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Multiple Replicons Constituting the Genome of *Pseudomonas cepacia* 17616

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Macrorestriction fragment analysis of DNA from *Pseudomonas cepacia* 17616, in conjunction with Southern hybridization experiments using junction fragments containing rare restriction enzyme sites as probes, indicated that this bacterium contains three large circular replicons of 3.4, 2.5, and 0.9 megabases (Mb). Inclusion of the 170-kb cryptic plasmid present in this strain gave an overall estimate of genome size of 7 Mb. Other Southern hybridization experiments indicated that the three large replicons contained rRNA genes as well as insertion sequence elements identified previously in this strain. The distribution of *Swa*I, *Pac*I, and *Pme*I sites on the three replicons was determined. A derivative of Tn*5-751* carrying a *Swa*I site was used to inactivate and map genes on the 2.5- and 3.4-Mb replicons. Mutants were isolated in which the 2.5- and 0.9-Mb replicons had been reduced in size to 1.8 and 0.65 Mb, respectively. The loss of DNA from the 2.5-Mb replicon was associated with lysine auxotrophy, β-lactamase deficiency, and failure to utilize ribitol and trehalose as carbon and energy sources. DNA fragments corresponding in size to randomly linearized forms of the different replicons were detected in unrestricted DNA by pulsed-field gel electrophoresis. The results provide a framework for further genetic analysis of strain 17616 and for evaluation of the genomic complexities of other *P. cepacia* isolates.

Pseudomonas cepacia has attracted attention because of its extraordinary biodegradative abilities and its potential as an agent of bioremediation (2, 13, 22, 25, 35, 44, 45, 51, 56). The genome of this bacterium contains a large number of insertion sequences (ISs) (25, 27), which were identified on the basis of their abilities to promote genomic rearrangements (4, 12) and to activate the expression of neighboring genes (15, 43, 60). Such elements have been implicated in the recruitment of foreign genes for novel catabolic functions (15, 43, 54, 60). Although there is considerable information about the biochemical activities of *P. cepacia* (2, 3, 11, 25, 26, 35, 40, 45, 51) and although certain genes and IS elements have been isolated and characterized (5, 7, 10, 15, 16, 33, 36, 42, 54, 59, 61), little is known about the overall organization of genes or the genomic distribution of IS elements. To gain such information we undertook the construction of a macrorestriction map of the genome of *P. cepacia* 17616 by using recently developed techniques for the manipulation of large DNA fragments (8, 17, 19, 46–48). We report here that *P. cepacia* 17616 contains three large replicons. Similar chromosome multiplicities have been reported recently for several other bacteria (1, 21, 23, 32, 50, 52, 53). We also have demonstrated the feasibility of mapping the distribution of various *P. cepacia* genes by transposon mutagenesis, using a derivative of Tn*5-751* (39) carrying a recognition site for the restriction endonuclease *Swa*I.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacteria and plasmids used for this study are listed in Table 1. For preparation of agarose plugs containing bacterial DNA, the bacteria were grown at 37°C in inorganic salts medium consisting of 50 mM phosphate buffer (16 mM Na_2HPO_4 and 34 mM KH_2PO_4) (pH 6.5), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.01 mM $FeSO_4$, and 0.2% (wt/vol) $(NH_4)_2SO_4$ supplemented with 1% (wt/vol) yeast extract. To test the phenotypes of auxotrophic strains, 0.5% potassium phthalate or mannitol was substituted for the yeast extract and the medium was supplemented with 50 μg of each required amino acid per ml. For growth in liquid medium, 10- to 20-ml cultures were shaken in 125-ml flasks. For growth on plates, the medium was supplemented with 2% (wt/vol) agar.

Preparation of genomic DNA for pulsed-field gel electrophoresis (PFGE). Agarose plugs containing intact chromosomal DNA were prepared essentially as described by Smith and Cantor (47). Approximately 5×10^8 bacteria embedded in 100-μl agarose plugs containing TE buffer (10 mM Tris and 1 mM EDTA) (pH 8.0) and 1% (wt/vol) Incert agarose (FMC Co., Rockland, Maine) were digested for ca. 12 h in 5 ml of pH 9.5 lysis buffer consisting of 10 mM Tris buffer, 0.45 M EDTA (sodium salt), 1% (wt/vol) laurylsarcosine, and 1 mg of proteinase K per ml. Laurylsarcosine and proteinase K were obtained from Sigma Chemical Co. (St. Louis, Mo.). After lysis of the bacteria, the plugs were washed repeatedly in TE buffer and then stored at 4°C.

Macrorestriction fragment analysis of *P. cepacia* DNA. DNA samples (10-μl slices of agarose containing between 300 and 400 ng of DNA) were treated with *Swa*I, *Pac*I, *Pme*I, or other restriction enzymes in 100-μl reaction mixtures. Reaction buffers and conditions were those recommended by the supplier, except in the case of *Pac*I, for which KGB buffer (30) was used. *Swa*I was obtained from Boehringer Mannheim Co. (Indianapolis, Ind.). *Pac*I and *Pme*I were obtained from New England Biolabs (Beverly, Mass.). The agarose slices were transferred to sample wells in agarose gels (13 by 13 by 0.5 cm) containing 1:2-diluted TBE buffer (pH 8.3) (41) and 0.8 or 1% (wt/vol) Fastlane agarose (FMC Co.) and sealed in place with 1% SeaPlaque agarose (FMC Co.). For experiments in which DNA fragments were to be recovered from the gels, SeaPlaque

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TABLE 1. *P. cepacia* strains and plasmids

Strain or plasmid	Pertinent characteristics	Reference o source
Strains		
249 (ATCC 17616)	Prototroph; contains three large circular replicons of 3.4, 2.5, and 0.9 Mb as well as the 170-kb cryptic plasmid pTGL1	12
249-UM	Prototrophic derivative of strain 249 with 0.25 Mb of DNA deleted from the 0.9-Mb replicon; contains the pTGL1 variant pTGL6	12
249-2	Derivative of strain 249-UM with a 0.8-Mb deletion from the 2.5-Mb replicon associated with lysine auxotrophy, β-lactamase deficiency, and failure to utilize trehalose and ribitol	12
249-2(pTGL6$^-$)	Derivative of strain 249-2 cured of pTGL6	12
249-110	Phthalate-negative mutant with Tn*5-751S* inserted into the 2.5-Mb replicon	This study
249-111	Histidine auxotroph generated by insertion of Tn*5-751S* into the 3.4-Mb replicon of strain 249	This study
249-112	Arginine auxotroph generated by insertion of Tn*5-751S* into the 3.4-Mb replicon of strain 249	This study
249-113	Isoleucine auxotroph generated by insertion of Tn*5-751S* into the 3.4-Mb replicon of strain 249	This study
249-114	Threonine auxotroph with Tn*5-751* inserted into the 3.4-Mb replicon of strain 249	This study
249-115	Derivative of strain 249 with Tn*5-751* inserted into the 3.4-Mb replicon; requires phenylalanine, tyrosine, and tryptophan for growth	This study
Plasmids		
pBlueKSP	3-kb vector containing multiple cloning sites within its *lacZ*α region; Confers resistance to penicillin	Stratagene Co.
pME9	53-kb, broad-host-range IncP1 plasmid; temperature sensitive with respect to its replication because of a lesion in *trfA*; contains Tn*5-751*, which confers resistance to trimethoprim and kanamycin, as well as nontransposable penicillin and tetracycline resistance markers	39
pTGL166	pME9 derivative in which the *Spe*I site within Tn*5-751S* was replaced by a *Swa*I site	This study
pTGL53	pRP1 derivative carrying IS*403*	43
pTGL59	pBlueKSP derivative carrying IS*402*	A. Ferrante
pTGL223	pACYC184 derivative carrying IS*401*	12
pTGL274	pBlueKSP derivative carrying IS*407*	59
pTGL275	pBlueKSP derivative carrying IS*415*	59
pTGL401	pBlueKSP derivative carrying IS*406*	59
pTGL403	pBlueKSP derivative carrying IS*401* and IS*408*	7

DNA fragments were resolved electrophoretically with a contour-clamped homogeneous electric field (CHEF) gel apparatus (9) obtained from Owl Scientific Products, Inc. (Cambridge, Mass.). Electrophoresis was carried out at 12°C by using programs in which the pulse time was progressively increased to allow separation of a wider range of fragment sizes. For resolution of fragments generated by treatment of *P. cepacia* DNA with *Swa*I, *Pac*I, and *Pme*I (0.3 to 2.2 megabases [Mb]), electrophoresis was at 120 V for ca. 48 h with ramping increments of 4 s over a range of pulse times of 50 to 450 s. For resolution of smaller fragments generated by treatment of the DNA with enzymes such as *Ase*I, *Afl*II, *Dra*I, *Spe*I, and *Xba*I, electrophoresis was at 300 V with increments of 0.05 or 0.2 s and respective ranges of pulse times of 0.1 to 3.1 or 2 to 18 s. For resolution of linearized replicons in preparations of unrestricted *P. cepacia* DNA, electrophoresis was at 50 V for 150 h with 240-s ramping increments over a range of pulse times of 600 to 3,480 s. Gels were stained with ethidium bromide (0.5 μg/ml) and photographed by UV transillumination. Estimates of DNA fragment size were made by including coliphage lambda multimers and *Saccharomyces cereviseae* and *Schizosaccharomyces pombe* chromosomal DNA reference markers in the gels.

Southern hybridization analyses. DNA fragments were transferred from agarose gels to hybridization membranes by capillary blotting (41), using either GeneScreen Plus nylon membranes from New England Nuclear Co. (Boston, Mass.) or Immobilon N polyvinylidene difluoride membranes from Millipore Co. (Bedford, Mass.). The gels first were soaked in 0.25 N HCl to reduce fragment size and facilitate DNA transfer. Prior to transfer the DNA was denatured in 0.5 N NaOH containing 0.15 M NaCl and then neutralized in 1 M Tris HCl (pH 7.4) containing 1.5 M NaCl. Preparation o membranes and transfer conditions were as recommended b the supplier. DNA immobilized on the membranes was hybrid ized with radiolabelled RNA or DNA in QuikHyb solutio (Stratagene Co., La Jolla, Calif.) by protocols provided by th supplier. Homologous fragments were detected by radioautog raphy at −70°C with X-OMAT AR film (Eastman Kodak Co. Rochester, N.Y.) and Quanta III-T intensifying screens (Du pont Co., Wilmington, Del.). DNA probes (either plasmid carrying IS elements or DNA fragments recovered from low melting-point agarose gels) were labelled with [α-^{32}P]dCTP b using a random-primer DNA labelling kit (U.S. Biochemica Co., Cleveland, Ohio). rRNA probes were end labelled witl [γ-^{32}P]ATP by using T4 polynucleotide kinase (U.S. Biochem ical Co.) and 16 and 23S RNAs which had been isolated fron *P. cepacia* 17616 by sucrose gradient centrifugation (24).

Plasmid manipulations. Plasmid DNA was isolated fron bacteria by the modified alkaline lysis procedure of Birnboin (6). Plasmid preparations used as DNA probes in Southern hybridization experiments were purified by CsCl-ethidiun bromide density gradient centrifugation (41). To obtain transposon carrying a rare macrorestriction site suitable fo gene mapping, we modified the broad-host-range plasmi pME9 (39) by inserting a short DNA fragment containing *Swa*I site into its copy of Tn*5-751*. The site of insertion was th unique *Spe*I site of the plasmid located upstream of th trimethoprim resistance determinant within the transposon Plasmid pME9 was linearized by treatment with *Spe*I an ligated in the presence of T4 DNA ligase (41) with the DN fragment containing the *Swa*I site. The inserted DNA frag ment, which had ends compatible with those of the plasmid, i

TABLE 2. Sizes of DNA fragments generated by digestion of *P. cepacia* 17616 DNA with various combinations of *Pac*I, *Swa*I, and *Pme*I[a]

Enzyme(s)	Sizes (Mb) of DNA fragments
*Pac*I	2.2, 1.4, 0.9,[b] 0.8, 0.6, 0.56, 0.27
*Pac*I-*Swa*I	1.4, 1.2,[c] 1.1,[c] 0.9, 0.8, 0.52,[c] 0.47,[c] 0.27, 0.14,[c] 0.04[c]
*Swa*I	2.5,[b] 1.7, 1.2, 0.9, 0.5
*Pme*I-*Swa*I	2.5, 1.7, 1.15,[c] 0.9, 0.3,[c] 0.2,[c] 0.05[c]
*Pme*I	3.15, 2.5, 0.9,[b] 0.25
*Pac*I-*Pme*I	2.2, 1.4, 0.9,[b] 0.56, 0.5,[c] 0.32,[c] 0.3,[c] 0.27, 0.25, 0.05[c]

[a] The sum of the sizes of the fragments for the *Pac*I-*Swa*I, *Pme*I-*Swa*I, and *Pac*I-*Pme*I digestions was ca. 6.8 Mb.

[b] Fragment present in trace amounts and generated by random double-strand breakage of replicons not cleaved by the indicated restriction enzyme(s).

[c] Fragment unique to the double digest.

pTGL166, was introduced into *Escherichia coli* DH5α by electroporation, using an *E. coli* gene pulser apparatus from Bio-Rad Co. (Richmond, Va.) and protocols recommended by the supplier. Electroporants were isolated on plates containing 100 μg of trimethoprim per ml. Plasmid pTGL166 was transferred from strain DH5α to *P. cepacia* by conjugation (59). *P. cepacia* transconjugants were isolated on plates containing 100 μg of tetracycline per ml to select for the nontransposable tetracycline resistance marker on the plasmid and containing potassium phthalate as the sole carbon source to counterselect the donor strain.

RESULTS

Size of the genome of *P. cepacia* 17616. To obtain an estimate of the size of the *P. cepacia* genome, we identified restriction enzymes which cleaved DNA from this bacterium infrequently and determined the sum of the sizes of the fragments generated. Since *P. cepacia* DNA contains ca. 68% G+C residues (2), we focused on enzymes which recognize 6- or 8-bp sequences composed exclusively or primarily of A and T residues or whose recognition sequences contain rare tetranucleotides such as CTAG (31). *Afl*II, *Ase*I, *Dra*I, *Spe*I, *Xba*I, *Ssp*I, *Nhe*I, *Pac*I, *Swa*I, and *Pme*I were among the enzymes tested. Macrorestriction fragments were resolved by CHEF gel electrophoresis (9), and their sizes were estimated by comparison of their electrophoretic mobilities with those of coliphage lambda multimers and yeast chromosomal DNA markers.

*Dra*I, *Ase*I, *Afl*II, *Spe*I, and *Xba*I (which recognize 6-bp sequences) cleaved DNA from strain 17616 into between 50 and 80 fragments in the range of 6 kb to 1 Mb. For each of these five enzymes, the sum of the sizes of the fragments generated was close to 7 Mb of DNA. We failed to identify any enzymes with 6-bp recognition sequences that cleaved *P. cepacia* 17616 DNA into fewer than ca. 50 fragments. The majority of enzymes we tested, for example, *Ssp*I and *Nhe*I, cleaved the DNA into more than 100 fragments, all with sizes of less than 200 kb.

*Swa*I, *Pac*I, and *Pme*I, three restriction endonucleases which recognize 8-bp sequences, cleaved the DNA into a more manageable number of fragments. Treatment of DNA from strain 17616 with *Swa*I, *Pac*I, and *Pme*I generated, respectively, four, six, and three fragments, whose sizes are indicated in Table 2. The *Pac*I and *Pme*I digests also contained traces of 0.9-Mb DNA, which corresponded to a major fragment present in *Swa*I digests, and *Swa*I digests contained traces of 2.5-Mb DNA, which corresponded to a major fragment in *Pme*I digests. When the sizes of the minor DNA species were

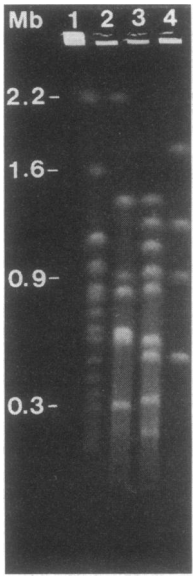

FIG. 1. Resolution of DNA fragments from *Pac*I-, *Pac*I- and *Swa*I-, and *Swa*I-digested DNA from *P. cepacia* 17616 by PFGE. Lane 1, *S. cerevisiae* chromosomal DNAs as DNA size markers. Lanes 2 to 4, DNA from strain 17616 digested with *Pac*I, with *Pac*I and *Swa*I, and with *Swa*I, respectively. *Pac*I-digested DNA contained six fragments of 2.2, 1.4, 0.8, 0.6, 0.56, and 0.27 Mb, as well as a faint band corresponding to the linearized 0.9-Mb replicon. DNA digested with *Pac*I and *Swa*I contained nine fragments of 1.4, 1.2, 1.1, 0.9, 0.8, 0.52, 0.47, 0.27, and 0.14 Mb, as well as a 0.04-Mb fragment not visible in this photograph. *Swa*I-digested DNA contained four fragments of 1.7, 1.2, 0.9, and 0.5 Mb and occasionally a faint band corresponding to the linearized 2.5-Mb replicon. DNA fragments were resolved in 1% Fastlane agarose at 120 V and 12°C for 46 h by ramping with increments of 4 s over 100 steps for a range of pulse times of 50 to 450 s.

was equivalent to 6.8 Mb of DNA. When the DNA was digested with a combination of *Swa*I and *Pac*I or *Swa*I and *Pme*I, there were no minor DNA species, and the sum of the sizes of the fragments generated also was equivalent to 6.8 Mb of DNA (Table 2). Figure 1 compares the patterns of DNA fragments resolved by CHEF gel electrophoresis from *Swa*I and *Pac*I digests with that obtained after double digestion of the DNA with *Swa*I and *Pac*I. On the basis of the macrorestriction fragment analyses with *Swa*I and *Pac*I and with *Swa*I and *Pme*I, we estimated that the size of the *P. cepacia* genome is 6.8 Mb.

Nature of the minor DNA species present in *Swa*I, *Pac*I, and *Pme*I digests and in preparations of unrestricted DNA. The genomes of *Rhodobacter sphaeroides* (52, 53), *Rhizobium meliloti* (21, 50), and *Brucella melitensis* (32) have been found to consist of multiple large circular replicons. Preparations of unrestricted DNA from *R. meliloti* and *B. melitensis* were reported to contain populations of linear DNA corresponding in size to each of the circular replicons identified in these bacteria. Unlike the intact circular replicons, which were retained in the sample wells, the linear species were resolvable by PFGE.

It seemed reasonable that the minor species of DNA detected in *Pac*I, *Pme*I, and *Swa*I digests of DNA from strain 17616 might represent randomly linearized forms of circular replicons that were not cleaved by these enzymes. This proved to be the case. As is shown in lane 2 of Fig. 2, when

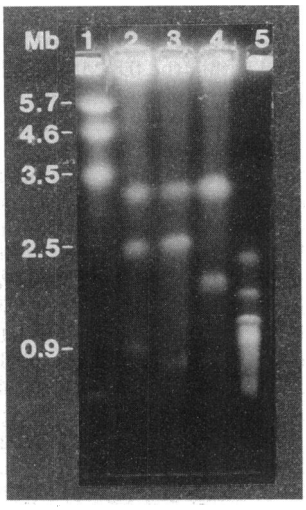

FIG. 2. Resolution of the linearized forms of three large replicons from *P. cepacia* 17616, 249-UM, and 249-2(TGL6$^-$). Unrestricted DNA from the three strains was resolved for 150 h at 50 V in 0.8% Fastlane agarose by ramping with increments of 250 s over 12 steps for a range of pulse times of 600 to 3,400 s. Lanes 1 and 5, yeast chromosomal DNA markers from *S. pombe* and *S. cerevisiae*, respectively. Lanes 2 to 4, ca. 5 μg of unrestricted DNA from strains 17616, 249-UM, and 249-2(TGL6$^-$), respectively. The agarose plugs containing the bacterial DNA samples used in this experiment had been stored for ca. 1 year at 4°C. The unrestricted DNA from strains 249-UM and 249-2(pTGL6$^-$) contained linear DNA fragments of ca. 0.65 Mb in place of the 0.9-Mb DNA present in strain 17616. Strain 249-2(pTGL6$^-$) contained linear DNA fragments of 1.8 Mb in place of the 2.5-Mb DNA present in strains 17616 and 249-2(pTGL6$^-$). The majority of the DNA remained in the sample wells, indicating that the randomly sheared DNA represented only a small fraction of the total population of 3.4-, 2.5-, and 0.9-Mb replicons.

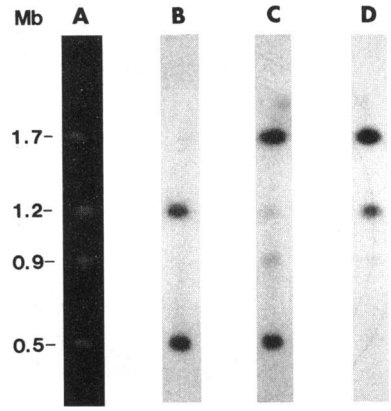

FIG. 3. Use of junction fragment probes to confirm that the 1.2-, 1.7-, and 0.5-Mb DNA fragments in *Swa*I digests of strain 17616 DNA constitute a 3.4-Mb circular replicon. *Swa*I fragments were resolved at 50 V for 46 h with ramping over 100 steps and increments of 4 s for a range of pulse times of 50 to 450 s. Lane A, ethidium bromide-stained DNA fragments generated by digestion of the DNA with *Swa*I. Lanes B through D, corresponding radioautograms probed with the radiolabelled junction fragments *Spe*I-310, *Spe*I-132, and *Xba*I-350, respectively. The respective pairs of *Swa*I fragments overlapped by these probes were the 1.2- and 0.5-Mb fragments, the 0.5- and 1.7-Mb fragments, and the 1.7- and 1.2-Mb fragments.

populations of DNA fragments of 3.4, 2.5, and 0.9 Mb were detected. These results in conjunction with those of experiments described below were consistent with the interpretation that 6.8-Mb genome of *P. cepacia* 17616 is composed of three replicons of 3.4, 2.5, and 0.9 Mb.

The 1.7-, 1.2-, and 0.5-Mb fragments in *Swa*I digests of DNA from strain 17616 constitute a circular replicon of 3.4 Mb. We were able to identify junction fragments that overlapped the different pairs of *Swa*I fragments constituting the 3.4-Mb replicon. The set of junction fragments consisted of two *Spe*I fragments, *Spe*I-130 and *Spe*I-310, and one *Xba*I fragment, *Xba*I-350, with respective sizes of 130, 310, and 350 kb. Each of these fragments contained a single *Swa*I site. *Swa*I cleaved fragment *Spe*I-130 into two subfragments of 80 and 50 kb and cleaved fragment *Spe*I-310 into subfragments of 150 and 160 kb. It cleaved fragment *Xba*I-350 into two subfragments of 270 and 80 kb. As is shown in Fig. 3, when the three junction fragments were used as probes in Southern hybridization experiments, *Xba*I-350 hybridized to the 1.2- and 1.7-Mb *Swa*I fragments. *Spe*I-310 hybridized to the 0.5- and 1.7-Mb *Swa*I fragments, and *Spe*I-130 hybridized to the 0.5- and 1.2-Mb *Swa*I fragments. The results indicated that the 1.7-, 1.2-, and 0.5-Mb *Swa*I fragments were organized within a 3.4-Mb circular replicon as depicted in Fig. 4.

To identify *Pac*I fragments that were part of the 3.4-Mb replicon, the three *Swa*I fragments constituting this replicon were isolated and used as probes in Southern hybridization experiments. The results indicated that three of the six fragments present in *Pac*I digests of DNA from strain 17616, the 2.2-, 0.6-, and 0.56-Mb fragments, were part of the 3.4-Mb replicon. In Southern hybridization experiments, the 1.2- and 1.7-Mb *Swa*I fragments exhibited strong homology with the 2.2-Mb *Pac*I fragment. The 1.7-Mb *Swa*I fragment also hybridized with the 0.56-Mb *Pac*I fragment. The 0.5-Mb *Swa* fragment hybridized only with the 0.6-Mb *Pac*I fragment, and the 1.2-Mb *Swa*I fragment exhibited weak homology with the 0.6-Mb *Pac*I fragment.

Further information about the distribution of *Swa*I, *Pac*I and *Pme*I sites within the 3.4-Mb replicon was obtained by analysis of the subfragments generated when various macrorestriction fragments were isolated from the gels and then treated with a second enzyme. For example, *Pac*I cleaved the 1.7-Mb *Swa*I fragment into two fragments of ca. 1.2 and 0.5 Mb, and *Pme*I cleaved the 0.6-Mb *Pac*I fragment into three subfragments of 0.3, 0.25, and 0.05 kb. The results were consistent with the arrangement of fragments indicated in Fig. 4.

The 1.4-, 0.8-, and 0.27-Mb fragments in *Pac*I digests of DNA from strain 17616 constitute a circular replicon of 2.5 Mb. Southern hybridization experiments using the 2.5-Mb DNA species from unrestricted preparations of 17616 DNA and a probe indicated that this DNA exhibited strong homology with the 1.4-, 0.8-, and 0.27-Mb DNA fragments in *Pac*I digests of DNA from strain 17616 but not with the 2.2-, 0.6-, and 0.56-Mb fragments constituting the 3.4-Mb replicon or with the small amounts of 0.9-Mb DNA present in the digests (results not shown). Southern hybridization experiments using as probes *Afl*II and *Dra*I fragments containing *Pac*I sites indicated that the 2.2-, 0.8-, and 0.27-Mb fragments were arranged within the 2.5-Mb replicon in the order shown in Fig. 4. For example, the linking fragments *Afl*II-400 and *Afl*II-230 hybridized, respectively, to the 0.8- and 1.4-Mb *Pac*I fragments and to the 0.8- and 0.27-Mb *Pac*I fragments. A third linking fragment *Dra*I-194, also hybridized to the 0.8- and 0.27-Mb *Pac*I fragments. No fragment which overlapped the 1.4- and 0.27-Mb fragments was identified.

The linkage between the 0.8- and 1.4-Mb *Pac*I fragments was

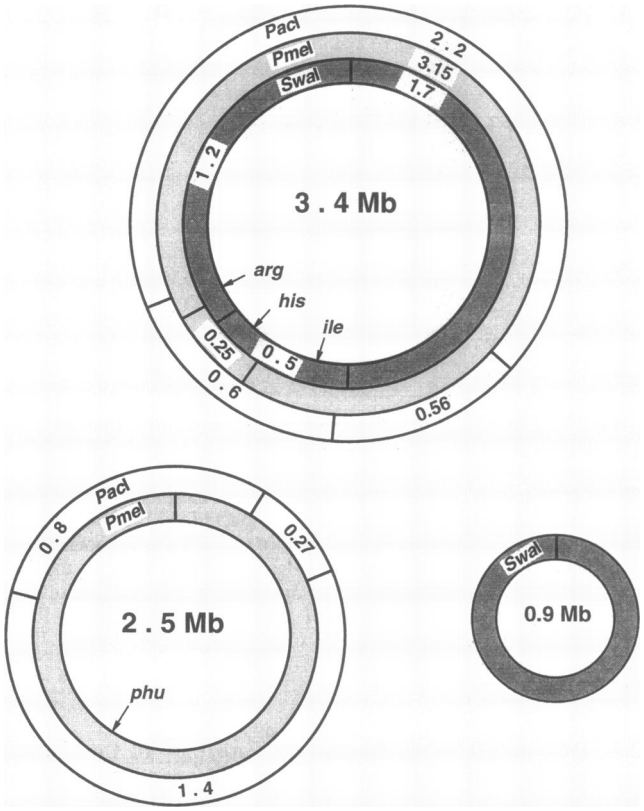

FIG. 4. Arrangement of macrorestriction fragments constituting the three large replicons of *P. cepacia* 17616. The arrangements of *Swa*I, *Pac*I, and *Pme*I fragments within the 3.4-Mb replicon and of *Pac*I and *Pme*I fragments within the 2.5-Mb replicon are shown. The 2.5-Mb replicon was not cleaved by *Swa*I, nor was the 0.9-Mb replicon cleaved by *Pac*I or *Pme*I. All three replicons were cleaved by treatment of DNA from strain 17616 with a combination of *Swa*I, *Pac*I, and *Pme*I. The concentric circles indicate the locations of *Swa*I, *Pac*I, and *Pme*I sites and fragments. The arrows show the distribution of selected genes on the 3.4- and 2.5-Mb replicons, as determined by analysis of the sites of insertion of Tn*5-751S* in mutants impaired in biosynthesis of Arg, Ile, or His or in phthalate utilization.

confirmed by analysis of a mutant strain, 249-2(pTGL6$^-$), in which the *Pac*I site between these two fragments had been eliminated by the deletion of 0.7 Mb of DNA from the 2.5-Mb replicon. This resulted in the replacement of the 1.4- and 0.8-Mb *Pac*I fragments by a new fusion fragment of 1.5 Mb. The deletion event left intact the unique *Pme*I site of the original replicon. Accordingly, *Pme*I digests of DNA from strain 249-2(pTGL6$^-$) contained a 1.8-Mb *Pme*I fragment instead of the 2.5-Mb *Pme*I fragment present in digests of DNA from strain 17616. Likewise, preparations of unrestricted DNA from strain 249-2(pTGL6$^-$) contained a 1.8-Mb DNA species in place of the 2.5-Mb species detected in similar preparations of DNA from strain 17616 (Fig. 2).

The distribution of *Pac*I sites relative to the unique *Pme*I site of the 2.5-Mb replicon was determined by analysis of macrorestriction fragments generated by double digestion of DNA from strains 17616 and 249-2(pTGL6$^-$) with *Pac*I and *Pme*I as well as by treatment of the 2.5-Mb fragment from *Pme*I digests with *Pac*I. *Pac*I cleaved the 2.5-Mb *Pme*I fragment into four fragments of 0.2, 0.27, 0.6, and 1.4 Mb. Double digests of DNA from strain 249-2(pTGL6$^-$) contained the 0.2- and 0.27-Mb fragments but were missing the 0.6- and 1.4-Mb fragments. The latter two fragments were replaced by a new fragment of 1.3 Mb. The results indicated that the *Pme*I site of the 2.5-Mb replicon was located within the 0.8-Mb *Pac*I fragment, 0.2 Mb away from the junction between the 0.8- and 0.27-Mb *Pac*I fragments and 0.6 Mb away from the junction between the 0.8- and 1.4-Mb *Pac*I fragments (Fig. 4). The fact that the *Pme*I site of the 2.5-Mb replicon was located within the 0.8-Mb *Pac*I fragment indicated that the 1.4-, 0.8-, and 0.27-Mb *Pac*I fragments were part of a circular replicon. (Hybridization experiments had established that the 0.8-Mb *Pac*I fragment was situated between the 1.4- and 0.27-Mb *Pac*I fragments.)

Characteristics of the 0.9-Mb replicon. We were able to identify a 420-kb *Spe*I fragment from digests of DNA from strain 17616 which contained the unique *Swa*I fragment of the 0.9-Mb replicon. When this fragment, *Spe*I-420, was isolated from a low-melting-point agarose gel and digested with *Swa*I, two new fragments of 350 and 70 kb were generated. When Southern hybridization experiments were carried out with fragment *Spe*I-420 or either the 350- or 70-kb subfragment to probe *Swa*I fragments of DNA from strain 17616, only the 0.9-Mb fragment exhibited homology. The most reasonable explanation for the failure of these probes to hybridize with other *Swa*I fragments is that the 0.9-Mb fragment was derived by cleavage of a circular replicon containing a single *Swa*I site.

Strain 249-UM is a derivative of strain 17616 in which pTGL1, a 170-kb cryptic plasmid present in strain 17616, has undergone several IS element-dependent rearrangements to produce pTGL6 (12). Comparison of *Swa*I digests of DNA from strains 17616 and 249-UM indicated that the latter had lost 0.25 Mb of DNA from the 0.9-Mb replicon. The 0.9-Mb *Swa*I fragment present in *Swa*I digests of strain 17616 was replaced in strain 249-UM by a new *Swa*I fragment of 0.65 Mb. Likewise, the 0.9-Mb DNA species detected in preparations of unrestricted DNA from strain 17616 was replaced in the case of strain 249-UM by a new DNA species of 0.65 Mb (Fig. 2). Strain 249-2(pTGL6), a lysine auxotroph isolated from strain 249-UM, and strain 249-2(pTGL6$^-$), a derivative cured of pTGL6 which we have used in most of our experiments, contained the same 0.65-Mb replicon.

All three of the large replicons from strain 17616 contain rRNA genes and IS elements. To determine the distribution of rRNA genes and IS elements among the various DNA fragments constituting the 3.4-, 2.5-, and 0.9-Mb replicons of strain 17616, we carried out Southern hybridization experiments, using as probes radiolabelled preparations of 16 and 23S RNA and plasmid vectors carrying representative IS elements. DNA from strain 17616 was digested with both *Swa*I and *Pac*I, and the resulting fragments were resolved on CHEF gels, transferred to hybridization membranes, and reacted with the appropriate probe RNAs or DNAs. Table 3 lists the fragments generated after digestion of the DNA with *Swa*I and *Pac*I and indicates those which exhibited homology with 16 and 23S RNAs and with the specified IS elements. The fragments are grouped according to their association with each replicon. Three fragments from the 3.4-Mb replicon, one fragment from the 2.5-Mb replicon, and the 0.9-Mb *Swa*I fragment representing the 0.9-Mb replicon hybridized with 16 and 23S rRNAs. Seven different IS elements, IS*401*, IS*402*, IS*403*, IS*406*, IS*407*, IS*408*, and IS*415*, were also used as probes. All seven IS elements exhibited homology with the 0.9-Mb *Swa*I fragment representing the 0.9-Mb replicon. Five of the elements, IS*403*, IS*406*, IS*407*, IS*408*, and IS*415*, hybridized to representative fragments from all three replicons. IS*402* failed to hybridize to fragments constituting the 2.5- and 3.4-Mb replicons. IS*401* failed to hybridize with fragments constituting the 3.4-Mb replicon.

TABLE 3. Distribution of rRNA genes and IS elements among macrorestriction fragments associated with the 3.4-, 2.5-, and 0.9-Mb replicons of *P. cepacia* 17616

Replicon [enzyme(s)]	Macrorestriction fragment (Mb)	Homology with:		
		16S RNA	23S RNA	IS elements
3.4 Mb (*SwaI-PacI*)	1.2	+	+	IS*403*, IS*406*, IS*408*, IS*415*
	1.1	+	+	IS*403*
	0.52	−	−	IS*403*, IS*406*, IS*415*
	0.47	+	+	IS*403*, IS*407*
	0.14	−	−	IS*403*
	0.04	−	−	
2.5 Mb (*PacI*)	1.4	+	+	IS*403*, IS*408*
	0.8	−	−	IS*403*
	0.27	−	−	IS*401*, IS*403*, IS*406*, IS*407* IS*408*, IS*415*
0.9 Mb (*SwaI*)	0.9	+	+	IS*401*, IS*402*, IS*403*, IS*406* IS*407*, IS*408*, IS*415*

Use of Tn*5-751S* to assign selected auxotrophic markers to the 3.4-Mb replicon. To develop a general approach for defining functions specified by the different replicons in strain 17616, we explored the usefulness of insertional mutagenesis with a derivative of transposon Tn*5-751* (39) engineered to contain a *SwaI* site. The appropriate transposon, Tn*5-751S*, was constructed by modifying the copy of Tn*5-751* within the 53-kb broad-host-range plasmid pME9 (Fig. 5). The strategy was to replace the unique *SpeI* site of the plasmid, which is located within Tn*5-751*, with a *SwaI* site. The oligonucleotide 5′-CTAGATTTAAAT-3′ was self-annealed to form a DNA duplex consisting of a *SwaI* recognition site with cohesive ends compatible with those of *SpeI*-digested pME9. This DNA was ligated with *SpeI*-digested pME9 to form plasmid pTGL166, which was cleaved by *SwaI* but not by *SpeI*.

Plasmid pTGL166, like its parent pME9, was temperature sensitive with respect to its replication and included a non-transposable tetracycline resistance marker as well as the trimethoprim and kanamycin resistance markers associated with Tn*5-751*. A pTGL166-containing derivative of strain 17616 was propagated for ca. 30 generations at 47°C in medium containing 1% yeast extract and 100 μg of trimethoprim per ml. Appropriate dilutions were then plated on medium containing 0.5% mannitol, 100 μg of trimethoprim per ml, and a concentration of Casamino Acids (0.02%) on which amino acid auxotrophs would form smaller colonies than the wild type. Trimethoprim-resistant auxotrophs were isolated and screened to ensure that they were tetracycline sensitive and devoid of pTGL166. Three such auxotrophs, which required L-Ile, L-Arg, or L-His, were further characterized to examine whether the auxotrophy was related to the insertion of Tn*5-751S*. First, prototrophic revertants of all three auxotrophs were selected, and these were tested to determine if they had regained sensitivity to trimethoprim. In all cases reversion to prototrophy was accompanied by the loss of trimethoprim resistance, consistent with the interpretation that restoration of prototrophy was a consequence of precise excision of Tn*5-751S*. In addition, the wild-type pattern of *SwaI* fragments, which was altered in the mutants (Fig. 6), was restored in the revertant strains. For example, in the His$^-$ derivative 249-111, the 0.5-Mb *SwaI* fragment of the 3.4-Mb replicon was replaced by two new *SwaI* fragments of 0.1 and 0.4 Mb, and in prototrophic revertants derived from this strain, the latter two fragments were replaced by a 0.5-Mb fragment. In the arginine-requiring mutant 249-112, Tn*5-751S* inserted into the 1.2-Mb fragment of the 3.4-Mb replicon. In the isoleucine auxotroph 249-113, the 0.5-Mb *SwaI* fragment was the target. Further restriction fragment mapping experimen were carried out to locate the sites of insertion of Tn*5-75* relative to the *PacI* sites on the 3.4-Mb replicon. The sites c insertion of Tn*5-751* in the three mutants are indicated in Fi 4. Megabase coordinates measured in a clockwise directio from the junction of the 1.2- and 1.7-Mb *SwaI* fragments wer 1.85, 2.1, and 2.3, respectively, for the *ile*, *his*, and *arg* gene inactivated by Tn*5-751* insertion.

Functions associated with the 2.5-Mb replicon. We als isolated a mutant strain in which Tn*5-751S* had inserted int the 2.5-Mb replicon. This mutant, 249-110, was impaired in th utilization of phthalate as a carbon and energy source. The sit of Tn*5-751* insertion was 1.4 Mb, in a clockwise direction, awa from the unique *PmeI* site on the 2.5-Mb replicon, as indicate in Fig. 4. It should be noted that strain 249-2(pTGL6$^-$), whic had suffered a deletion affecting the same 1.4-Mb *PacI* fra ment that was the target of Tn*5-751S* insertion in strai 17616-110, was also impaired in phthalate utilization. Th mutant had also irreversibly lost the ability to utilize penicilli G, ribitol, and trehalose, as well as the ability to synthesiz lysine—all presumably as a consequence of the same deletio The results suggest that the 2.5-Mb replicon contains gen related to lysine biosynthesis and to the degradation of phth late, penicillin, ribitol, and trehalose.

DISCUSSION

The macrorestriction fragment analysis described here wa undertaken to gain information about the genomic organiz tion of *P. cepacia* 17616. The first objective was to obtain a estimate of genome size; the second was to construct a physic map of the genome of this strain that could serve as framework for further genetic analysis. Although gene e change in certain *P. cepacia* strains by transduction (29) an conjugation (38) had been reported, no systematic analysis the genetic organization of this bacterium had been carried ou by classical approaches. We decided to take an alternati approach and test the feasibility of using a transposon carryin a rare restriction enzyme site for insertional inactivation an mapping of representative genes, as had been described f enteric bacteria (49, 58).

PacI, *SwaI*, and *PmeI*, three enzymes which recognize 8-b sequences, cleaved DNA from strain 17616 into three to s large fragments, which were resolved readily by pulsed-field g electrophoresis with a CHEF gel aparatus. On the basis of th sizes of macrorestriction fragments obtained by double dige tion of the DNA with combinations of *SwaI* and *PacI* or *Swa*

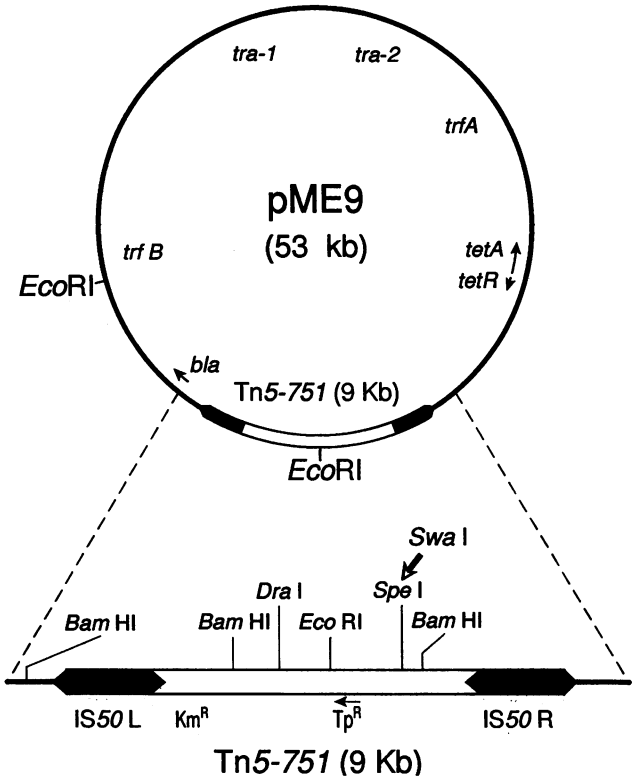

FIG. 5. Pertinent features of the Tn*5-751*-containing plasmid pME9. The distribution of selected restriction enzyme sites and drug resistance markers within the plasmid and transposon is shown. Note the unique *Spe*I site, which is located within Tn*5-751*. This site was exploited in construction of plasmid pTGL166, where it was converted to a *Swa*I site. Plasmid pME9 is temperature sensitive with respect to its replication as a consequence of a lesion within the *trfA* gene (39). Designations: *bla*, β-lactamase gene; *trfA* and *trfB*, genes encoding *trans*-acting proteins required for plasmid replication; *tra-1* and *tra-2*, genes encoding functions required for conjugal transfer; *tetA*, gene encoding a membrane protein associated with tetracycline effux; *tetR*, gene encoding a repressor that blocks *tetA* expression; Km^R, gene encoding an ATP-dependent aminoglycoside-phosphorylating enzyme.

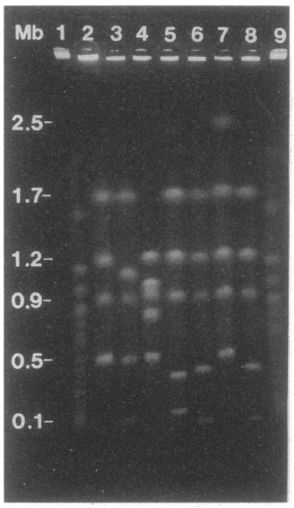

FIG. 6. Identification of *Swa*I fragments disrupted by the insertion of Tn*5-751S* in mutant derivatives of strain 17616 isolated by transposon mutagenesis. DNA from strain 17616 and several Tp^r Tc^s transposants isolated from strain 17616(pTGL166) was digested with *Swa*I, and the resulting fragments were resolved by PFGE by ramping with increments of 4 s over 100 steps for a range of pulse times of 50 to 450 s. Lanes 1 and 9, chromosomal DNA markers from *S. cerevisiae*. Lane 2, DNA from the wild-type strain 17616. Lanes 3, 4, 5, 6, and 8, DNA from the amino acid auxotrophs 249-112, 249-115, 249-111, 249-113, and 249-114, respectively. Lane 7, DNA from 249-110, a strain impaired in phthalate utilization. Characteristics of the mutant strains are summarized in Table 1. In each of the amino acid auxotrophs, one of the *Swa*I fragments from the 3.4-Mb replicon was replaced by two new fragments generated by the insertion of the *Swa*I site on Tn*5-751S*. In the mutant impaired in phthalate utilization (lane 7), a new *Swa*I fragment was generated as a consequence of insertion of Tn*5-751S* into the 2.5-Mb replicon, which ordinarily is not cleaved by *Swa*I.

and *Pme*I, we estimate that the size of the *P. cepacia* genome is 6.8 Mb. Taking into account the 170-kb cryptic plasmid present in strain 17616, the overall genome size of strain 17616 was close to 7 Mb. This was in general agreement with the estimate of 6.5 Mb reported for another *P. cepacia* strain, DSM 50180 (14), and was significantly larger than the value of 5.8 Mb obtained for *Pseudomonas aeruginosa* (14, 18, 23, 37).

Bacteria containing multiple chromosomes have to date been restricted to members of the alpha group of proteobacteria (1, 32). The finding that *P. cepacia*, a member of the beta group of proteobacteria (34), exhibits a similar multiplicity indicates that this may be a more common feature of bacterial genomes. In the case of *R. sphaeroides*, the two large replicons (3.0 and 0.9 Mb) constituting the genome of this bacterium each contained rRNA as well as other essential genes (53). On this basis Suwanto and Kaplan (53) designated both replicons chromosomes. On the other hand, *R. meliloti* 1021 was reported to contain a single, 3.5-Mb chromosome bearing rRNA genes and other indispensible genes and to contain two megaplasmids of 1.7 and 1.4 Mb specifying functions related to symbiosis and nitrogen fixation (21, 50). The two megaplasmids were devoid of rRNA genes. *Agrobacterium tumefaciens* C58 represents an interesting case; rRNA genes appeared to be present on both a 2.1-Mb linear chromosome and a 3-Mb circular chromosome. In the present study, macrorestriction fragments representative of each of the three large replicons in *P. cepacia* 17616 were found to contain rRNA genes. Neither the number nor the locations of rRNA cistrons on the different macrorestriction fragments were determined. However, since we know the locations of *Swa*I, *Pac*I, and *Pme*I sites on the different replicons, it may be feasible to obtain more-detailed information about the distribution of rRNA genes by mapping the relative positions of *Ceu*I cleavage sites. The enzyme *Ceu*I appears to cleave bacterial DNA only within genes (*rrl*) encoding 23S RNA and has proved useful in confirming or establishing the locations of *rrl* genes on the chromosomes of several bacteria (21, 28).

On the basis of our analysis of genes inactivated by insertion of Tn*5-751* into the 3.4-Mb replicon of strain 17616 or lost by deletion of DNA from the 2.5-Mb replicon, it would appear that, in addition to rRNA genes, both of these replicons carry genes essential for amino acid biosynthesis. For example, different insertions of Tn*5-751S* into the 3.4-Mb replicon inactivated genes related to the synthesis of isoleucine, arginine, histidine, and threonine. Functions lost by deletion of DNA from the 2.5-Mb replicon included the ability to synthesize lysine as well as the capacity to utilize penicillin, trehalose, and ribitol as carbon and energy sources. To date we have been unable to identify any functions lost as a consequence of deletion of DNA from the 0.9-Mb replicon, and no mutations

resulting from insertion of Tn*5-751S* have mapped to the 0.9-Mb replicon.

Whether the presence of multiple replicons is characteristic of the majority of *P. cepacia* isolates remains to be determined. Our group is in the process of screening preparations of unrestricted DNA from other *P. cepacia* isolates for the presence of randomly linearized replicons similar to those identified in strain 17616. To date we have detected DNA species with similar sizes in two other strains, G4 (45) and DB01 (3, 61). One problem in using this approach is that preparations of unrestricted DNA often contain insufficient amounts of the linearized replicons to ensure their detection. However, it should be feasible to increase the relative amounts of such linear species by treatment of the DNA with ionizing radiation or by limited digestion of the DNA with restriction enzymes or DNase I, which in the presence of Mn^{2+} produces double-strand breaks in DNA (41, 55, 57).

B. Holloway and his collaborators have proposed that the unusual catabolic potential of pseudomonads evolved by the addition of blocks of DNA specifying biodegradative functions to a primitive chromosome containing primarily genes for housekeeping functions (17–20). In considering such an accretion model of genome evolution, the importance of the acquisition and incorporation of plasmid DNA into the chromosome in expanding degradative capabilities is evident. Now that a number of bacteria which contain multiple large replicons or "chromosomes" have been identified, it seems reasonable that genome evolution might also involve cases in which bacteria incorporate into their genomes entire "chromosomes" from other bacteria. In this context it would be of interest to compare rRNA genes on the different replicons within a bacterium such as *P. cepacia*. Assuming that such large replicons can be acquired by lateral transfer and maintained, then rRNA sequence data should provide some insight into their origin.

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