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Size of the Chromosome of *Pseudomonas aeruginosa* PAO

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Electron microscope examination and velocity sedimentation analysis of the deoxyribonucleic acid released from *Pseudomonas aeruginosa* spheroplasts indicate that this organism carries the bulk of its genetic determinants in a single duplex deoxyribonucleic acid molecule having a molecular mass of 2.1×10^9 daltons.

Genetic and physical evidence shows that *Escherichia coli* has a single, circular chromosome (4, 10). This characteristic is shared by such organisms as *Salmonella typhimurium* (21), *Streptomyces coelicolor* (9), and *Mycoplasma hominis* (2). Whether this structure is universal among bacteria is not known. However, it is important to determine whether other species of bacteria carry their genetic determinants on a single, circular chromosome because of the implications that this structure has for replication and genetic mapping. The organism examined in this study is the human pathogen *Pseudomonas aeruginosa*.

Since genetic data suggest that *P. aeruginosa* PAO carries the bulk of its genetic determinants on a single linkage group (18) and initial data presented here show that the amount of deoxyribonucleic acid (DNA) per cell is only slightly less than that of *E. coli*, the size of the *P. aeruginosa* chromosome should be approximately that of *E. coli*. In view of the anticipated size of the chromosome, it was decided to determine its molecular mass by the velocity sedimentation technique of Petes and Fangman (20) and the protein-film electron microscope technique of Kleinschmidt and Zahn (12).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used were the adenine-requiring FP$^-$ strain of *P. aeruginosa* JC9006, which carries the mutation *pur-600*[20], and the multiply auxotrophic F$^-$ strain of *E. coli* AB1157, which carries the mutations *lac-1*, *his-4*, *thr-4*, *leu-8*, *proA2*, *arg-3 thi*$^-$, Sm^r (25).

Labeling procedure and preparation of lysate. A 1-ml sample of an overnight Luria broth culture of JC9006 (*pur-600*) FP$^-$ was added to 5 ml of minimal medium containing 50 μCi of [2-^{3}H]adenine (specific activity 22 Ci/mmol; Schwarz/Mann). In the case of AB1157, the 1-ml sample of the overnight Luria broth culture was added to 5 ml of minimal medium containing 50 μCi of [^{3}H]thymidine (specific activity 28 Ci/mmol; Schwarz/Mann). Minimal medium contains 100 ml of 56/2 buffer (R. V. Miller, J. M. Pemberton, and K. E. Richards, Virology, in press) plus 1 ml of 40% glucose. The initial turbidity was approximately 15 to 20, as measured on a Klett-Summerson colorimeter with a red filter. The cultures were incubated with aeration at 37 C until a Klett reading of 60 to 70 was reached (early stationary phase, ca. 10^9 to 2×10^9 cells per ml). The cultures were harvested by centrifugation at $10,000 \times g$ for 10 min, and the pellet was suspended in 1.5 ml of 25% sucrose solution made up in 0.05 M tris(hydroxymethyl)aminomethane (pH 8.0) containing 200 μg of pancreatic ribonuclease (rendered deoxyribonuclease-free by heating at 80 C for 10 min) per ml. After addition of 1 ml of freshly made lysozyme solution [1 mg/ml in 0.25 M tris(hydroxymethyl)aminomethane, pH 8.0], the culture was incubated on ice for 5 min. The spheroplast suspension was layered directly onto a linear 15 to 30% sucrose gradient (4.7 ml) so that each gradient carried 1 to 2 μg of bacterial DNA. The sucrose solutions contained 1% sodium dodecyl sarcosinate, 0.01 M sodium ethylenediaminetetraacetic acid (pH 8.0), 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0), and 1 M NaCl. The sedimentation marker used, coliphage T4 labeled with [^{14}C]thymine, was lysed gently by incubating with 1% Sarkosyl at 60 C for 10 min. A large-bore pipette was used to transfer the T4 DNA (10^{-3} to 2×10^{-3} μg) to the gradient to minimize breakage by shear. The sucrose gradients were incubated at room temperature for 15 min after addition of the spheroplasts and marker DNA to allow for complete lysis of the bacterial cells.

Centrifugation and collection of gradients. Centrifugation was carried out at 5 C in a Spinco SW50 rotor for 24 h at 8,000 rpm using an L-2-65 Beckman ultracentrifuge. After centrifugation, 40 fractions were collected in tubes. In the case of JC9006, the fractions were collected in tubes containing 0.3 M NaOH, and these were then incubated for 24 h at 37 C to hydrolyze the ribonucleic acid (17). For both AB1157 and JC9006, the tubes were incubated on ice for 1 h; the trichloroacetic acid was then added to each tube to a final concentration of 5%. The precipi-

tated DNA was collected on filters which were dried and washed twice in ETOH and once in ET_2O. When dry, the filters were immersed in 10 ml of toluene-2,5-diphenyloxazole, and the samples were counted in a Packard Tri-Carb scintillation counter.

Electron microscopy. The method employed for electron microscopy is derived in part from that of MacHattie et al. (15). The spheroplasts were prepared by incubating washed, stationary-phase cells of JC9006 (ca. 2×10^9 cells per ml) in 27% sucrose containing 500 μg of lysozyme [in 0.01 M tris(hydroxymethyl)aminomethane, pH 8.0] per ml and 0.01 M sodium ethylenediaminetetraacetic acid at 37 C for 2 h. The spheroplasts were lysed by adding 0.05 ml of the spheroplast suspension to the chamber of a large-bore 2-ml syringe containing 1 ml of distilled water. Then 0.4 ml of 0.01% cytochrome *c* in 3 M NaCl was added to the chamber, and the solution was incubated at 37 C for 2 h.

The DNA molecules were spread by slowly expelling a drop of the solution from the syringe onto a glass slide which had just been washed in distilled water. A few particles of talc were sprinkled on the water surface to follow the spreading of the monofilm. The monofilm was picked up onto a 50-mesh, nickel electron microscope grid (Ladd Research Industries) which had been coated with a support film of Formvar. The grid was washed in ethanol for 5 s, allowed to dry on a tissue, and then rotary-shadowed with uranium. Molecules were photographed at magnifications of ×6,000 and ×7,000, and molecular lengths were determined by a map-measuring device from tracings protected at an overall magnification of ×44,000.

Chemical determination of DNA per cell. A 100-ml volume of stationary-phase cells was inoculated into 1 liter of minimal medium. After the cells grew into early stationary phase, they were washed twice with water and suspended in 5 ml of 5% trichloroacetic acid. Supernatant fractions were titered at each step to determine the loss of cells. The suspension was boiled at 100 C for 5 min to extract the DNA and cooled under running water, and the precipitated material was centrifuged out. The amount of DNA in the supernatant was determined by the method of Webb and Levy (24), with calf thymus DNA as the standard.

RESULTS

Chemical determination of the DNA per cell. The amount of DNA in stationary-phase cells was determined by the method of Webb and Levy (24). Results from three different determinations on *P. aeruginosa* and *E. coli* show that the amount of DNA per cell for JC9006 was $4.0 \pm 0.6 \times 10^{-9}$ μg ($2.5 \pm 0.4 \times 10^9$ daltons) and for AB1157 it was $5.2 \pm 0.8 \times 10^{-9}$ μg ($3.3 \pm 0.5 \times 10^9$ daltons). The figures obtained for both *E. coli* and *P. aeruginosa* are in good agreement with data obtained by Park and De Ley (16) for *E. coli* and three other *Pseudomonas* species.

Velocity sedimentation analysis. In performing this type of analysis, we made a number of assumptions. First, it is possible to extrapolate the equation of Burgi and Hershey (3) to include DNA molecules as large as the *E. coli* chromosome (i.e., 2×10^9 to 3×10^9 daltons). Second, the DNA molecules being measured in this way are linear molecules free from ribonucleic acid and nucleoproteins. Recent work on yeast chromosomes (20) and the chromosomes of *Bacillus subtilis* (11) and *E. coli* (23, 26) suggests that these two assumptions are valid under the particular conditions employed in these experiments.

The results of the sedimentation analyses of DNA molecules obtained from stationary-phase cells of both *E. coli* (Fig. 1A) and *P. aeruginosa* (Fig. 1B) indicate the presence of large DNA molecules. In the case of *E. coli*, the largest

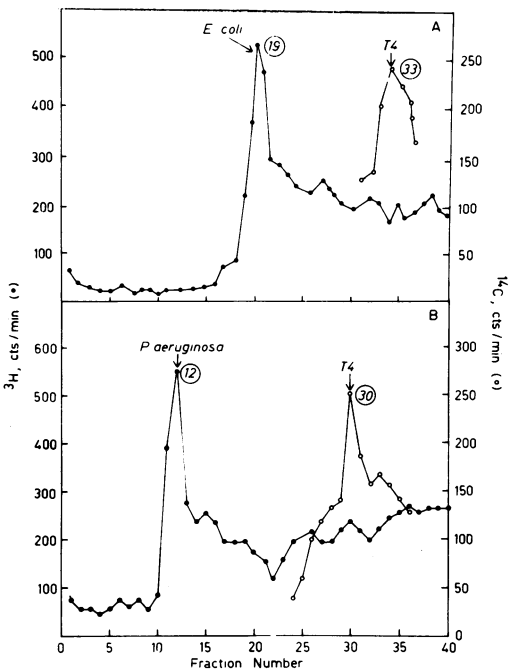

FIG. 1. *Sucrose gradient centrifugation of DNA released from stationary-phase cells of (A) E. coli and (B) P. aeruginosa. A suspension of spheroplasts containing 1 to 2 μg of ^{3}H-labeled DNA was layered directly onto a linear 15 to 30% sucrose gradient. Centrifugation was carried out in a Beckman L-2-65 ultracentrifuge with a Spinco SW50 rotor at a speed of 8,000 rpm for 24 h at 5 C. After centrifugation, fractions were collected from the bottom of the tube. In the case of P. aeruginosa, the ribonucleic acid was hydrolyzed with 0.3 N NaOH for 24 h at 37 C. Then, the samples from both E. coli and P. aeruginosa were chilled, and the DNA was precipitated by 5% trichloroacetic acid. Precipitates were collected on filters, washed, dried, and counted in a scintillation counter.*

molecules occur in fraction 19 (Fig. 1A); by extrapolation of the equation of Burgi and Hershey (3) using the internal marker T4, which has a molecular mass of 1.2×10^8 daltons (7, 14), the molecules in this fraction have a calculated molecular mass of 2.8×10^9 daltons. The largest DNA molecules of *P. aeruginosa* occur in fraction 12 (Fig. 1B) and, by reference to the internal marker, have a calculated molecular mass of 2.2×10^9 daltons. The presence of little or no radioactivity in the pellet fractions (0 to 5) indicates that cell lysis on the gradient was almost complete. The fractions containing the largest molecules constitute only 10 to 15% of the radioactivity present in the sedimentation profile, the remainder of the radioactivity being distributed among molecules that are somewhat smaller in size. This size distribution probably results from shearing of the DNA molecules during lysis and centrifugation; however, such a distribution could represent molecules at different stages of replication. In an attempt to obtain more information about the size of these DNA molecules and their state of replication, we performed electron microscope studies on DNA molecules derived from stationary-phase cells.

Electron microscopy of the DNA from P. aeruginosa stationary-phase cells. The initial method employed by MacHattie et al. (15) to measure the DNA content of a bacterial cell by using an electron microscope involved an examination of DNA partially extruded from disrupted spheroplasts of *Haemophilus influenzae*. This approach has a number of limitations. First, it is difficult to determine whether the molecule under examination is replicating; second, there is no measure of exactly how much of the cellular DNA has been extruded and hence visible. Bode and Morowitz (2) overcame these particular problems simply by lysing the bacterial cells prior to the formation of the protein monofilm. Since this procedure enables the DNA molecule to separate from the cell debris, it provides an opportunity to examine any replicating forks which may be present in the molecule; its essential features were incorporated into the method employed to examine DNA molecules from *P. aeruginosa*.

One of the major difficulties encountered in these experiments was breakage of the DNA molecules during preparation and spreading, not a surprising fact considering that the size of these molecules varied from 1,000 to 1,300 μm, which is some four to five times as large as the chromosomes isolated intact from *Mycoplasma hominis* (2). After careful lysis and spreading, it was found that a small fraction (0.2 to 0.5%) of the total DNA molecules examined were completely free of cell debris, contained no visible branching, and possessed only small areas of supercoiling. An example of the degree of spreading of one of these molecules is given in the overlapping electron micrographs of Fig. 2. The three largest unbranched molecules had measured lengths of 970,990 and 1,040 μm. All three molecules appeared to have "circular" configurations, but areas of supercoiling and light metal shadowing precluded an unequivocal answer to the question of physical circularity.

The bulk of the DNA molecules (>95%) obtained from stationary-phase cells of *P. aeruginosa* were free of cell debris but contained large areas of tangled supercoils. Although the overall lengths of such molecules could be measured, little information about their struc-

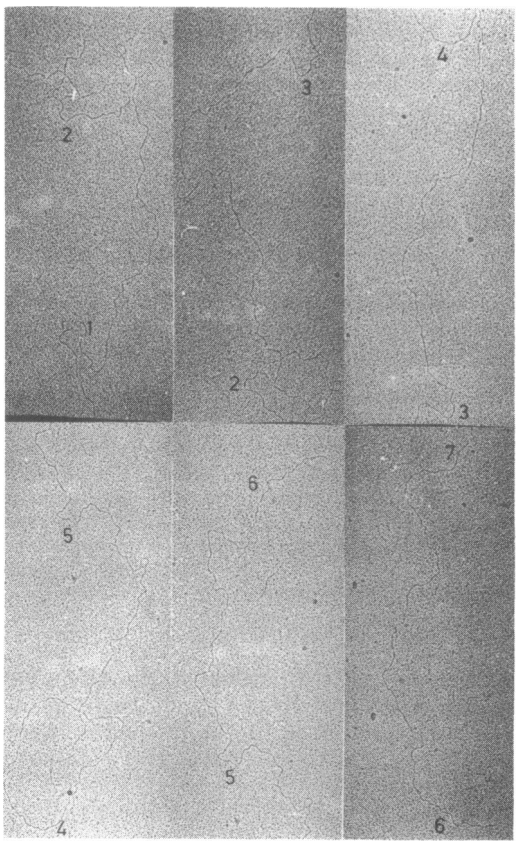

FIG. 2. *Overlapping electron micrographs of a section of a DNA molecule which was completely free of cell debris, contained no visible branching or ends, and possessed only small areas of supercoiling. The degree of spreading pictured here was typical of that obtained for the entire length of this DNA molecule, which was close to 1,000 μm. Magnification: ×4,000.*

ture or state of replication could be derived from the electron micrographs. The histogram pictured in Fig. 3 shows the molecular lengths of 23 such molecules chosen at random. The majority of these molecules measured between 1,000 and 1,300 μm; by the relationship between molecular mass and molecular length (13) (2.07×10^6 daltons per μm), these molecules have calculated molecular masses of 2.1×10^9 to 2.7×10^9 daltons. If it is assumed that the smaller molecular mass 2.1×10^9 daltons represents that of the nonreplicating chromosome, then the larger molecules probably contain partially replicated segments.

Only in a few instances were clearly defined forks detected (Fig. 4A, B), and these occurred in DNA molecules whose lengths were greater than 1,200 μm, suggesting that the nonreplicating molecule was somewhat smaller. All of the forks observed were single; no double forks of the type described by Bode and Morowitz (2) were found; this was almost certainly due to the extensive supercoiling which prevented an unambiguous view of the region being replicated. Since single forks may represent either the initiation point of replication or the point at which replication is proceeding, the failure to observe double forks precludes any conclusion as to the mode of replication of the chromosome.

DISCUSSION

From the data presented here it has been concluded that *P. aeruginosa* PAO contains a single chromosome per cell and this chromosome has a molecular mass of 2.1×10^9 daltons. The data from which this conclusion has been drawn have been summarized as follows.

DNA per cell. (i) Chemical determination of the DNA per cell gives a figure of $2.5 \pm 0.4 \times 10^9$ daltons. (ii) Electron microscope measurements of the DNA released from spheroplasts of stationary-phase cells show that there is between 2.1×10^9 and 2.7×10^9 daltons of DNA per cell. It is likely that the smaller number, 2.1×10^9 daltons, represents the molecular mass of a single, nonreplicating chromosome (2, 15).

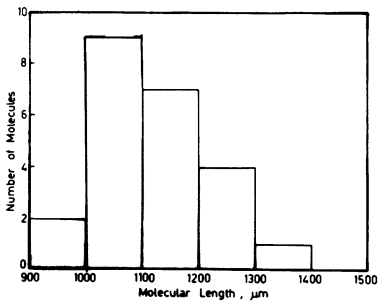

FIG. 3. *Histogram showing the distribution of lengths of DNA obtained from stationary-phase cells of P. aeruginosa. The DNA molecules were prepared by the method described in Materials and Methods; the spreading and shadowing was performed by the method of Kleinschmidt and Zahn (12).*

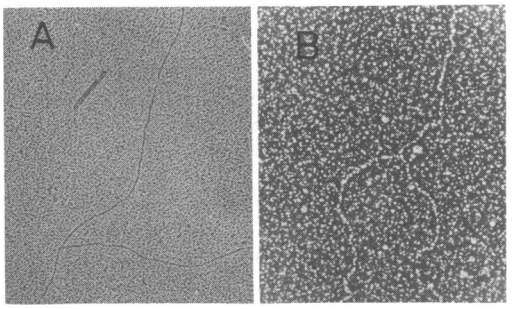

FIG. 4. *Electron micrographs of single forks in DNA molecules obtained from stationary-phase cells of P. aeruginosa. Each arm of the fork is the thickness of double-stranded DNA. Magnification: (A) ×5,250, (B) ×15,000.*

Size of individual DNA molecules. (i) Velocity sedimentation studies reveal that linear molecules as large as 2.2×10^9 daltons can be isolated from stationary-phase cells of this bacterium. (ii) Single, unbranched DNA molecules are large as 1,000 μm have been observed, photographed, and measured.

Since the amount of DNA per cell and the size of individual molecules obtained from such cells are approximately equal, it can be concluded that there is a single chromosome per cell.

Using renaturation techniques, Bak et al. (1) obtained figures of between 4.0×10^9 and 7.0×10^9 daltons for the size of the bacterial genomes of several *Pseudomonas* species. These data conflict with those obtained by myself, Park and De Ley (16), Gillis et al. (6), and De Ley et al. (5). Since the nature of this discrepancy remains unresolved, it must be pointed out that the data in this communication have been derived by a variety of techniques and are consistent with a genome size of 2.1×10^9 daltons.

It is useful to know the size of the genome of *P. aeruginosa* for several reasons. First, it is a measure of the average number of cistrons and the phenotypic potential of this organism. By comparison with the genome of *E. coli* (molecular mass 2.8×10^9 daltons) which contains approximately 3,000 cistrons, *P. aeruginosa*, which has a molecular mass of 2.1×10^9 daltons, should contain approximately 2,250

cistrons. Second, using the data provided in this and other papers (18; Miller et al., in press) an estimate can be made of the time taken to transfer the entire chromosome from the donor to the recipient cell. The generalized transducing phage F116 is known to transduce 1.5 to 2.0 min (in transfer time) of the chromosome (8, 18). Assuming that the transduced fragment is approximately the size of the viral genome (3.8×10^7 daltons; Miller et al., in press) then the chromosome, which has a molecular mass of 2.1×10^9 daltons, contains 55 F116 equivalents. Therefore, in terms of transfer time, the entire chromosome should be ($55 \times 1.5 - 2.0$) 80 to 110 min long. Further genetic analyses of this organism using the R factors (22) and FP sex factors (19) should provide a more precise measurement of the length of the chromosome in terms of transfer time.

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