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Localization of the Virulence-Associated Genes *pilA*, *pilR*, *rpoN*, *fliA*, *fliC*, *ent*, and *fbp* on the Physical Map of *Pseudomonas aeruginosa* PAO1 by Pulsed-Field Electrophoresis

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Seven virulence-associated genes have been placed on a genomic map of *Pseudomonas aeruginosa* PAO1, using pulsed-field electrophoresis, on the basis of the previous physical maps of Romling et al. (U. Romling, M. Duchene, D. Essar, D. Galloway, C. Guidi-Rontani, D. Hill, A. Lazdunski, R. Miller, K. Schleifer, D. Smith, H. Toschka, and B. Tummler, J. Bacteriol. 174:327–330, 1992; U. Romling, D. Grothues, W. Bautsch, and B. Tummler, EMBO J. 8:4081–4089, 1989) and Ratnaningsih et al. (E. Ratnaningsih, S. Dharmsthiti, V. Krishnapillai, A. Morgan, M. Sinclair, and B. W. Holloway, J. Gen. Microbiol. 136:2351–2357, 1990). The new locations for the outer membrane enterobactin iron-siderophore receptor *ent* gene (41 to 42 min) and the *fliA* gene (59 to 61 min), which encodes a minor sigma factor of RNA polymerase, are given. The *pilA* (the pilin structural gene), *pilR* (a pilin regulatory gene), and *rpoN* (encoding another minor sigma factor of RNA polymerase) genes map together at 71 to 75 min, locations correcting the previously reported values (V. Shortridge, M. Pato, A. Vasil, and M. Vasil, Infect. Immun. 59:3596–3603, 1990). The *fbp* gene (28 to 29 min), which encodes an outer membrane ferripyochelin-binding protein of low molecular weight, and the *fliC* gene (64 to 66 min), the flagellin structural gene, were determined to lie in the previously reported locations.

Although *Pseudomonas aeruginosa* is a common organism in soil and water, it is also an important opportunistic pathogen of humans. Because of the naturally high resistance of *P. aeruginosa* to a wide spectrum of antibiotics, it has been isolated frequently in severe nosocomial infections, especially from immunocompromised patients, burn victims, and those intensive-care patients fitted with indwelling devices (11). *P. aeruginosa* is also the most frequently isolated agent in chronic infections of the lungs of cystic fibrosis patients and is associated with the highest mortality rate for this common genetic disorder (4).

P. aeruginosa PAO utilizes capsular alginate exopolysaccharide and surface appendages such as pili (fimbriae) as principal adhesins to facilitate attachment to human respiratory epithelial cells (6). The pili of *P. aeruginosa* (type 4; *N*-methyl-Phe) are composed entirely of monomeric pilin protein subunits encoded by the *pilA* structural gene (14). Pili mediate the cellular locomotion termed twitching motility and promote adherence to human epithelial cells. Antibodies directed against pili impede adherence to human buccal epithelial cells, and consequently, the development of pilus-specific vaccines is an active area of current research. A regulatory gene of pilin production, *pilR*, has recently been cloned and sequenced and shown to possess substantial homology with the NtrC family of response regulators of the two-component regulatory systems (8). The discovery of this 50-kDa protein suggests that pilin (*pilA*) transcription, which requires the substitution of the vegetative sigma factor (σ^{70}) by the alternate sigma factor σ^{54} (RpoN), shares a common feature with other RpoN-transcribed genes in the need for additional transcriptional activator proteins (9). RpoN (9) allows for initial promoter recognition and for the selective transcription of a subset of genes by *P. aeruginosa* RNA polymerase, including those involved in pilus and flagellum biogenesis. This class of sigma factors is also involved with the recognition of promoters associated with nitrogen assimilation genes, including amino acid transport and catabolism (7, 24).

Flagella are the most obvious surface appendages on bacterial cells and are also implicated in virulence (10), in addition to their primary function of locomotion. *P. aeruginosa* cells are motile by means of a single polar flagellum composed of flagellin subunits encoded by the *fliC* gene. The *fliC* gene has previously been mapped genetically at 59 to 61 min on the 75-min map (13) and mapped physically at 61 to 63 min by Shortridge et al. (21). The promoter region of the *fliC* gene does not resemble a sequence recognized by σ^{70} or by σ^{54} but rather has a sequence related to those recognized by the σ^{28} class of sigma factors (*sigD*, *fliA*, and *rpoF*), which have been shown to be necessary for the expression of motility and chemotaxis in many bacteria (12). The *fliA* gene in *P. aeruginosa* has recently been cloned and sequenced and demonstrates a high degree of homology with the *fliA* gene from *Salmonella typhimurium* (23).

Most bacteria require iron for growth, and in particular, pathogenic organisms must effectively compete with their host for available iron, which is mainly sequestered by iron-binding glycoproteins (transferrin in serum and lactoferrin in secretory fluids; 20). The majority of successful pathogens produce low-molecular-weight iron chelators (siderophores) along with the corresponding cell surface receptors, which are capable of removing iron from host sequestering proteins. In order to acquire iron from its surrounding environment, *P. aeruginosa* is known to produce two characterized siderophores, pyochelin and pyoverdine (1, 2, 15). *P. aeruginosa* is also able to take up iron bound to the

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TABLE 1. Sizes of *Dpn*I restriction fragments of PAO1 chromosomal DNA[a]

Fragment	Fragment size (range) in:	
	kb	min
A	806 (3814–4620)	10.2 (61.1–71.3)
B	757 (2361–3118)	9.5 (42.8–52.3)
C	696 (3118–3814)	8.8 (52.3–61.1)
D	580 (1781–2361)	7.3 (35.5–42.8)
E	506 (5928–486)	6.3 (12.8–19.1)
F_1	394 (486–880)	5.0 (19.1–24.1)
F_2	394 (5534–5928)	5.0 (7.8–12.8)
H	302 (1095–1397)	3.8 (26.8–30.6)
I	290 (4620–4910)	3.7 (71.3–75.0)
J_1	269 (4910–5179)	3.3 (75.0–3.3)
J_2	269 (5179–5448)	3.4 (3.3–6.7)
L	215 (880–1095)	2.7 (24.1–26.8)
M	215 (1397–1612)	2.7 (30.6–33.3)
N	169 (1612–1781)	2.2 (33.3–35.5)
O	86 (5448–5534)	1.1 (6.7–7.8)

[a] The lettering system used for restriction fragments is the one previously suggested (17), and the fragment sizes and ranges are given in map minutes from *ilv* and in kilobases from *oriC* on the basis of a total genome length of 5,940 kb.

TABLE 2. Sizes of *Spe*I restriction fragments of PAO1 chromosomal DNA[a]

Fragment	Fragment size (range) in:	
	kb	min
A	517 (687–1204)	6.5 (21.7–28.2)
B	451 (4970–5421)	5.7 (0.9–6.6)
C	412 (1463–1875)	5.2 (31.5–36.7)
D_1	377 (2888–3265)	4.7 (49.6–54.3)
D_2	377 (4548–4925)	4.7 (70.6–0.3)
F	326 (4192–4518)	4.1 (66.1–70.2)
G	304 (5421–5728)	3.9 (6.6–10.5)
H	293 (272–565)	3.8 (16.4–20.2)
I	258 (1890–2148)	3.3 (36.9–40.2)
J	234 (2597–2831)	2.9 (45.9–48.8)
K	231 (5725–25)	2.8 (10.5–13.3)
L	211 (3265–3476)	2.7 (54.3–57.0)
M	201 (3815–4016)	2.5 (61.3–63.8)
N	169 (3646–3815)	2.1 (59.2–61.3)
O	169 (103–272)	2.1 (14.3–16.4)
P	160 (2290–2450)	2.0 (42.0–44.0)
Q	147 (2450–2597)	1.9 (44.0–45.9)
R	147 (3499–3646)	1.9 (57.3–59.2)
S	133 (1204–1337)	1.7 (28.2–29.9)
T	126 (1337–1463)	1.6 (29.9–31.5)
U	114 (4078–4192)	1.5 (64.6–66.1)
V	82 (2148–2230)	1.0 (40.2–41.2)
W	82 (565–647)	1.0 (20.2–21.2)
X	57 (2831–2888)	0.8 (48.8–49.6)
Y	50 (2240–2290)	0.6 (41.4–42.0)
Z	45 (4925–4970)	0.6 (0.3–0.9)
AA	40 (647–687)	0.5 (21.2–21.7)
AB	34 (25–59)	0.4 (13.3–13.7)
AC	34 (69–103)	0.4 (13.9–14.3)
AD	30 (4041–4071)	0.3 (64.2–64.5)
AE	30 (4518–4548)	0.4 (60.2–60.6)
AG	23 (3476–3499)	0.3 (57.0–57.3)
AH	25 (4016–4041)	0.4 (63.8–64.2)
AI	15 (1875–1890)	0.2 (36.7–36.9)
AJ	10 (59–69)	0.2 (13.7–13.9)
AK	10 (2230–2240)	0.2 (41.2–41.4)
AL	7 (4071–4078)	0.1 (64.5–64.6)

[a] The lettering system used for restriction fragments is the one previously suggested (17), and the fragment sizes and ranges are given in map minutes from *ilv* and in kilobases from *oriC* on the basis of a total genome length of 5,940 kb.

siderophore enterobactin (enterochelin) produced by some members of the *Enterobacteriaceae* family (*Shigella* spp., *Klebsiella* spp., and *Escherichia coli*) via an 80-kDa outer membrane receptor (*ent*). The location of this gene has been determined on the physical map of *P. aeruginosa*. In addition, the *fbp* gene, encoding a low-molecular-weight outer membrane pyochelin-binding protein has been confirmed by mapping with *Spe*I and *Dpn*I to lie in the location previously reported by Shortridge et al. (21).

DNA manipulations. *P. aeruginosa* PAO1 cells, embedded in 0.75% low-melting-point agarose, were lysed with detergents and digested with proteinase K as previously described (18). After being washed extensively with TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA; 5 to 10 changes), the chromosomal DNA was digested in situ with *Dpn*I, *Spe*I, and *Hpa*I restriction endonucleases, and the fragments were resolved by pulsed-field gel electrophoresis with Bio-Rad contour-clamped homogeneous electric field equipment (Fig. 1). The gels were stained with ethidium bromide, dried, and subsequently probed with 5′-end-labelled synthetic oligonucleotides (polynucleotide kinase and [γ-^{32}P]ATP) or random primed double-stranded DNA probes by hexamer primer extension (Klenow fragment of DNA polymerase I and [α-^{32}P]ATP) by the unblot technique of Wallace and Miyada (25).

In order to facilitate the task of locating mapped genes, a circular map of the genome was constructed (Fig. 2) from the linear physical map and by using the fragment designations proposed by Romling et al. (17). This map correlates well with the map generated by Ratnaningsih et al. (16) and with the map generated with the PAO1 strain used in this laboratory. There are slight differences in the very small (<50-kb) *Spe*I fragments, but these differences have not been a hindrance in mapping. The small fragments placed on the map are those of Romling et al. (17) who have best located these fragments on their map. The locations of genes have been reported previously by map minutes based on the 75-min map proposed by O'Hoy and Krishnapillai (13), which uses *ilv* as the point of entry (0.0 min). Romling et al. (17) have determined that *oriC* (chromosomal origin of replication) lies at 13 min on the 75-min map and should be considered the point of entry for physical mapping. The length of the genome has been determined to be 5,940 kb, which corresponds to 79 kb per map minute. A compilation of all of the *Spe*I and *Dpn*I fragments and the range of their locations on the physical map, in kilobases from the *oriC* gene and in map minutes from *ilv*, is presented in Tables 1 and 2.

***rpoN*.** Sequence analysis has shown that *rpoN* homologs from a wide variety of bacteria show considerable amino acid sequence conservation, particularly at the C-terminal end of the protein (*rpoN* box; ARRTVAKYR; 5, 19). Using a synthetic oligonucleotide (5′GCCCGTCGCACCGTCGCC AAGTACCG3′) derived from the *rpoN* gene sequence of

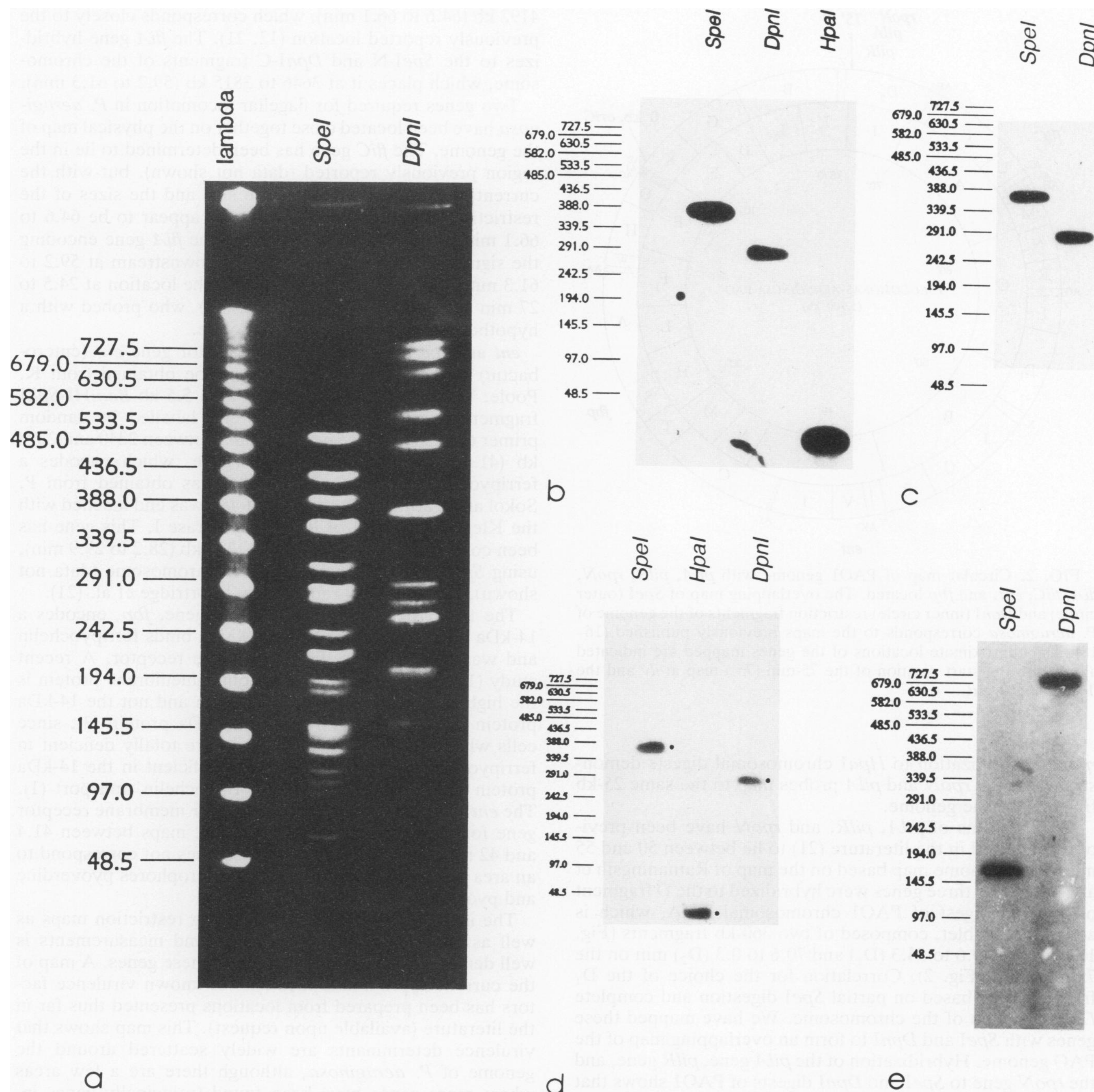

FIG. 1. Pulsed-field separation of *Spe*I and *Dpn*I fragments of *P. aeruginosa* PAO1 genome and corresponding Southern hybridization autoradiographs for *pilA*, *pilR*, *rpoN*, and *fliA*. The ethidium bromide-stained 1.5% agarose gel was electrophoresed at 180 V and 12°C with a switching rate of 1 to 40 s for 14 h and then by a second ramp of 20 to 80 s for 20 h. The gel was then processed as described in the text for Southern hybridization and autoradiography (a). The autoradiographs show the various hydridization patterns of *pilA* (b), *pilR* (c), *rpoN* (d), and *fliA* (e).

Pseudomonas putida (5, 26), we have probed pulsed-field gels of PAO1 DNA and predict that the *P. aeruginosa* homolog of this gene lies between 71.3 and 75.0 map minutes (4620 to 4910 kb). The probe hybridized to the *Spe*I-D_2 and *Dpn*I-I fragments (Fig. 1).

***pilA* and *pilR*.** Pulsed-field gels of the PAO chromosome digested with *Spe*I, *Dpn*I, and *Hpa*I were probed with a random primed 1.2-kb *Hin*dIII fragment of the PAO genome containing the entire *pilA* open reading frame. The *pilA* gene maps to the *Dpn*I-I and *Spe*I-D_2 fragments of the PAO chromosome (Fig. 1). The *pilR* gene maps in the same location as *pilA*, using a 1.6-kb gene probe in plasmid pKI21 (8). This mapping places the *pilA* gene and the *pilR* gene between 4620 and 4910 kb (71.3 to 75 min) and very close to

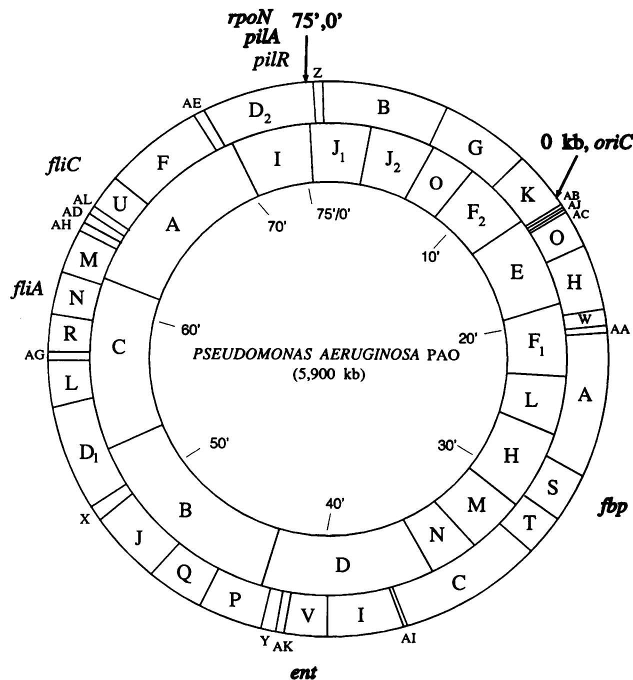

FIG. 2. Circular map of PAO1 genome with *pilA*, *pilR*, *rpoN*, *fliA*, *fliC*, *ent*, and *fbp* located. The overlapping map of *Spe*I (outer circle) and *Dpn*I (inner circle) restriction fragments of the genome of *P. aeruginosa* corresponds to the maps previously published (16–18). The approximate locations of the genes mapped are indicated along with the start position of the 75-min (75′) map at *ilv* and the 0-kb point at *oriC*.

rpoN. Hybridization to *Hpa*I chromosomal digests demonstrate that the *rpoN* and *pilA* probes map to the same 25-kb fragment of the genome.

The locations of *pilA*, *pilR*, and *rpoN* have been previously reported in the literature (21) to lie between 50 and 55 min on the genome map based on the map of Ratnaningsih et al. (16). These three genes were hybridized to the D fragment of an *Spe*I digest of PAO1 chromosomal DNA, which is actually a doublet, composed of two 360-kb fragments (Fig. 1) spanning 49.6 to 54.3 (D_1) and 70.6 to 0.3 (D_2) min on the 75-min map (Fig. 2). Correlation for the choice of the D_1 fragment was based on partial *Spe*I digestion and complete *Dra*I digestion of the chromosome. We have mapped these genes with *Spe*I and *Dpn*I to form an overlapping map of the PAO genome. Hybridization of the *pilA* gene, *pilR* gene, and the *rpoN* gene to *Spe*I and *Dpn*I digests of PAO1 shows that all three gene probes map to the *Spe*I-D fragment and the *Dpn*I-I fragment. The *pilA* and *rpoN* genes also map to a small 25-kb fragment of the *Hpa*I chromosomal digest, making them closely linked (Fig. 1). Since the *Dpn*I-I fragment maps between 71.3 and 75.0 map minutes, the corresponding *Spe*I-D fragment must be the D_2 fragment (70.6 to 0.3 min). This mapping places these genes between 71.3 and 75.0 min in an area near other virulence-associated genes for alginate production and hemolysin and exotoxin A regulation.

***fliA* and *fliC*.** The flagellin structural gene *fliC* and the minor sigma factor gene *fliA* were obtained from S. Lory as plasmids and were labelled directly by random primer extension. The flagellin gene hybridized to the *Spe*I-U and *Dpn*I-A fragments, placing it on the physical map at 4078 to 4192 kb (64.6 to 66.1 min), which corresponds closely to the previously reported location (12, 21). The *fliA* gene hybridizes to the *Spe*I-N and *Dpn*I-C fragments of the chromosome, which places it at 3646 to 3815 kb (59.2 to 61.3 min).

Two genes required for flagellar locomotion in *P. aeruginosa* have been located close together on the physical map of the genome. The *fliC* gene has been determined to lie in the region previously reported (data not shown), but with the current estimation of the genome size and the sizes of the restriction fragments, the coordinates appear to be 64.6 to 66.1 min, rather than 61 to 63 min. The *fliA* gene encoding the sigma factor maps some 200 kb downstream at 59.2 to 61.3 min, which is quite distant from the location at 24.5 to 27 min suggested by Ronald et al. (19), who probed with a hypothetical consensus oligonucleotide.

***ent* and *fbp*.** The siderophore receptor gene *ent* (enterobactin) was mapped with a gene probe obtained from K. Poole. The *ent* gene was purified as a 5.5-kb *Bam*HI-*Sph*I fragment from a plasmid clone and labelled by random primer extension. The *ent* gene maps between 2240 and 2290 kb (41.4 to 42.0 min). The *fbp* gene, which encodes a ferripyochelin-binding protein (22), was obtained from P. Sokol as a purified DNA fragment and was end labelled with the Klenow fragment of RNA polymerase I. This gene has been confirmed to map at 1204 to 1337 kb (28.2 to 29.9 min), using *Spe*I and *Dpn*I digests of the chromosome (data not shown), as previously reported by Shortridge et al. (21).

The ferripyochelin-binding protein gene, *fbp*, encodes a 14-kDa outer membrane protein which binds ferripyochelin and was suggested as the siderophore receptor. A recent study (1) suggests that a 75-kDa outer-membrane protein is the high-affinity receptor for pyochelin and not the 14-kDa protein or a previously reported 90-kDa protein (3), since cells which lack the 75-kDa protein are totally deficient in ferripyochelin transport while cells deficient in the 14-kDa protein only slightly diminish ferripyochelin transport (1). The *ent* gene, encoding an 80-kDa outer membrane receptor gene for the siderophore enterobactin, maps between 41.4 and 42 min (data not shown), which does not correspond to an area where genes for the native siderophores pyoverdine and pyochelin are clustered (13).

The importance of using overlapping restriction maps as well as common map nomenclature and measurements is well demonstrated by the mapping of these genes. A map of the current approximate locations of known virulence factors has been prepared from locations presented thus far in the literature (available upon request). This map shows that virulence determinants are widely scattered around the genome of *P. aeruginosa*, although there are a few areas where more genes have been found (principally genes involved in alginate, phospholipase C, and exotoxin A production). This scattering is in contrast with the arrangement of housekeeping and biosynthetic genes, which are predominantly located in one-half of the genome, and seems to suggest that virulence in this organism has been acquired over a long period of time (21).

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