integrated RP4-2-Tc::Mu-Km::Tn*7*; mobilizer of plasmid carrying the R68-derived Mob[a] region	44
P. aeruginosa		
PAO1	Prototroph	19
PAO25	*leu-10 argF10*	17
PAO1304	*nar-9011 cnu-9001 gcu-2*::Tn*5*	57
PAO1305	*nar-9011 cnu-9001 gcu-3*::Tn*5*	57
PAO2196	*met-9020 catA1 nar-9011 chu-9002 trpB9029 lys-9015*	H. Matsumoto
PAO2384	*met-9020 catA1 nar-9011 mtu-9002 tyu-9030 dcu-9001*	52
MT1	PAO1 *str-8006*	46
MT2503	*trpA,B*::pME319[b] *his-301 str-1 recA301*; Hfr strain	47
AMT72	*lys-9015*$^+$ *recA301* derivative of PAO2196; MT2503 × PAO2196	This study
AMT74	*met-9020*$^+$ *recA301* derivative of PAO2384; MT2503 × PAO2384	This study
Plasmids		
pME305	Cb^r Tc^r Tra$^+$ Rep(Ts); R68 derivative	40
R68.45	Cb^r Tc^r Km^r Tra$^+$ Cma$^+$[c]; R68 derivative	17
pMO1983	Tc^r, pLA2917 derivative carrying the *gcu-3* gene of *P. aeruginosa* PAO	57
pBR322	Ap^r Tc^r	8
pRU869	Ap^r Km^r, pJOE676::Tn*1737*Km	53
pNOT322	Ap^r Tc^r; pBR322 derivative with the *Nde*I-*Not*I adaptor in the *Nde*I site of pBR322 (Fig. 1)	42
pMOB3	Km^r Cm^r; pHHS21 derivative with *Not*I-flanked mobilization cassette (the cassette contains the Cm^r gene, the *sacB* locus, and the R68-derived Mob region)	42
pSUP203	Ap^r Tc^r Cm^r; pBR325 derivative carrying the R68-derived Mob region	44
pHP45ΩHg	Ap^r Hg^r; pBR322 derivative carrying *Hin*dIII-flanked Hg^r genes	16
pBend2	Ap^r; pBR322 derivative carrying directly repeated copies of multiple cloning sites (Fig. 1)	22
pNM21	Cb^r Tc^r Km^r Tra$^+$; R68.45 derivative carrying the *argC*, *trpCD*, *prtA-N*, and *trpE* genes of *P. aeruginosa* PAO	43
pNM108	Cb^r Tc^r Km^r Tra$^+$; R68.45 derivative carrying the *prtN*, *argC*, and *trpCD* genes of *P. aeruginosa* PAO	43
pMT267	Ap^r; pBR322 derivative whose *Eco*RI-*Hin*dIII fragment is replaced by that derived from pUC19 (Fig. 1)	This study
pMT1288	Ap^r Tc^r; pBR322::Tn*1722* (Fig. 1)	50
pMT5056	Ap^r Tc^r; pBend2 derivative carrying the *Kpn*I-flanked Tc^r gene cartridge (Fig. 1)	This study
pMT5059	Ap^r; pBend2 derivative carrying the multiple cloning site and *Not*I site (Fig. 1)	This study
pMT7009	Cb^r Tc^r Km^r Tra$^+$; R68.45 derivative carrying the *catA*, *pvd*, and *mtu-9002* genes of *P. aeruginosa* PAO; PAO25(R68.45) × AMT72	This study

[a] Plasmid mobilization.
[b] Stable chromosomal copy of an R68 derivative temperature sensitive for replication.
[c] Cma, chromosome mobilization ability.

mosome and (ii) genetic regulation of this system. In this study, we isolated the *pvd* mutations of *P. aeruginosa* PAO by use of a type I transcription probe transposon, and these mutations were genetically and physically mapped on the chromosome. We also show that some *pvd* genes were transcriptionally regulated in response to ferric ion concentrations.

MATERIALS AND METHODS

Organisms and media. Plasmids and bacterial strains used are listed in Table 1. The *pvd* allele numbers are the same as the strain numbers after the prefix AMT. Strains were, unless otherwise stated, grown at 37°C. For conjugal transfer and mobilization of plasmids from *Escherichia coli* to *P. aeruginosa*, the recipient cells were grown overnight at 43°C to minimize host restriction (57). Bacteriophage G101*h2* (10) was employed for generalized transduction.

Bacterial cells were cultivated in Luria broth (LB) or M9 minimal medium (6). The carbon source added to the latter medium was 10 mM either glucose, sodium succinate, sodium benzoate, mannitol, or glycine, and amino acids (1 mM) were added as required. Solid media were prepared by the addition of agar (1.5%). The iron-deficient minimal medium contained 400 μg of ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDA) per ml as a nonmetabolizable iron chelator. Selective agents added to media were as follows (in micrograms per milliliter): ampicillin, 50 for *E. coli*; carbenicillin, 500 for *P. aeruginosa*; $HgCl_2$ (Hg), 15; kanamycin, 50 for *E. coli* and 250 for *P. aeruginosa*; streptomycin, 1,000; and tetracycline, 10 for *E. coli* and 50 for *P. aeruginosa.*

Genetic methods. The procedures described previously (46–48, 52) were employed for transduction, conjugation on an LB agar plate, in vivo construction of R-prime plasmids from R68.45, and the complementation test with the resulting R-prime plasmids. The R-prime plasmids carrying the *catA* locus were constructed by mating of PAO25(R68.45) and AMT72 followed by selection of the transconjugants on M9 benzoate minimal agar plates supplemented with tryptophan, methionine, kanamycin, and tetracycline. The resulting transconjugants were used as the donors for the subsequent mating with AMT74 to investigate the presence of the *mtu-9002* locus on the R-prime plasmids. Plasmid pMT7009 is one of such R-prime plasmids carrying both loci (Table 1).

Recombinant DNA techniques. Established procedures (6, 51) were employed for preparation of plasmid and chromosomal DNAs, their in vitro manipulation, agarose gel electrophoresis, transformation of *E. coli*, and Southern hybridization.

Construction of Tn*1737*KH and its use for mutagenizing *P. aeruginosa*. Tn*1737*Km is a Tn*1722*-based, type I transcription probe transposon carrying a promoterless *lacZ* gene as a reporter (53). Since *P. aeruginosa* is intrinsically resistant to high concentrations of kanamycin, a 4.7-kb *Hin*dIII fragment of pHP45ΩHg containing an Hg^r determinant (16) was inserted in one of the *Hin*dIII sites of Tn*1737*Km on pRU869 (53) to construct Tn*1737*KH (Fig. 1A). A mating-out procedure (49) was employed for *E. coli* to transpose Tn*1737*KH at 30°C from the pRU869 derivative to pME305, a broad-host-range, transmissible R68 derivative temperature sensitive for replication (40). One of the re-

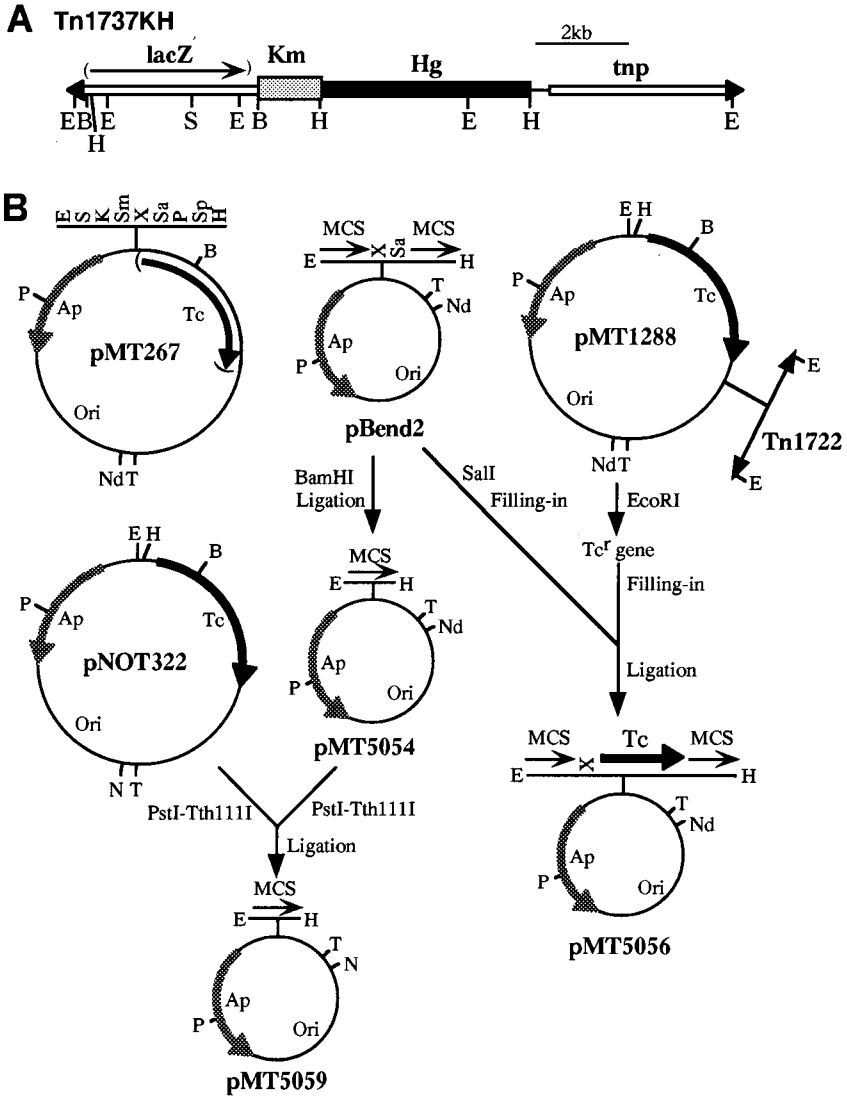

FIG. 1. Transposon and plasmids constructed in this study. (A) Structure of Tn*1737*KH. The arrow in parentheses indicates the transcriptional direction of the promoterless *lacZ* gene. (B) Construction of plasmids. See Table 1 and Materials and Methods for details of construction. The arrow in parentheses indicates the transcriptional direction of the promoterless Tc^r gene. "MCS" with an arrow indicates the synthetic oligonucleotide containing the 17 restriction sites in the order *Mlu*I-*Bgl*II-*Nhe*I-*Cla*I-*Sty*I-*Spe*I-*Xho*I-*Dra*I-*Eco*RV-*Pvu*II-*Sma*I-*Stu*I-*Nru*I-*Ssp*I-*Kpn*I-*Nco*I-*Bam*HI. Abbreviations for restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Not*I; Nd, *Nde*I; P, *Pst*I; S, *Sac*I; Sa, *Sal*I; Sp, *Sph*I; Sm, *Sma*I; T, *Tth*111I; and X, *Xba*I. tnp, genes required for transposition; ori, replication origin of plasmid.

sulting plasmids, pMT6121, was used to mutagenize the *P. aeruginosa* chromosome with Tn*1737*KH. The donor *E. coli* strain, DH5α(pMT6121), and the recipient *P. aeruginosa* strain, MT1, were spotted on the same place on an LB agar plate and incubated overnight at 30°C. The mixture was suspended in LB, and the diluted suspension was plated on LB agar containing Hg, kanamycin, and streptomycin. The transconjugants obtained at 42°C were purified under the same selective conditions and examined for resistance to carbenicillin and tetracycline.

Construction of pBend2 derivatives. Plasmid pBend2 (Fig. 1B) (22) is derived from pBR322 by (i) deleting the *Eco*RV-*Pvu*II fragment and (ii) replacing the *Eco*RI-*Hin*dIII fragment with a 244-bp *Eco*RI-*Hin*dIII segment. This segment contains directly repeated copies of 17 unique restriction sites separated by *Xba*I and *Sal*I sites. Plasmid pMT1288 is a pBR322 derivative carrying a Tn*1722* insert at kb 1.6 on pBR322 (50). Since Tn*1722* possesses the *Eco*RI sites 13 bp apart from its leftmost and rightmost ends (2), it was possible to excise the pBR322-derived Tc^r gene as a 1.6-kb *Eco*RI cartridge. This cartridge was blunt ended by T4 DNA polymerase and inserted into pBend2 that had been digested with *Sal*I and blunt ended with T4 DNA polymerase. One of the plasmids thus obtained was pMT5056 (Fig. 1B). Digestion of pBend2 with *Bam*HI followed by intramolecular ligation removed, in conjunction with the *Sal*I and *Xba*I sites, one copy of the repeat. The *Pst*I-*Tth*111I fragment of the resulting plasmid (pMT5054) containing the multiple cloning sites was next ligated to the *Pst*I-*Tth*111I fragment of pNOT322 containing the *Not*I site, and the resulting plasmid was designated pMT5059 (Fig. 1B).

Gene replacement mutagenesis. Allelic replacement mutagenesis of the *P. aeruginosa* chromosome was carried out by the system of Schweizer (42) except that the ColE1-type vector used was pMT5059 (Fig. 1B). An appropriate *P. aeruginosa*-derived *Eco*RI fragment on pMT7072 was first subcloned into the *Eco*RI on pMT5059, and the *Kpn*I-flanked, Tc^r gene cartridge was next inserted into most of the *P. aeruginosa*-derived *Kpn*I sites on the resulting pMT5059 derivative. A 5.8-kb *Not*I fragment containing the mobilization cassette of pMOB3 (Table 1) was subsequently inserted into the *Not*I site on each pMT5059 derivative carrying the Tc^r gene cartridge. The plasmids thus constructed were conjugally mobilized from an *E. coli* strain, S17-1, to MT1, and selection for Tc^r was made on M9 glucose agar plates. The transconjugants that were resistant to 5% sucrose but sensitive to carbenicillin were chosen, and their chromosomal DNAs were analyzed by Southern hybridization to confirm the occurrence of gene replacement.

Biochemical assays. Secretion of pyoverdin was measured by determining absorption spectra of the culture supernatants from the succinate minimal medium-grown, stationary-phase cells (18). Before determination of the absorption spectra, we adjusted the supernatants to pH 5.5 with acetic acid or pH 9.0 with

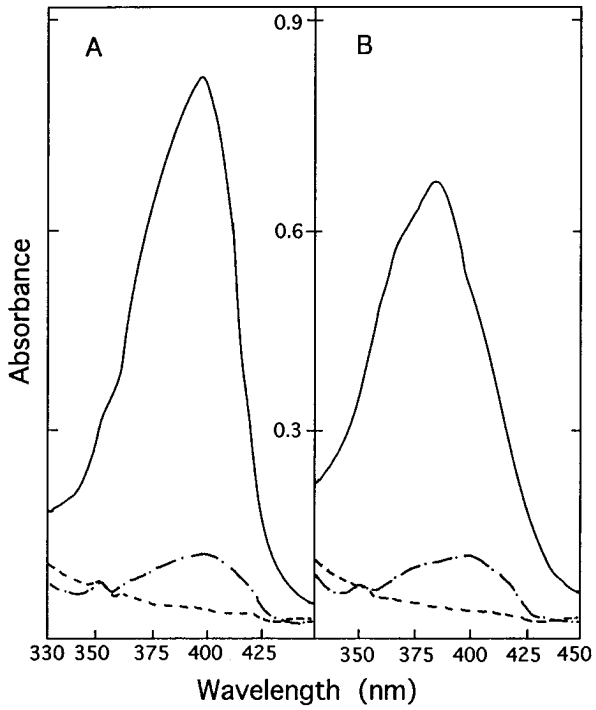

FIG. 2. Absorption spectra of culture supernatants from SM9-grown cells of strains MT1 (—), AMT22 (Flu$^-$ mutant) (----), and AMT41 (Flu$^\pm$ mutant) (-·--·-). Absorption spectra were determined after the culture supernatants were adjusted to pH 9.0 (A) and pH 5.5 (B).

NaOH. To assay the β-galactosidase activity, cells were cultivated for 16 h in M9 succinate minimal medium with or without the addition of 100 μM $FeCl_3$. Enzyme activity was determined by the method of Miller (30) and expressed as Miller units.

RESULTS

Isolation of mutants deficient in pyoverdin production. Intergeneric matings between DH5α(pMT6121) and MT1 yielded at 42°C the Hg^r Km^r transconjugants at frequencies of around 8×10^{-5} per donor cell. More than 99% of these transconjugants were Cb^s Tc^s, indicating that Tn*1737*KH was inserted into the MT1 chromosome with concomitant loss of the pME305 region. Among the 2,424 derivatives of MT1 with Tn*1737*KH insertions in more than a hundred matings, 25 independent mutants (AMT22 to AMT46) that grew normally on succinate M9 (SM9) agar plates but did not grow on EDDA-supplemented SM9 (EDDA-SM9) agar plates were isolated. To determine the genetic linkage between the mutation and the transposon insert, the G101*h2* lysate prepared on each mutant was used to transduce MT1 to Hg^r. In all 25 mutants except for AMT27, the Hg^r marker was completely linked to the mutant phenotype, indicating that these mutations were due to the insertion of Tn*1737*KH.

The 24 mutants were divided into two groups on the basis of the nonfluorescent (Flu$^-$) or slightly fluorescent (Flu$^\pm$) phenotype under UV excitation after growth on SM9 agar plates. While the culture supernatants from the 20 Flu$^-$ mutants (represented by AMT22) contained no pyoverdin at all, the culture supernatants from the 4 Flu$^\pm$ mutants, AMT39, AMT41, AMT42, and AMT44, showed residual absorption spectra with a peak at 400 nm (Fig. 2). There was, however, no shift of such spectra after acidification of the supernatants. This contrasted with the shift of absorption spectrum of the culture supernatant from a wild-type strain, MT1, that contained the native pyoverdin (18). It was therefore suggested that the slight fluorescence emitted by the four mutants on the agar plates was not due to the native pyoverdin molecule.

All 24 mutants appeared to have defects in production of pyoverdin but not in the step of the ferripyoverdin uptake or later ferric ion assimilation. This was based on the observation that addition of the culture supernatant from the SM9-grown cells of MT1 supported normal growth of all mutants on EDDA-SM9 agar plates.

Cloning of chromosomal regions involved in pyoverdin production. Tn*1737*KH carries a unique *Sac*I site at the left side of the Km^r determinant (Fig. 1A). It was therefore expected that digestion with *Sac*I of the chromosome with the Tn*1737*KH insertion followed by cloning of the Km^r determinant into *E. coli* led to isolation of the chromosomal region located at the right side of the transposon insert. The chromosomal region located at both sides of the insert was also expected to be cloned by use of *Kpn*I because of the absence of this site in Tn*1737*KH. The vector plasmid employed for this purpose was a pBR322 derivative, pMT267 (Fig. 1B), carrying the *Sac*I and *Kpn*I sites. Using this strategy, we cloned, in conjunction with all or some part of the Tn*1737*KH sequence, the chromosomal regions flanking all 24 insertion mutations. The presence of the *Eco*RI sites 13 bp apart from each end of Tn*1737*KH (Fig. 1A) (2) was especially advantageous for delineation of the Tn*1737*KH-derived region on each recombinant plasmid. The maps of the chromosomal regions thus cloned were compared with one another, and in some cases, more detailed comparison of the regions was made by Southern hybridization (data not shown). These analyses showed that the 7 insertion mutations were located in a 25.8-kb region (region A) and the remaining 17 mutations were located in a 77.6-kb region (region B) (Fig. 3).

To clarify whether these two regions are contiguous or separated on the *P. aeruginosa* chromosome, an attempt to clone the chromosomal region situating at the right side of region B was made. Plasmid pMT7030 (Fig. 3) contained the rightmost 3.0-kb *Eco*RI-*Sac*I fragment of region B, so the *Sac*I site is adjacent to the pMT267-derived *Eco*RI site. This *Eco*RI fragment was subcloned in the *Eco*RI site of pSUP203, the resulting plasmid (pMT7103) was mobilized from an *E. coli* strain, S17-1, to MT1, and selection for Cb^r Tc^r was made. In the transconjugants represented by AMT107, the plasmid was integrated into the chromosome by a single crossover through homologous recombination (Fig. 4). The chromosomal DNA prepared from AMT107 was digested with *Bam*HI, self-ligated, and used to transform *E. coli* DH5α to recover the plasmid carrying the Ap^r determinant. The recovered plasmid, pMT7105, possessed a contiguous 18.6-kb *Eco*RI-*Bam*HI fragment of the MT1 chromosome that consisted of the rightmost 3.0-kb *Eco*RI-*Sac*I fragment of region B and the leftmost 15.6-kb *Sac*I-*Bam*HI fragment of region A (Fig. 3). We therefore concluded that regions A and B are contiguous on the *P. aeruginosa* chromosome, and the contiguous 103-kb region is hereafter referred to as the *pvd* region.

No inserted mutations with Tn*1737*KH insertions were localized within a 37.7-kb segment spanning from kb 22.5 to 60.2 (Fig. 3 and Table 2). To investigate whether this DNA segment carries some additional *pvd* genes, five of the six *Kpn*I sites in this chromosomal segment of MT1 were subjected to insertion of the Tc^r gene cartridge by the gene replacement mutagenesis technique described in Materials and Methods. Among the five insertion derivatives, AMT122 and AMT123, which carry the Tc^r inserts at the *Kpn*I sites of kb 34.3 and 39.9, respectively, showed no difference from MT1 in pyoverdin production. The

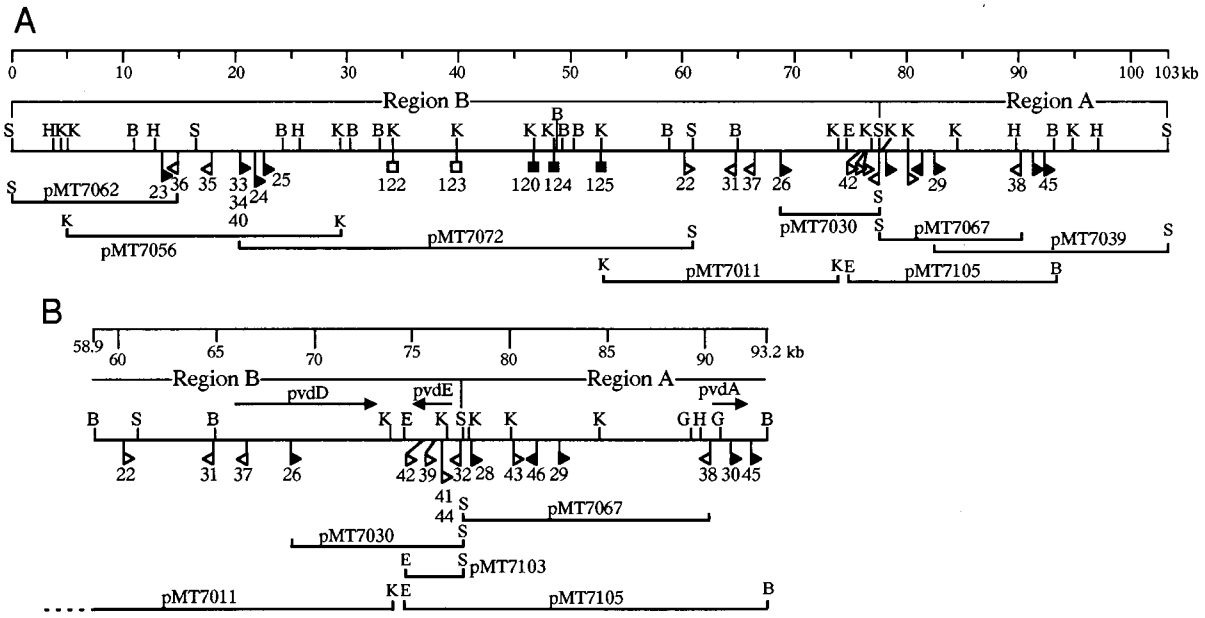

FIG. 3. Physical map of the *pvd* region and distribution of mutations. Depicted are the map of the 103-kb *pvd* region (A) and a detailed portion from kb 58.9 to 93.2 (B). Abbreviations for restriction sites are the same as those described in the legend of Fig. 1 except for G (*Bgl*II). The *Eco*RI site depicted in panel A is limited to that at kb 74.6, and there are a unique *Eco*RI and two *Bgl*II sites in the detailed map (B). Regions A and B are located to the right and left, respectively, of the *Sac*I site at kb 77.6. ▷ and ◁, orientations of the Tn*1737*KH inserts (the transcriptional directions of the promoterless *lacZ* genes are right and left, respectively). The inserts conferring the type I phenotypes to the mutant cells (see text and Table 2 for details) are represented by solid arrowheads. Solid and open squares represent the inserts of a Tc^r gene cartridge resulting in the Pvd^- and Pvd^+ phenotypes, respectively. The numerals below the symbols indicate strain names with the prefix AMT. The arrow above the physical map in panel B indicates the transcriptional direction of the gene (24, 55). Drawn below the physical maps are representatives of the cloned chromosomal DNA fragments. The Tn*1737*KH insertion mutants and the restriction sites used for construction of the pMT267-derived plasmids are as follows: AMT22 and *Kpn*I for pMT7011; AMT26 and *Sac*I for pMT7030; AMT29 and *Sac*I for pMT7039; AMT35 and *Kpn*I for pMT7056; AMT36 and *Sac*I for pMT7062; AMT38 and *Sac*I for pMT7067; and AMT40 and *Sac*I for pMT7072. See the text for construction of pMT7103 and pMT7105.

remaining three derivatives, AMT120, AMT124, and AMT125, having the inserts at the *Kpn*I sites of coordinates 46.7, 48.5, and 52.8, respectively, had no ability to produce pyoverdin. These results indicated that at least the right one-third of the 37.7-kb segment also carries additional *pvd* genes.

Genetic and physical mapping of the *pvd* region on the chromosome map. According to the studies by other groups (3, 18, 41, 56), the chemically induced *pvd* mutations have been genetically localized at the two distinct regions on the chromosome map, namely, the 23- and 47-min regions (20). In this study, we first attempted to map the *pvd* region by using three R-prime plasmids derived from R68.45. Two R-prime plasmids carrying the 23-min region of the chromosome (pNM21 and pNM108) (43) could not complement any of the 24 mutations. On the other hand, all the mutations were complemented by pMT7009 that carried the wild-type alleles of the *catA* and *mtu-9002* genes located at the 46- to 48-min region, indicating that the *pvd* region is located in this chromosomal region. Zhang and Holloway (57) have constructed the physical map of a 125-kb chromosomal region containing the *catA* gene (the *catA* region), and a cosmid, pMO1983, carries the rightmost part of the *catA* region. We noticed that physical map of this part showed similarity to that of the leftmost part of the *pvd* region cloned on pMT7062 (Fig. 5A and C). Southern analysis clearly revealed that the 8.5-kb *Xho*I fragments residing in both plasmids were identical (Fig. 5B), and more detailed analysis led to the conclusion that the *pvd* and *catA* regions are overlapped by 10 kb in size with each other (Fig. 5C).

The restriction enzyme *Spe*I has been used for construction of the physical map of the *P. aeruginosa* PAO chromosome (21). There was no *Spe*I site in the *pvd* region. Considering that the chromosomal DNA on pMO1983 resides within the *Spe*I-J fragment on the physical map, we concluded that the 103-kb *pvd* region is also located in this *Spe*I fragment.

Transductional analysis of the *pvd* region. The two Tn*5* insertion mutations *gcu-2* and *gcu-3*, which lead to a defect in glycine utilization, have been mapped within the *catA* region in the order *catA gcu-2 gcu-3 mtu-9002* (57). The two *gcu* markers, which are very closely linked with each other on the physical map, are more than 50 kb away from *catA*, and pMO1983 has been shown to carry the *gcu-3* but not the *gcu-2* marker (57). These facts and our data obtained by Southern analysis (Fig. 5) suggested that the *gcu-3* marker was located at the left side of the *pvd* region. G101*h2*-mediated transduction was therefore carried out to investigate the linkage of the *pvd* mutations with the two *gcu* mutations as well as the *catA* and *mtu-9002* mutations (Fig. 6). The Tn*1737*KH-encoded Hg^r marker in the four representative *pvd* mutations (*pvd-22*, *-23*, *-25*, and *-45*) was used as the positively selectable donor marker. None of the four Hg^r markers showed any linkage with *catA* or *mtu-9002*. Only the Hg^r marker in *pvd-23*, the leftmost marker in the *pvd* region, exhibited linkage with *gcu-2* and *gcu-3* (8 and 18%, respectively), whereas the remaining three Hg^r markers were not cotransducible with either of the two *gcu* markers. We also examined the linkage of the AMT123-loaded Tc^r marker with their flanking markers. This Tc^r marker revealed transductional linkage with *pvd-23*, *-25*, and *-22* but not with *pvd-45*, *catA1*, *gcu-2*, *gcu-3*, or *mtu-9002*. These results clearly indicated that all of the *pvd*::Tn*1737*KH mutations are located between *gcu-3* and *mtu-9002.*

Transcriptional activities of the Tn*1737*KH insertion sites in the *pvd* region. The promoterless *lacZ* gene in Tn*1737*Km

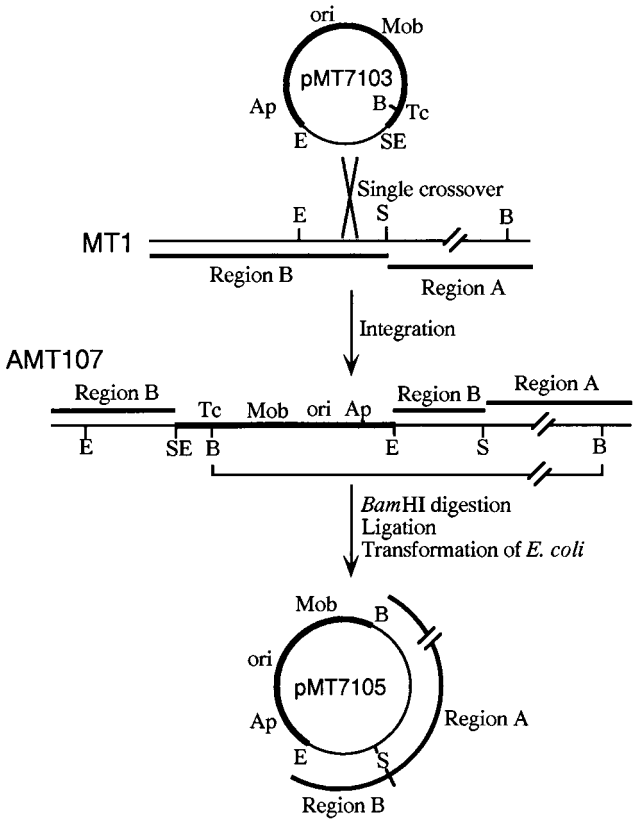

FIG. 4. Strategy for cloning the *P. aeruginosa* chromosomal DNA located at the right side of region B. The thick line represents the pSUP203 DNA, and the sizes of the DNA fragments are arbitrary. Abbreviations for restriction sites are the same as those in the legend to Fig. 1. Note that the *Eco*RI-*Sac*I fragment of region B is duplicated in the AMT107 chromosome. Mob, region required for plasmid mobilization; ori, replication origin of plasmid.

can be expressed upon its insertion into the target gene only when the disrupted gene is transcribed in the direction of the reporter *lacZ* gene and the β-galactosidase activity corresponds to the activity of the promoter located upstream of the inserted *lacZ* gene (53). This feature is also retained in Tn*1737*KH, and we expected that some *pvd*::Tn*1737*KH mutants exhibited β-galactosidase activities that might be further modulated in response to the ferric ion in culture media. Strain AMT3 is an MT1 derivative with a chromosomal Tn*1737*KH insert that was randomly chosen as a control strain with no apparent defects in its iron uptake systems. The 24 *pvd*::Tn*1737*KH mutants along with AMT3 were cultivated in SM9 with or without the addition of 100 μM $FeCl_3$, and the β-galactosidase activities were measured.

On the basis of the β-galactosidase activities under iron-limiting conditions together with the ratios of reduction of the activities by the addition of $FeCl_3$, 22 of the 24 *pvd*::Tn*1737*KH mutants (except for AMT22 and AMT31) were classified into two types, types I and II (Table 2 and Fig. 3). Type I mutants consisted of 12 mutants showing β-galactosidase activities of more than 512 U under iron-limiting conditions, and the addition of $FeCl_3$ resulted in reduction of the activities by more than 7.4-fold. The typical mutants were AMT28, AMT46, and AMT30, which expressed very high levels (>2,300 U) of β-galactosidase activity under iron-limiting conditions. Ten type II mutants showed similarity to AMT3 in that (i) the levels of β-galactosidase activities under iron-limiting conditions were

TABLE 2. β-Galactosidase activities of *pvd*::Tn*1737*KH mutants

Strain	Tn*1737*KH insert[a]		β-Galactosidase activity[b]			Type[d]
			At following concn of $FeCl_3$		Ratio[c]	
	Site	Orientation	0 μM	100 μM		
AMT3	ND[e]	ND	127	54	2.4	ND
AMT23	13.5	R	619	66	9.4	I
AMT36	14.7	L	105	44	2.4	II
AMT35	17.9	L	123	44	2.8	II
AMT33	20.4	R	976	60	16.3	I
AMT34	20.4	R	865	93	9.3	I
AMT40	20.4	R	512	69	7.4	I
AMT24	21.7	R	570	68	8.4	I
AMT25	22.5	R	677	55	12.3	I
AMT22	60.2	R	775	319	2.4	ND
AMT31	64.8	L	242	37	6.5	ND
AMT37	66.5[f]	L[g]	101	73	1.4	II
AMT26	68.8[f]	R[g]	957	93	10.3	I
AMT42	75.7[f]	R[g]	104	40	2.6	II
AMT39	76.2[f]	R[g]	84	48	1.8	II
AMT41	76.4[f]	R[g]	166	69	2.4	II
AMT44	76.4[f]	R[g]	130	68	1.9	II
AMT32	77.5	L	126	54	2.3	II
AMT28	78.0	R	2,368	247	9.6	I
AMT43	80.1	R	150	44	3.4	II
AMT46	81.4	L	3,674	165	22.3	I
AMT29	82.5	R	961	60	16.0	I
AMT38	90.2	L	176	44	4.0	II
AMT30	91.3[f]	R[g]	3,932	210	18.7	I
AMT45	92.3	R	863	90	9.6	I

[a] The insertion site is expressed as kilobase coordinates in Fig. 3. R, Tn*1737*KH in the *pvd* region (Fig. 3) is inserted so that the transcriptional direction of the promoterless *lacZ* gene is to the right; L, Tn*1737*KH is in the opposite orientation.

[b] The values, in Miller units, are the means of at least two independent experiments.

[c] The β-galactosidase activity without the addition of $FeCl_3$ divided by that with the addition of $FeCl_3$.

[d] See text for classification.

[e] ND, not determined.

[f] The inserts are at *pvdD* in AMT37 and AMT26; at *pvdE* in AMT42, AMT39, AMT41, and AMT44; and at *pvdA* in AMT30.

[g] The transcriptional directions of the Tn*1737*KH-loaded *lacZ* gene and the disrupted *pvd* gene are the same in AMT26 and AMT30 and are opposite in AMT37, AMT42, AMT39, AMT41, and AMT44.

low (<176 U) and (ii) reduction of the activity levels by the addition of $FeCl_3$ was not drastic (reduction by less than 4.0-fold). Exceptions of the two types of mutants were AMT22 and AMT31. AMT22 expressed a moderate level of β-galactosidase activity (775 U) under an iron-limiting condition, and only a 2.4-fold reduction of the activity was observed after the addition of $FeCl_3$. The level of β-galactosidase activity of AMT31 under an iron-limiting condition was low (242 U), and the addition of $FeCl_3$ resulted in reduction of the activity level by 6.5-fold.

DISCUSSION

Tn*5*, the most frequently used transposon in various bacteria (7), and its derivatives have been indicated not to be stably maintained in *P. aeruginosa* chromosomes (11). In contrast, Tn*501*, a Tn*1722*-related transposon (2, 7), has been demonstrated to be stably maintained and not to induce further undesirable rearrangement of the DNA regions flanking the transposon inserts in this bacterium (46, 47). For this reason, we employed in this study Tn*1737*KH to isolate the mutants of *P. aeruginosa* defective in iron acquisition systems, and all the

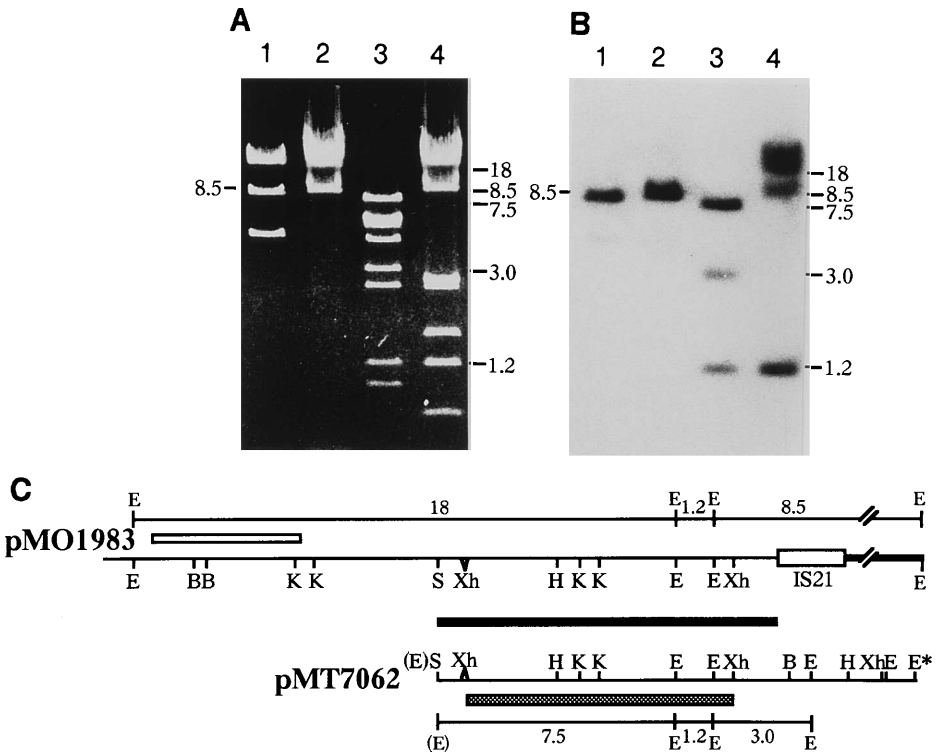

FIG. 5. Overlap of the *pvd* region with the *catA* region. (A) Agarose gel electrophoresis of the restricted DNA fragments of the two plasmids, pMT7062 and pMO1983, that carry the leftmost and rightmost parts of the *pvd* and *catA* regions, respectively. (B) Southern hybridization of the restricted DNA fragments of the two plasmids by use of an 8.5-kb *Xho*I fragment of pMT7062 as the probe (shaded box in panel C). The samples in panels A and B are as follows: lane 1, *Xho*I digest of pMT7062; lane 2, *Xho*I digest of pMO1983; lane 3, *Eco*RI digest of pMT7062; and lane 4, *Eco*RI digest of pMO1983. The sizes (in kilobases) of the hybridized bands are shown beside the photographs. (C) Comparison of the two plasmids. In the pMO1983 map is drawn the right part of the chromosomal *catA* region in conjunction with IS*21* (open box) and part of the vector plasmid pLA2917 (thick bar). The open bar above the map is the DNA segment inferred to contain the *gcu-3* locus (57). The pMT7062 map is limited to the *pvd* region, and the vector (pMT267) and Tn*1737*KH portions are omitted for simplicity. Abbreviations for restriction sites are the same as those described in the legend to Fig. 1, except for Xh (*Xho*I). The *Eco*RI sites derived from the vector and Tn*1737*KH are shown in parentheses and marked by an asterisk, respectively. The *Eco*RI fragments of pMO1983 and pMT7062 that were hybridized with the probe are schematically shown above and below the maps, respectively. The 10-kb segment shared by the two plasmids is depicted by the solid bar between the two maps.

pvd::Tn*1737*KH mutants also showed stability in the mutant phenotypes and in the physical structures of the inserted transposons as well as their insertion sites. An advantage of Tn*1737*KH insertion mutagenesis over chemical mutagenesis was the ability to provide the mutations with positively selectable markers. The Hg^r marker in Tn*1737*KH was suitable for demonstrating the transductional linkage of a *pvd* mutation with the *gcu-2* and *gcu-3* mutations. The Km^r marker was proven to be valuable for molecular cloning of the mutated sites, leading to construction of the physical map of the 103-kb *pvd* region. There was a 10-kb overlap between this *pvd* region and the 125-kb *catA* region (Fig. 5). We could therefore draw the physical map of the 46- to 47-min region of the chromosome with a size of 218 kb, which corresponds to 3.7% of the 5,850-kb genome (21).

The 24 (1.0%) mutants in our collection of mutants with Tn*1737*KH insertions were all defective in pyoverdin production. It was anticipated that our screening would lead to isolation of mutants defective in the components involved in siderophore-mediated transport. The most likely reason for our lack of success in finding such mutants might be nonrandom manner of the insertion of the transposon into the chromosome. This agrees with the nonrandom manner of the Tn*501* insertion into the chromosomal auxotrophic genes (46). Our *pvd* mutations were clustered in the 9.0-kb (kb 13.5 to 22.5) and 32.1-kb (kb 60.2 to 92.3) DNA segments within the 103-kb *pvd* region (Fig. 3 and Table 2). It was indicated by gene replacement mutagenesis that additional *pvd* genes are also located within the 37.7-kb DNA segment that intervenes the two clusters (Fig. 3). It is unknown at present (i) whether the 78.8-kb DNA segment between *pvd-23* (in AMT23) and *pvd-45*

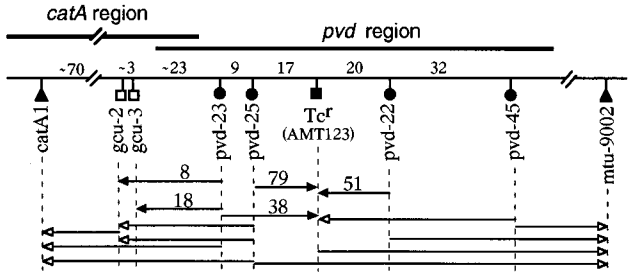

FIG. 6. Linkage of the *pvd* region by G101*h2*-mediated transduction. The strains used were PAO1304 (*gcu-2*), PAO1305 (*gcu-3*), PAO2384 (*catA1 mtu-9002*), AMT22 (*pvd-22*), AMT23 (*pvd-23*), AMT25 (*pvd-25*), AMT45 (*pvd-45*), and AMT123. ▲, point mutation; □, Tn*5* insertion; ●, Tn*1737*KH insertion; and ■, insertion of a Tc^r gene cartridge. The distance (in kilobases) between the markers is shown above the map. Combinations of the makers showing and not showing the linkage are depicted as the arrows with solid and open arrowheads, respectively, and the percent linkage is, in cases in which it was detected, indicated above the arrow. The markers in the recipient are represented by arrowheads. Selections were made for Cat^+ or Mtu^+ when PAO2384 was used as the recipient strain and for Hg^r when the mutants with Tn*1737*KH insertions were used as the donor strains.

(in AMT45) is occupied solely by the *pvd* genes and (ii) whether other unidentified *pvd* genes are located outside of the 78.8-kb segment in the *pvd* region. In our preliminary experiments, no housekeeping or auxotrophic genes appeared to be located in the 103-kb *pvd* region, since SM9 agar plates supported growth of the mutants carrying large deletions in this *pvd* region. The *P. aeruginosa pvd* genes show extensive and strong homology with the pseudobactin synthesis genes from *Pseudomonas* sp. strain B10 and *P. putida* WCS358 (41), and such genes have been considered to occupy far more than 100 kb in size on each chromosome (26, 31). It might be feasible that synthesis and secretion of mature pyoverdin by *P. aeruginosa* require much longer DNA segments than the 78.8-kb segment in the *pvd* region or another genetically unlinked region. The latter situation is likely to be the case, because the recently identified DNA fragment possibly responsible for secretion of pyoverdin (36) appears, so far judged from its physical map, not to reside in the *pvd* region.

Visca et al. (55) and Lamont et al. (24, 29, 41) have recently succeeded in cloning and sequencing some *pvd* genes of *P. aeruginosa* PAO. Detailed comparison of their physical maps with that of our *pvd* region revealed that their DNA segments cover the *pvd* region of Fig. 3 ranging from 75.1 to 95.0 and from 64.5 to 77.3. Some *pvd*::Tn*1737*KH mutations could be consequently mapped in specific loci: *pvd-30* (in AMT30) in *pvdA* encoding L-ornithine N^5-oxygenase involved in an early step of pyoverdin synthesis that hydroxylates L-Orn to form L-N^5-OH-Orn (55); *pvd-37* (in AMT37) and *pvd-26* (in AMT26) in *pvdD* encoding an enzyme probably involved in a step to form peptide moiety of pyoverdin by joining the amino acid components in a nonribosomal manner (29); and *pvd-42*, *pvd-39*, *pvd-41*, and *pvd-44* (in AMT42, AMT39, AMT41, and AMT44, in that order) in *pvdE* encoding a product with an uncharacterized function (24). We found that the culture supernatants from these four *pvdE* mutants showed absorption spectra with residual peaks at 400 nm which were not shifted by acidification (Fig. 2). This suggests that the *pvdE* mutants have secreted a compound that might be the precursor of pyoverdin or its degraded form. If this is the case, the *pvdE* product would be involved in a later step of pyoverdin synthesis, e.g., formation of the functional domain important not only for emission of fluorescence but also for chelation of the ferric ion.

According to the sequence data of the above-mentioned two groups (24, 55), the transcriptional directions of the reporter *lacZ* gene in *pvdA30* and in *pvdD26* are the same as those of *pvdA* and *pvdD*, respectively, whereas those of the reporter gene in *pvdD37* and in the four *pvdE* mutations are opposite of those of *pvdD* and *pvdE*, respectively (Fig. 3). By our classification of the *pvd*::Tn*1737*KH mutants (Table 2), AMT30 and AMT26 were designated type I mutants, which showed higher levels of β-galactosidase activity under iron-limiting conditions and a higher degree (>7.4-fold and up to 22.3-fold) of reduction of the activities after the addition of $FeCl_3$; AMT37 and the four *pvdE* mutants were designated type II mutants, which showed lower levels of activity and a lower degree (<4.0-fold) of reduction. It is conceivable from this correlation that (i) type I mutants other than AMT30 and AMT26 also have the Tn*1737*KH inserts in the orientations appropriate for the reporter gene to be expressed from the *pvd* promoters located upstream of the insertion sites of the transposon and (ii) transcription from many, if not all, of such promoters is repressed by $FeCl_3$. The levels of β-galactosidase activity of the type II mutants under iron-rich conditions were all lower than those under iron-limiting conditions, and a 2.6-fold reduction of the level of activity after the addition of $FeCl_3$ was observed for a *pvdE* mutant, AMT42. Taking into consideration that the transcriptional direction of the reporter gene in this mutant is opposite of that of *pvdE*, a reduction ratio of up to 2.6-fold would not reflect the repression of transcription by $FeCl_3$. Our measurement of the β-galactosidase activities in this study would not be enough to rule out another possibility, that the transcriptions at some mutated sites in the type II mutants are not regulated by iron.

Prince et al. (39) have recently isolated a regulatory mutation, *fur*, which led to constitutive production of pyoverdin even in the presence of $FeCl_3$. Our assay of the β-galactosidase activities of the 24 *pvd*::Tn*1737*KH mutants suggested that there were a number of transcriptional units of the *pvd* genes (Fig. 3 and Table 2). It is of interest whether (i) Fur directly represses expression of all or many of such transcriptional units and (i) there exists additional regulatory systems for transcription of such units.

ACKNOWLEDGMENTS

We thank J. Frey, B. W. Holloway, H. Matsumoto, R. Schmitt, P. H. Schweizer, and T. Shinomiya for their gifts of bacterial strains and plasmids.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. **Abdallah, M. A.** 1991. Pyoverdins and pseudobactins, p. 139–153. *In* G. Winkelmann (ed.), CRC handbook of microbial iron chelates. CRC Press, Boca Raton, Fla.
2. **Allmeier, H., B. Cresnar, M. Greck, and R. Schmitt.** 1992. Complete nucleotide sequence of Tn*1721*: gene organization and a novel gene product with features of a chemotaxis protein. Gene **111:**11–20.
3. **Ankenbauer, R., L. F. Hanne, and C. D. Cox.** 1986. Mapping of mutations in *Pseudomonas aeruginosa* defective in pyoverdin production. J. Bacteriol. **167:**7–11.
4. **Ankenbauer, R., S. Sriyosachati, and C. D. Cox.** 1985. Effects of siderophores on the growth of *Pseudomonas aeruginosa* in human serum and transferrin. Infect. Immun. **49:**132–140.
5. **Ankenbauer, R. G., and H. N. Quan.** 1994. FptA, the Fe(III)-pyochelin receptor of *Pseudomonas aeruginosa*: a phenolate siderophore receptor homologous to hydroxamate siderophore receptors. J. Bacteriol. **176:**307–319.
6. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1991. Current protocols in molecular biology. John Wiley & Sons, New York.
7. **Berg, C. M., D. E. Berg, and E. A. Groisman.** 1989. Transposable elements and the genetic engineering of bacteria, p. 879–925. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
8. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
9. **Briskot, G., K. Taraz, and H. Budzikiewicz.** 1989. Pyoverdin-type siderophores from *Pseudomonas aeruginosa*. Liebigs Ann. Chem. **37:**375–384.
10. **Chandler, P. M., and V. Krishnapillai.** 1977. Characterization of *Pseudomonas aeruginosa* derepressed R-plasmids. J. Bacteriol. **130:**596–603.
11. **Cornelis, P., V. Anjaiah, N. Koedam, P. Delfosse, P. Jacques, P. Thonart, and L. Neirinckx.** 1992. Stability, frequency and multiplicity of transposon insertions in the pyoverdine region in the chromosomes of different fluorescent pseudomonads. J. Gen. Microbiol. **138:**1337–1343.
12. **Cox, C. D.** 1980. Iron uptake with ferripyochelin and ferric citrate by *Pseudomonas aeruginosa*. J. Bacteriol. **142:**581–587.
13. **Cox, C. D., and P. Adams.** 1985. Siderophore activity of pyoverdin for *Pseudomonas aeruginosa*. Infect. Immun. **48:**130–138.
14. **Cox, C. D., and R. Graham.** 1979. Isolation of an iron-binding compound from *Pseudomonas aeruginosa*. J. Bacteriol. **137:**357–364.
15. **Crosa, J. H.** 1989. Genetics and molecular biology of siderophore-mediated iron transport in bacteria. Microbiol. Rev. **53:**517–530.
16. **Fellay, R., J. Frey, and H. Krisch.** 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of Gram-negative bacteria. Gene **52:**147–154.
17. **Haas, D., and B. W. Holloway.** 1976. R factor variants with enhanced sex factor activity in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **144:**243–251.
18. **Hohnadel, D., D. Haas, and J.-M. Meyer.** 1986. Mapping of mutations affecting pyoverdine production in *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **36:**195–199.
19. **Holloway, B. W.** 1969. Genetics of *Pseudomonas*. Bacteriol. Rev. **33:**419–443.

20. **Holloway, B. W., and E. Carey.** 1993. *Pseudomonas aeruginosa* PAO, p. 2.98–2.105. *In* S. J. O'Brien (ed.), Genetic maps: locus maps of complex genomes, 6th ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. **Holloway, B. W., E. Ratnaningsih, V. Krishnapillai, B. Tümmler, and U. Römling.** 1993. Physical and genetic map of *Pseudomonas aeruginosa* PAO, p. 2.93–2.97. *In* S. J. O'Brien (ed.), Genetic maps: locus maps of complex genomes, 6th ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Kim, J., C. Zwieb, C. Wu, and S. Adhya.** 1989. Bending of DNA by gene-regulatory proteins: construction and use of a DNA bending vector. Gene **85:**15–23.
23. **Koster, M., W. van Klompenburg, W. Bitter, J. Leong, and P. Weisbeek.** 1994. Role for the outer membrane ferric siderophore receptor PupB in signal transduction across the bacterial cell envelope. EMBO J. **13:**2805–2813.
24. **Lamont, I. L.** 1994. Pseudomonas aeruginosa OT11 pyoverdine synthetase D (pvdD), ferripyoverdine receptor (fpvA), and pyoverdine synthetase E (pvdE) genes, complete cds. GenBank database release 82.0, accession number U07359.
25. **Litwin, C. M., and S. B. Calderwood.** 1993. Role of iron in regulation of virulence genes. Clin. Microbiol. Rev. **6:**137–149.
26. **Marugg, J. D., M. van Spanje, W. P. M. Hoekstra, B. Schippers, and P. J. Weisbeek.** 1985. Isolation and analysis of genes involved in siderophore biosynthesis in plant-growth-stimulating *Pseudomonas putida* WCS358. J. Bacteriol. **164:**563–570.
27. **Marugg, J. D., and P. J. Weisbeek.** 1991. Molecular genetics of siderophore biosynthesis in fluorescent pseudomonads, p. 155–175. *In* G. Winkelmann (ed.), CRC handbook of microbial iron chelates. CRC Press, Boca Raton, Fla.
28. **Merriman, T. R., and I. L. Lamont.** 1993. Construction and use of a self-cloning promoter probe vector for Gram-negative bacteria. Gene **126:**17–23.
29. **Merriman, T. R., and I. L. Lamont.** 1993. Characterization of a gene required for synthesis of pyoverdine by *Pseudomonas aeruginosa*, p. 112. *In* R. E. W. Hancock (ed.), Abstracts of the 4th International Symposium on *Pseudomonas*. Vancouver, Canada.
30. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Moores, J. C., M. Magazin, G. S. Ditta, and J. Leong.** 1984. Cloning of genes involved in the biosynthesis of pseudobactin, a high-affinity iron transport agent of a plant growth-promoting *Pseudomonas* strain. J. Bacteriol. **157:**53–58.
32. **Neilands, J. B.** 1981. Iron absorption and transport in microorganisms. Annu. Rev. Nutr. **1:**27–46.
33. **O'Sullivan, D. J., and F. O'Gara.** 1990. Iron regulation of ferric ion uptake in a fluorescent pseudomonad: cloning of a regulatory gene. Mol. Plant Microbe Interact. **3:**86–93.
34. **O'Sullivan, D. J., and F. O'Gara.** 1991. Regulation of iron assimilation: nucleotide sequence analysis of an iron-regulated promoter from a fluorescent pseudomonad. Mol. Gen. Genet. **228:**1–8.
35. **O'Sullivan, D. J., and F. O'Gara.** 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiol. Rev. **56:**662–676.
36. **Poole, K., D. E. Heinrichs, and S. Neshat.** 1993. Cloning and sequence analysis of an EnvCD homologue in *Pseudomonas aeruginosa*: regulation by iron and possible involvement in the secretion of the siderophore pyoverdine. Mol. Microbiol. **10:**529–544.
37. **Poole, K., S. Neshat, and D. Heinrichs.** 1991. Pyoverdine-mediated iron transport in *Pseudomonas aeruginosa*: involvement of a high-molecular-mass outer membrane protein. FEMS Microbiol. Lett. **78:**1–6.
38. **Poole, K., S. Neshat, K. Krebes, and D. E. Heinrichs.** 1993. Cloning and nucleotide sequence analysis of the ferripyoverdine receptor gene *fpvA* of *Pseudomonas aeruginosa*. J. Bacteriol. **175:**4597–4604.
39. **Prince, R. W., C. D. Cox, and M. L. Vasil.** 1993. Coordinate regulation of siderophore and exotoxin A production: molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. J. Bacteriol. **175:**2589–2598.
40. **Rella, M., A. Mercenier, and D. Haas.** 1985. Transposon insertion mutagenesis of *Pseudomonas aeruginosa* with a Tn*5* derivative: application to physical mapping of the *arc* gene cluster. Gene **33:**293–303.
41. **Rombel, I. T., and I. L. Lamont.** 1992. DNA homology between siderophore genes from fluorescent pseudomonads. J. Gen. Microbiol. **138:**181–187.
42. **Schweizer, H. P.** 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis sacB* marker. Mol. Microbiol. **6:**1195–1204.
43. **Shinomiya, T., S. Shiga, A. Kikuchi, and M. Kageyama.** 1983. Genetic determinant of pyocin R2 in *Pseudomonas aeruginosa* PAO. II. Physical characterization of pyocin R2 genes using R-prime plasmids constructed from R68.45. Mol. Gen. Genet. **189:**382–389.
44. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology **1:**784–791.
45. **Sokol, P. A.** 1987. Surface expression of ferripyochelin-binding protein is required for virulence of *Pseudomonas aeruginosa*. Infect. Immun. **55:**2021–2025.
46. **Tsuda, M., S. Harayama, and T. Iino.** 1984. Tn*501* insertion mutagenesis in *Pseudomonas aeruginosa* PAO. Mol. Gen. Genet. **196:**494–500.
47. **Tsuda, M., and T. Iino.** 1983. Ordering of the flagellar genes in *Pseudomonas aeruginosa* by insertions of mercury transposon Tn*501*. J. Bacteriol. **153:**1008–1017.
48. **Tsuda, M., and T. Iino.** 1983. Transductional analysis of the flagellar genes in *Pseudomonas aeruginosa*. J. Bacteriol. **153:**1018–1026.
49. **Tsuda, M., and T. Iino.** 1987. Genetic analysis of a transposon carrying toluene degrading genes on a TOL plasmid, pWW0. Mol. Gen. Genet. **210:**270–276.
50. **Tsuda, M., K.-I. Minegishi, and T. Iino.** 1989. Toluene transposons Tn*4651* and Tn*4653* are class II transposons. J. Bacteriol. **171:**1386–1393.
51. **Tsuda, M., and T. Nakazawa.** 1993. A mutagenesis system utilizing a Tn*1722* derivative containing an *Escherichia coli*-specific vector plasmid: application to *Pseudomonas* species. Gene **136:**257–262.
52. **Tsuda, M., T. Oguchi, and T. Iino.** 1981. Analysis of flagellar genes in *Pseudomonas aeruginosa* by use of R*fla* plasmids and conjugations. J. Bacteriol. **147:**1008–1014.
53. **Ubben, D., and R. Schmitt.** 1987. A transposable promoter and transposable promoter probes derived from Tn*1721*. Gene **53:**127–134.
54. **Venturi, V., C. Ottevanger, J. Leong, and P. J. Weisbeek.** 1993. Identification and characterization of a siderophore regulatory gene (*pfrA*) of *Pseudomonas putida* WCS358: homology to the alginate regulatory gene *algQ* of *Pseudomonas aeruginosa*. Mol. Microbiol. **10:**63–73.
55. **Visca, P., A. Ciervo, and N. Orsi.** 1994. Cloning and nucleotide sequence of the *pvdA* gene encoding the pyoverdin biosynthetic enzyme L-ornithine N^5-oxygenase in *Pseudomonas aeruginosa*. J. Bacteriol. **176:**1128–1140.
56. **Visca, P., L. Serino, and N. Orsi.** 1992. Isolation and characterization of *Pseudomonas aeruginosa* mutants blocked in the synthesis of pyoverdin. J. Bacteriol. **174:**5727–5731.
57. **Zhang, C., and B. W. Holloway.** 1992. Physical and genetic mapping of the *catA* region of *Pseudomonas aeruginosa*. J. Gen. Microbiol. **138:**1097–1107.