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Detection and Characterization of Plasmids in *Pseudomonas aeruginosa* Strain PAO

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Using a variety of techniques, it has been established that the sex factor FP2 has a density of 1.717 g/cm^3 (corresponding to a guanine plus cytosine [G + C] content of 58%) and a mean circular contour length of 28.5 $\pm$ 0.6 μm (corresponding to a molecular weight of 59×10^6). Another sex factor FP39 has a density of 1.719 g/cm^3 (corresponding to a G + C content of 60%) and a mean circular contour length of 26.5 $\pm$ 0.5 μm (corresponding to a molecular weight of 55×10^6). It appears that all, or nearly all, of the FP sex factor deoxyribonucleic acid occurs as covalent circular molecules under the conditions employed. In addition, these procedures have been used to demonstrate that strain PAO, a naturally occurring female (i.e., FP$^-$) strain of *Pseudomonas aeruginosa*, harbors a number of cryptic plasmids having similar densities to the bulk of the cell deoxyribonucleic acid (1.726 g/cm^3) and occurring as covalent circular molecules.

Demonstration of the occurrence of plasmids in strains of bacterial species may be genetic or physical. *Pseudomonas aeruginosa* has been shown by genetic tests to harbor a variety of plasmids: sex factors (1, 2, 3); R factors (12); and metabolically important plasmids (Emerick, personal communication) originating in *Pseudomonas putida* (1). Genetic tests suffer severe limitations, however, and physical tests are required to characterize plasmids fully. Physical tests may also serve to detect plasmids which confer no known phenotypic properties on their hosts. For these reasons, we have developed methods for the detection, isolation, and characterization of two of the sex factors of *P. aeruginosa*, FP2 and FP39.

To the extent that plasmids differ in average density (i.e., base composition) from the bulk of their host deoxyribonucleic acid (DNA), they are separable from it by density gradient centrifugation in CsCl solution. Plasmids which are of the same density as the bulk of their host DNA cannot be detected by this means, but they can be detected if they occur as covalently closed circular duplex DNA at some stage in their multiplication. Closed circular duplexes are separable from linear duplex fragments of DNA of the same base composition in CsCl solutions containing saturating amounts of the intercalating dye, ethidium bromide (EtBr; 9).

Separation of the plasmid from bulk DNA is only half of the problem because the separate peaks must be detected. To detect them, absorption of DNA at 260 nm or the amount of radioactive label incorporated in the DNA may be measured. We chose the latter method because of its greater sensitivity. Tritiated thymidine (or tritiated thymine) is the standard specific DNA label, and efforts in various laboratories (4) have been made to isolate thymidine-requiring mutants of *P. aeruginosa*. In this laboratory, we tried unsuccessfully to employ selective and enrichment techniques as well as direct screening of survivors of mutagenic treatment for the isolation of three different types of mutants: (i) those requiring thymidine; (ii) those not requiring but still incorporating thymidine; and (iii) those stimulated by pyrimidine analogues to incorporate thymidine. Finally, we turned to a method of labeling both ribonucleic acid and DNA with tritiated adenine followed by digestion of the RNA with ribonuclease and base. Under the conditions used, the DNA remained acid insoluble. This method has been used successfully to label the DNA of *Saccharomyces cerevisiae* (8), and a report of this prompted our experiments.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are derivatives of *P. aeruginosa* strain PAO (4). The

adenine-requiring FP$^-$ strain JC9006 carrying the mutation *pur-600*, was obtained from the prototrophic female strain PAO1 after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Inc.). Male strains JC9005 FP2$^+$ and JC9007 FP39$^+$ were constructed by transferring the sex factors FP2 and FP39 to JC9006 FP$^-$ using the male strains PAO1227 FP2$^+$ and PAO1264 FP39$^+$ (J. M. Pemberton and B. W. Holloway, Genet. Res., in press) as donors. Basic genetic methods are described in detail by Pemberton and Holloway (J. M. Pemberton and B. W. Holloway, Genet. Res., in press). Luria broth, consisting of 1 liter of distilled water, 10 g of trptone (Difco), 5 g of yeast extract (Difco), and 20 g of NaCl, was used.

Labeling procedure and preparation of lysate. A 1-ml sample of an overnight broth culture of the adenine-requiring mutant was added to 10 ml of minimal medium containing 50 μCi of adenine-2-^3H (sp act, 22 Ci/mmol; Schwarz/Mann). Minimal medium contains 100 ml of 56/2 buffer plus 1 ml of 40% glucose. The initial turbidity was approximately 10 to 15 U as measured on a Klett-Summerson colorimeter using a red filter. The culture was incubated with aeration at 37 C until a Klett reading of 40 to 50 U was reached. The culture was then harvested by centrifugation at 10,000 $\times$ *g* for 10 min, and the pellet was suspended in 0.5 ml of 25% sucrose solution made up in 0.05 M tris(hydroxymethyl)amminomethane (Tris; pH 8.0). After adding 0.1 ml of freshly made lysozyme solution (1 mg/ml in 0.25 M Tris, pH 8.0) the culture was incubated on ice for 5 min. An addition of 0.1 ml of 0.25 M ethylenediaminetetraacetic acid (EDTA) was then made, and the culture was left on ice for 5 min before adding 0.1 ml of 0.62% sarcosyl nc97. The final mixture was gently swirled until lysis occurred. When lysis was complete, 0.3 ml of TES medium (0.05 M NaCl; 0.05 M Tris; 0.005 M EDTA, pH 5.0) containing 1 mg of ribonuclease/ml (rendered deoxyribonuclease-free by heating at 80 C for 10 min) was added, and the mixture was incubated at room temperature for 10 min. The mixture was then chilled and assayed for [^{3}H]adenine incorporation. Samples of 10 μliters of each lysate were spotted onto pieces of filter paper and dried. These were then washed two times in 5% trichloroacetic acid, two times in ethyl alcohol, and once in ET_2O. Only 0.5 ml of each lysate was used immediately for centrifugation, the rest were frozen for further use.

Preparation and handling of gradients. For gradients containing EtBr, 0.5 ml of the lysate was first added to 0.33 ml of EtBr solution (3 mg/ml dissolved in water), 0.33 ml of phosphate buffer (0.7 M, pH 7.3), and 4.1 ml of TES. This mixture was poured into a vial containing 5.1 g of CsCl. The EtBr-DNA mixture was protected from the light to prevent dye-mediated nicking of covalently closed circular DNA. When CsCl alone was used, 0.33 ml of TES replaced the 0.33 ml of EtBr solution, and 7.1 g of CsCl was used instead of 5.1 g. Only after all of the CsCl had dissolved was the mixture transferred to a polyallomer centrifuge tube, overlaid with light mineral oil, and spun. The centrifuge tubes were prepared by boiling in 1 mM EDTA for 10 to 15 min and soaked in TES plus 5 μg of bovine serum albumin per ml for 1 h before storage.

Centrifugation was carried out at 15 C in a type 50 fixed-angle rotor for 60 h at 44,000 rpm using a L-2-65 Beckman ultracentrifuge. Using a drop collection unit, 50 to 55 fractions of 10 drops each were collected in tubes containing 0.3 M NaOH. The tubes were incubated for 24 h at 37 C, after which they were placed on ice for 1 h, then trichloroacetic acid was added to each tube to a final concentration of 5%. The precipitated DNA was collected on filters which were dried, washed twice in ETOH, and once in ET_2O. When dry, the filters were immersed in 10 ml of toluene-2,5-diphenyloxazole (3 liters of toluene plus 13.7 g of 2,5-diphenyloxazole), and the samples were counted in a Packard Tri-Carb scintillation counter.

When CsCl solution densities were required, these were determined immediately after collection of the gradient using refractometer readings taken on small samples from each fraction. Calculations were based on the relationship between refractivity and density (7).

Electron microscopy. The positions of unlabeled satellite and main DNA bands were inferred from their densities as determined from centrifugation runs in which they were labeled. All of the plasmid DNA used for electron microscopy examination was obtained from CsCl gradients containing EtBr. DNA samples were used immediately after collection of the gradient or dialyzed for 2 h at 4 C against three changes of 0.2 M ammonium acetate (NH_4Ac). When it was eventually noted that there appeared to be no significant difference in contour lengths of circular molecules before or after dialysis, the dialysis step was not used. When fractions had to be stored, they were left in the CsCl-EtBr solution.

The grids used for electron microscopy were prepared by the method of Kleinschmidt and Zahn (5). The spreading solution (1.3 ml) contained 1 ml of 1.0 M NH_4Ac (pH 5.0), 0.1 ml of 1 mg of cytochrome *c* per ml (made up in 1.0 M NH_4Ac, pH 5.0), and 0.2 ml of an undialyzed sample of DNA. The hypophase was 0.3 M NH_4Ac, pH 5.0. All molecules were photographed with a Siemens Elmiskop 1A, and their lengths were obtained by making enlarged prints and measuring their contours with a map measurer. Usually 20 molecules having well-defined open contours were used to determine the length of a particular class of plasmids.

RESULTS

CsCl gradients in the absence and presence of EtBr. Data in Fig. 1A, B, and C are representative of the data obtained when the DNAs from both male and female strains of *P. aeruginosa* strain PAO (4) are centrifuged to equilibrium in CsCl gradients in the absence of EtBr. The DNA from the female strain JC9006 shows no detectable satellite bands, while that from the FP2$^+$ male strain, JC9005, gives a

satellite band (representing 5.0% of the total counts in DNA) at a density of 1.717 g/cm³. The DNA from the FP39⁺ male strain, JC9007, gives a satellite band (also representing 5.0% of the total counts in DNA) at a density of 1.719 g/cm³. In both cases, the bulk of the DNA banded at a density of 1.726 g/cm³.

In an attempt to obtain more information about the nature of the satellite bands, we used the method of Radloff et al. (9) and banded the DNAs from the female strain JC9006 and the two male strains JC9005 and JC9007 in CsCl containing saturating amounts of EtBr. Any closed circular molecules having densities close to that of the main chromosomal DNA in the absence of EtBr should form detectable bands at a higher density than the main chromosomal DNA in the presence of saturating amounts of EtBr.

The results (Fig. 2A, B, and C) indicate that the female strain JC9006 does contain at least one plasmid because a satellite band occurs at a density of 0.036 g/cm³ greater than that of the bulk of the DNA. The DNAs of the two male strains show a satellite band with the same density and in addition another satellite at a density closer to that of the main band. We designated the satellite that the three strains had in common as the P satellite band; its displacement from the main chromosomal band is close to the value expected if the DNA contained in this band has a density similar to that of the main chromosomal DNA in the absence of EtBr (i.e., 1.726 g/cm³). The average amount of the P plasmid DNA does not vary significantly between strains, and it is 2.9 ± 0.2% of the total counts incorporated into DNA.

The extra satellite bands found in the two male strains may be attributed to the sex factors FP2 and FP39. They form much closer to the main DNA band than the P plasmid peak (Fig. 2A, B, and C). Data from at least five different centrifugation runs for each male strain show that the DNA band which is attributed to FP2 contains 5.4 ± 0.5% of the radioactive label incorporated into DNA, while the DNA band which is attributed to FP39 is slightly lower in amount (containing 4.1 ± 0.4% of the total counts in DNA).

Electron microscopy. Plasmid DNA from the FP2, FP39, and P satellite bands was prepared and examined with the electron microscope. Since some 70 to 75% of all molecules

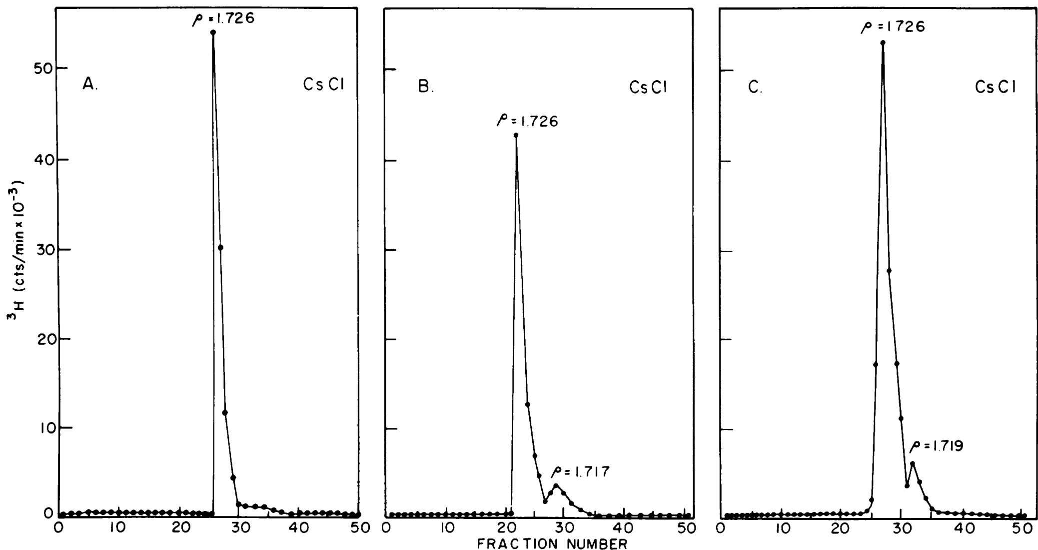

FIG. 1. *(A) CsCl-gradient analysis of lysates of JC9006 FP⁻; (B) JC9005 FP2⁺; (C) JC9007 FP39⁺. The density of each peak fraction is indicated near the appropriate place on each graph. Cultures (10 ml) of these strains were grown in the presence of adenine-2-³H to the log phase. The lysate was prepared by the lysozyme-sarcosyl procedure and centrifuged after addition of CsCl for 60 h at 15 C and 44,000 rpm in a Spinco fixed-angle rotor type 50. Fractions of 10 drops each were collected from the bottom of the tube in small glass tubes. Sodium hydroxide (0.3 M) was added to the tubes, and they were incubated for 24 h at 37 C to hydrolyze the RNA. Trichloroacetic acid was then added to each fraction to a final concentration of 5% to precipitate the DNA. The fractions were filtered, washed, dried, and counted in a scintillation counter according to the procedure described in Materials and Methods.*

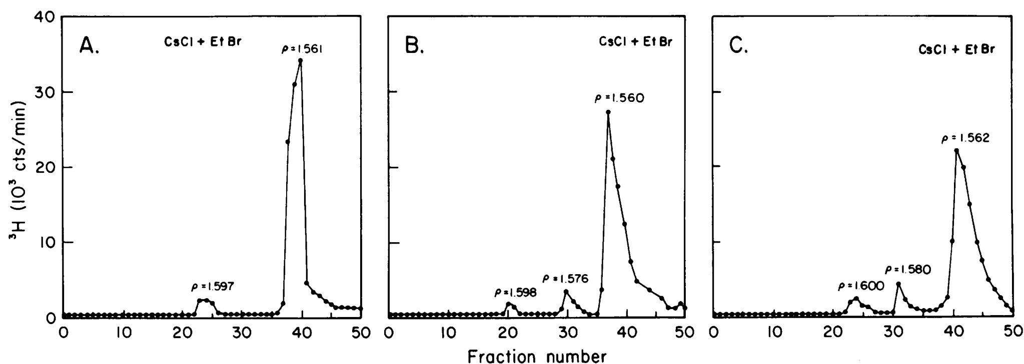

FIG. 2. *(A) Ethidium bromide/CsCl-gradient analysis of JC9006 FP⁻; (B) JC9005 FP2⁺; and (C) JC9007 FP39⁺. The density of each peak fraction is indicated near the appropriate place on each graph. Cultures (10 ml) of these strains were grown in the presence of adenine-2-³H to the log phase. The lysate was prepared by the lysozyme-sarcosyl procedure and centrifuged after addition of CsCl and ethidium bromide for 60 h at 15 C and 44,000 rpm in a Spinco fixed-angle rotor type 50. Fractions of 10 drops each were collected from the bottom of the tube in small glass tubes. Sodium hydroxide (0.3 M) was added to the tubes and they were incubated for 24 h at 37 C to hydrolyze the RNA. Trichloroacetic acid was then added to each fraction to a final concentration of 5% to precipitate the DNA. The fractions were filtered, washed, dried and counted in a scintillation counter according to the procedure described in Materials and Methods.*

from each preparation examined were in the supercoiled configuration, only those molecules having well-defined open coutours were used for measurement of molecular lengths.

Circular molecules obtained from the P satellite band are pictured in Fig. 3, 4, and 5. Some 50% of all molecules (both open and supercoiled forms) had an average contour length of 2.6 ± 0.1 μm (Fig. 2), 40% had a mean contour length of 0.8 ± 0.1 μm (Fig. 4), and 10% had a mean length of 4.9 ± 0.2 μm (Fig. 5).

The FP2 and FP39 bands each contain a single molecular species. The molecules of the FP2 band have an average contour length of 28.5 ± 0.6 μm, and those of the FP39 band have an average contour length of 26.5 ± 0.5 μm (Fig. 6 and 7).

DISCUSSION

By banding the DNAs from both male and female strains of *P. aeruginosa* strain PAO in CsCl gradients in the absence of EtBr, we found that FP2 had a density of 1.717 g/cm³, and FP39 had a density of 1.719 g/cm³, while the bulk of the host DNA has a density of 1.726 g/cm³. Using the relationship between density and guanine plus cytosine (G + C) content (10), FP2 has a calculated G + C content of 58%, and FP39 has a calculated G + C content of 60%, which can be compared with a 67% G + C content for the main chromosome. This difference in G + C content of the FP-type sex factors and main chromosome of *P. aeruginosa* strain PAO may explain why the large number of sex factors isolated by Pemberton and Holloway (J. M. Pemberton and B. W. Holloway, Genet. Res., in press) have only one or perhaps two origins of chromosome transfer. If homology between sex factor and chromosome in *P. aeruginosa* is a prerequisite for integration and chromosome mobilization, then the FP-type sex factors, because of their different G + C contents, may have homology with only very limited regions of the chromosome.

Further information about the size and structure of FP2 and FP39 was obtained by electron microscopy examination of the sex factor satellite DNA prepared by the method of Radloff et al. (9). In both cases, there appeared to be only a single size class of molecules. The FP2 DNA contained molecules which had a mean circular contour length of 28.5 ± 0.6 μm, while the FP39 molecules had a mean circular contour length of 26.5 ± 0.5 μm. Using the relationship between molecular length and molecular weight ($2.07 \pm 0.04 \times 10^6$/μm of DNA; reference 6), FP2 has a calculated molecular weight of 59.0×10^6, and FP39 has a calculated molecular weight of 55.0×10^6. Since the amount of sex factor satellite DNA (5% of the total counts incorporated into DNA) is similar when the DNA from a male strain is banded in CsCl with and without EtBr, it may be concluded that all, or nearly all, of the DNA of the sex factor occurs in a closed circular configuration.

In an attempt to obtain some idea of the number of copies of FP per main chromosome,

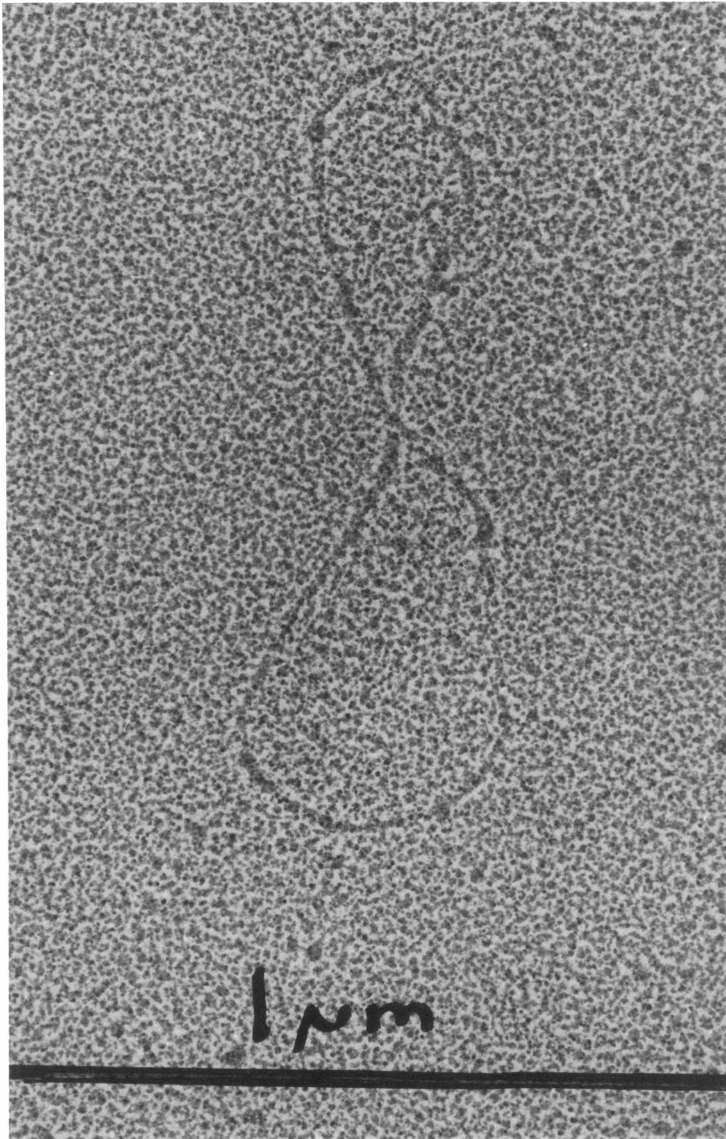

FIG. 3. *Electron micrograph of circular DNA molecule obtained from the P satellite band formed in an ethidium bromide/CsCl gradient and prepared by the method of Kleinschmidt and Zahn (5). The contour lengths of the molecule pictured is 2.7 μm.*

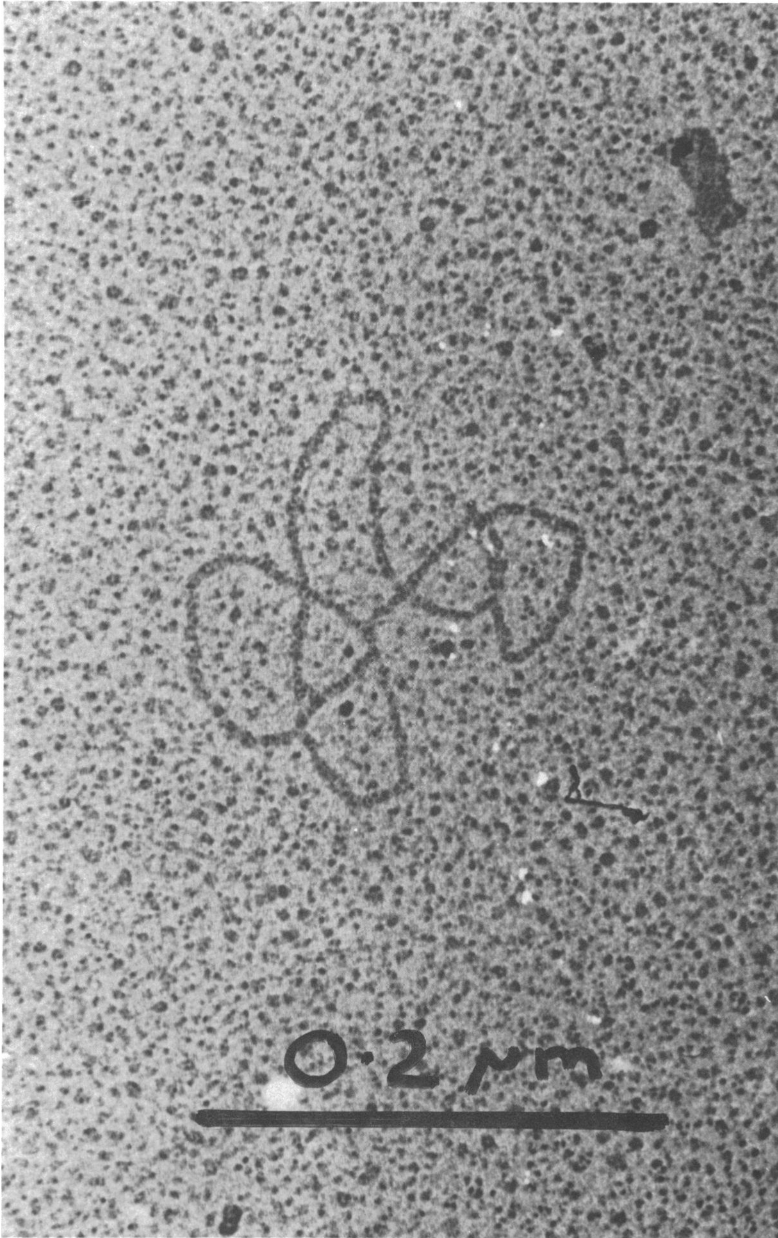

FIG. 4. *Electron micrograph of circular DNA molecule. Same as Fig. 3, except that contour length of the molecule pictured is 0.8 μm.*

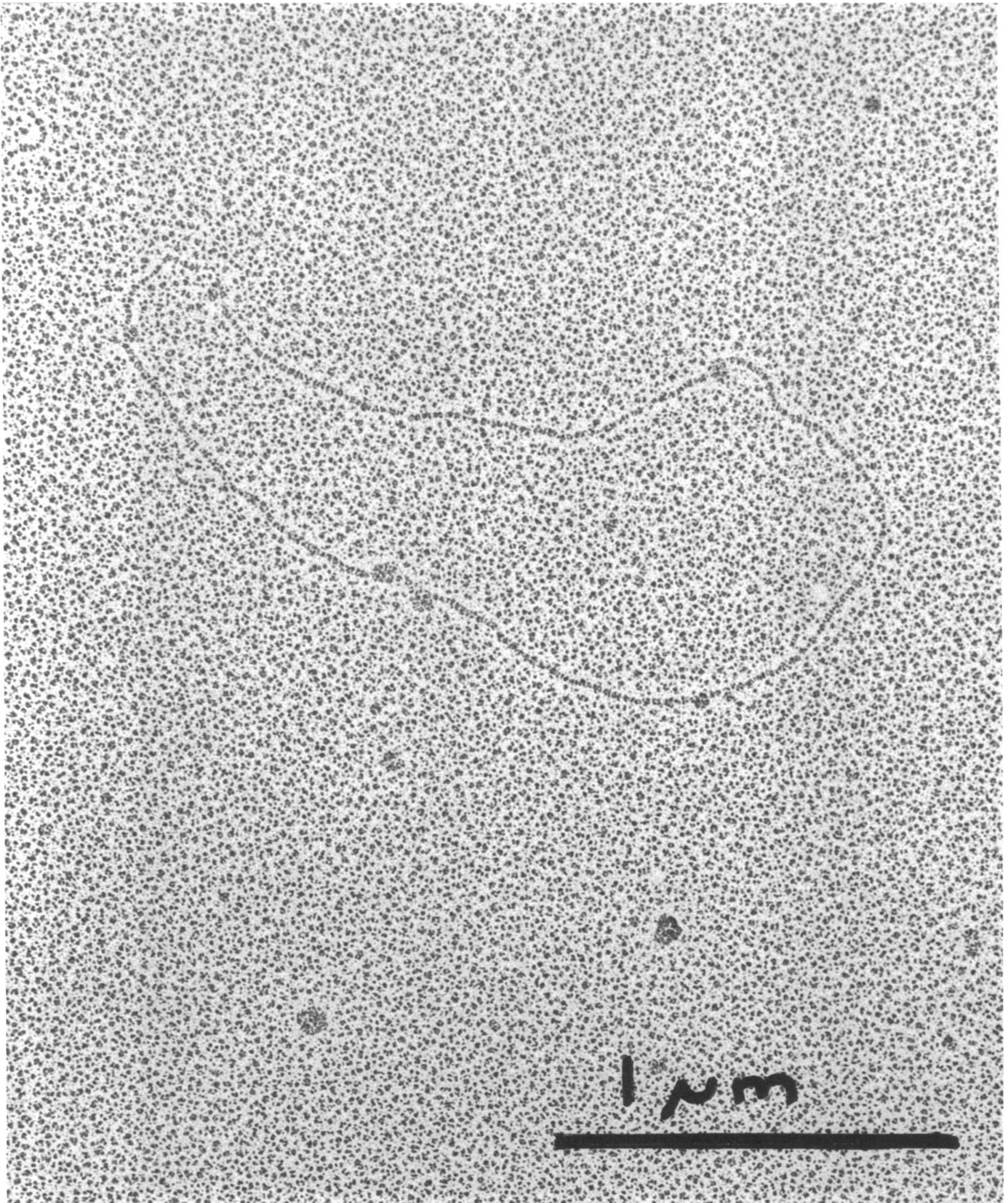

FIG. 5. *Electron micrograph of circular DNA molecule. Same as Fig. 3, except that contour length of the molecule pictured is 4.8 μm.*

FIG. 6. *Electron micrograph of an open circular molecule obtained from the FP2 satellite band formed in an ethidium bromide/CsCl gradient and prepared by the method of Kleinschmidt and Zahn (5). The molecule has a contour length of 28.0 μm.*

FIG. 7. *Electron micrograph of an open circular molecule obtained from the FP39 satellite band formed in an ethidium bromide/CsCl gradient and prepared by the method of Kleinschmidt and Zahn (5). The molecule has a contour length of 26.5 µm.*

we used the following data: (i) the main chromosome of *P. aeruginosa* has a molecular weight of approximately 2×10^9 (Pemberton, unpublished data); (ii) 5% of the total cellular DNA can be attributed to the FP-type sex factors; (iii) FP-type sex factors have molecular weights between 55×10^6 and 60×10^6. Using the above data, it was calculated that there are at least one to two autonomous copies of FP per chromosome, which is in agreement with the work of Stanisich and Holloway (11).

The presence of a smaller satellite band in both male and female strains indicates that strain PAO carries some number of cryptic plasmids. Electron microscopy examination of this DNA, whether from a male or a female strain, reveals that there are three distinct species of closed circular DNA. These species have circular contour lengths of 0.8 ± 0.1 μm, 2.8 ± 0.1 μm, and 4.6 ± 0.2 μm. Again using the relationship between molecular length and molecular weight, these species have calculated molecular weights of 1.7×10^6, 5.8×10^6, and 9.5×10^6.

An estimate of the number of copies per chromosome of each class of P plasmid has been made using the molecular weights of both the chromosome and plasmids, the relative percentage of each type of plasmid in the P satellite band, and the percentage of this type of DNA in the cell (3% of the cellular DNA, closed circular configuration only). Each plasmid is present in the following numbers per chromosome: 1.7×10^6 daltons, 15/chromosome; 5.8×10^6 daltons, 7/chromosome; 9.5×10^6 daltons, 1/chromosome. Whether each species represents a different plasmid cannot be assessed from these results.

As Clowes (2) has pointed out, plasmids having molecular weights of less than 20×10^6 often do not have a sex factor activity, and frequently replicate under "relaxed" control, which results in large numbers of copies per cell. It seems likely that the molecules of the P satellite band fall into this category. With these limitations in mind, many possibilities exist as to the nature of these cryptic plasmids, which range from R factors to lysogenic phages to plasmids involved in peripheral metabolism (e.g., degradation of camphor), or the production of aeruginocins (analogous to the colicins of *Escherichia coli*). Another possibility is that they may be responsible for the plasmid-like transfer and inheritance of recombination proficiency, ultraviolet resistance, and mitomycin resistance by certain recombination-deficient mutants of *P. aeruginosa* (Yajko and Clark, unpublished data).

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