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Size and Physical Map of the Chromosome of *Haemophilus influenzae*

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A variation of pulse-field electrophoresis, field-inversion gel electrophoresis, was used to determine the size and physical map of the chromosome of *Haemophilus influenzae*. The DNA of *H. influenzae* had a low G+C content (39%) and no restriction sites for the enzymes *Not*I or *Sfi*I. However, a number of restriction enzymes (*Sma*I, *Apa*I, *Nae*I, and *Sac*II) that recognized 6-base-pair sequences containing only G and C nucleotides were found to generate a reasonable number of DNA fragments that were separable in agarose gels by field-inversion gel electrophoresis. The sizes of the DNA fragments were calibrated with a lambda DNA ladder and lambda DNA restriction fragments. The sum of fragment sizes obtained with restriction digests yielded a value for the chromosome of 1,980 kilobase pairs. Hybridization of a labeled fragment with two or more fragments from a digest with a different restriction enzyme provided the information needed to construct a circular map of the *H. influenzae* chromosome.

Pulse-gel electrophoresis is now a well-established procedure for the analysis of large DNA fragments and has led to the physical mapping of the *Escherichia coli* chromosome (10, 11). It is apparent that this technique can be exploited to prepare physical maps of the chromosomes of bacteria and other organisms. Since the DNA content of *Haemophilus influenzae* is relatively low and only a partial genetic linkage map is available, field-inversion gel electrophoresis (FIGE) (4) was used to provide an accurate size and physical map of the *H. influenzae* chromosome. The strategy for mapping the *H. influenzae* chromosome involved cutting the chromosome into a relatively small number of fragments that could be separated by pulse-field gel electrophoresis. The map was constructed by hybridization of individually labeled restriction fragments, which were obtained with one restriction enzyme, to the array of fragments produced by other restriction enzymes. The hybridization of one labeled fragment with two or more fragments from a digest with another restriction enzyme provided the overlap needed to construct a map.

The size of the chromosome was determined by taking the sum of the sizes of individual fragments that were determined with a lambda ladder or lambda restriction fragments. These data yielded a reasonably precise value for the size of the wild-type *H. influenzae* chromosome of 1,980 kilobase pairs (kbp). Inasmuch as the size of the *H. influenzae* chromosome, or more precisely, the amount of DNA per viable cell, has been presented to have various values, we decided to reexamine the amount of DNA in *H. influenzae* cells. The first estimates of the amount of DNA per *H. influenzae* cell were made by Zamenhof et al. (15), who estimated it to be 1,800 kbp per cell. Berns and Thomas (2) obtained values of 1,900 to 2,400 kbp per cell and proposed that the amount of DNA per cell should be divided by two, the number of nucleoids per cell. Zoon and Scocca (16) reported DNA sizes corresponding to 4,200 to 5,060 kbp per cell. No matter how carefully the measurement of cells and DNA content were made, there was always a measure of uncertainty concerning the actual size of the chromosome. The advent of pulse-gel electrophoresis of large DNA fragments has provided the methodology to determine accurately the size and provide a complete circular map of the *H. influenzae* chromosome.

MATERIALS AND METHODS

Bacterial strains. *H. influenzae* V23 was a subculture from this laboratory, and Rd30 was a subculture of the strain Rd from the laboratory of A. Piekarowicz that was originally obtained from Alexander and Leidy (1). Strain RdA13N has been described previously (6a). Strain RdSCDGK carries five antibiotic resistance markers: those for streptomycin, cathomycin (novobiocin), dalacin (streptovaricin), gentamycin, and kanamycin. RdSCDGK was derived from Rd by successive transformations with the indicated markers.

Preparation of DNA in agarose beads. The preparation of DNA in agarose beads was compiled with some modifications from the method of Jackson and Cook (6) and Smith et al. (11). An overnight cell culture was diluted 1:50 into brain heart infusion broth supplemented with 10 μg of hemin per ml and 2 μg of NAD per ml and was grown to a density of about 2×10^9 cells per ml (optical density of 0.15 at 650 nm on a spectrophotometer [Coleman Junior]). Cultures were incubated at 37°C with rotatory shaking (G-77 shaker; New Brunswick Scientific Co., Inc., Edison, N.J.). The cells were centrifuged at 0°C at 5,000 rpm in a centrifuge (RC2B; Ivan Sorvall, Inc., Norwalk, Conn.) for 10 min, washed with P4 solution (1 M NaCl, 10 mM Tris hydrochloride [pH 8.0]), and suspended in one-fifth its original volume in P4 solution. The bacterial suspension was warmed to 42 to 45°C and mixed with an equal volume of 1% low-gelling, low-melting-temperature agarose (NuSieve; FMC Corp., Marine Colloids, Div., Rockland, Maine) that was prepared in P4 solution and prewarmed to 44°C. Paraffin oil prewarmed to 44°C was added to the cell-agarose mixture at a ratio of 2:1 and was thoroughly mixed for 15 s. The resulting emulsion was poured immediately, with stirring, into a 3× volume of P4 in an ice bath for 5 to 10 min. The paraffin oil was removed by centrifugation at 1,000 × *g* for 10 min, and the agarose bead pellet was suspended in a lysis buffer solution (1 M NaCl, 0.1 M EDTA, 10 mM Tris hydrochloride [pH 8.0], 0.5% Brij 58,

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0.2% deoxycholate, 0.5% Sarkosyl [CIBA-GEIGY Corp., Summit, N.J.], and 1 mg of lysozyme per ml). After a 60- to 120-min incubation at 37°C, the lysates were centrifuged at 1,000 × *g* and suspended in a solution of 0.25 M EDTA (pH 8.0), 1.0% Sarkosyl, and 100 μg of proteinase K per ml. The agarose bead suspension was incubated overnight at 50°C, centrifuged at 1,000 × *g*, and suspended in TE buffer (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride, incubated at room temperature for 1 to 2 h with gentle mixing, and washed five times with TE buffer. Agarose beads could be kept at 4°C in TE buffer for several weeks. To digest DNA the agarose beads were equilibrated with 1× restriction buffer before the addition of the restriction enzyme. The enzyme concentration necessary for chromosomal DNA digestion was similar to the concentration used for digestion of DNA in solution and represented a considerable savings in time and enzymes compared with those for gel plugs (11). In general, the agarose beads were treated with enzyme for 2 to 4 h, but on occasion, they were incubated overnight. The agarose beads were melted at 62°C for 5 to 10 min before they were loaded onto the gel.

FIGE. FIGE was performed in 1% agarose or 1.2% low-gelling, low-melting temperature agarose, usually for 16 h at 8°C and 220 to 240 V (80 to 100 mA) in a 0.5× solution of Tris borate and EDTA (pH 8.3) (8). Field inversion was supplied by a ramp (programmable power inverter; model PPI-100; MJ Research), which is a device that controls the time for the forward and reverse cycles and is capable of separating fragments of greater than 1 Mbp. For the most part two programs were used: program A was for separating fragments of up to 200 kbp and program B was for separating fragments above 200 kbp. Program A used for 0.03 to 9.0 s in the forward range and for 0.01 to 3.0 s in the reverse range for 300 cycles. Program B used 0.3 to 30 s in the forward range and 0.1 to 10 s in the reverse range for 100 cycles. Different zones of compression have been obtained with these programs (3, 6a), and it was sometimes necessary to extend the time of electrophoresis to obtain a good separation of fragments that had small size differences. Following electrophoresis the gel was stained with ethidium bromide and the DNA bands were visualized with a UV transilluminator. The DNA bands were measured for their distance from the origin and compared with DNA standards such as those of a lambda ladder (14). For labeling experiments or testing for biological activity, the DNA bands were removed from the gel by electroelution and concentrated by alcohol precipitation. If the DNA bands were to be used for transformation experiments, only a representative strip of the gel was stained with ethidium bromide to locate the position of the DNA fragments. In some experiments DNA electrophoresed in low-gelling, low-melting-temperature agarose was melted at 62°C, cooled to 40°C, and added directly to competent *H. influenzae* cells to measure the biological activity in the DNA.

DNA transfer and hybridization. To locate specific fragments of DNA with labeled probes, DNA was transferred from the agarose gel to a nylon membrane (Hybond-N; Amersham Corp., Arlington Heights, Ill.) by the Southern blotting technique (8). However, instead of fixing the DNA to the membrane by heating, the DNA was bonded to the membrane by irradiation at 254 nm for 4 to 5 min. The probes that were used for hybridization were labeled by nick translation (8) to a specific activity of approximately 10^7 cpm/μg of DNA. To reuse membranes for different probes, the previous probes were removed with 0.4 N NaOH by gently shaking at 45°C for 40 min. Subsequently, the membranes were neutralized for 40 min at 45°C with a solution that contained 0.2 M Tris hydrochloride (pH 7.5), 0.1% (wt/vol) sodium dodecyl sulfate, and 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Usually, all of the radioactivity was removed; however, on occasion, after extremely strong hybridization, residual faint hybridization bands were visible after a 20-h exposure to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) at −70°C. A second NaOH treatment for 20 min was sufficient to remove the remaining radioactivity completely.

Isolation of DNA for the diphenylamine assay. Cells were grown to an optical density of 0.2 to 1.2 measured at 650 nm in a spectrophometer (Coleman Junior). Samples of 10 ml were chilled on ice, centrifuged at 4°C for 8 min at 4,500 rpm in a centrifuge (RC2B; Sorvall), suspended in saline, recentrifuged, and resuspended in 1.0 ml of 0.5 N trichloroacetic acid. The cells were extracted in a boiling water bath for 15 min, cooled to room temperature, and centrifuged at 4°C for 15 min at 4500 rpm in a centrifuge (RC2B; Sorvall). The supernatant was carefully removed from the pellet and used directly in the diphenylamine assay (5).

RESULTS

DNA content per cell. The Rd strain of *H. influenzae* studied in our laboratory grew as long chains during the log phase of growth; and with increasing density it gradually converted to discrete cells in chains of four cells or more, and then to three cells, two cells, and ultimately a population of mostly single cells in the late lag phase. About 10% of the cells, even in the late lag phase, appeared as chains of two cells. Since the DNA content per viable cell obtained by a plating procedure might not be an accurate measure of the DNA content per cell, it was important to obtain a reasonably precise count of the number of individual cells in the culture. For this purpose liquid cultures of *H. influenzae* cells were grown and sampled for plating and counting in a bacterial counting chamber (Petroff-Hausser) to determine the number of cells as single cells, clusters of cells, and chains. The sum of the individual units provided a value for the total number of cells in the population. A minimum of 600 cells were counted for each sample, and the experiment was repeated. For each sample a viable count was obtained by diluting the cells in rich medium and plating the diluted cells in supplemented brain heart infusion agar. The DNA content was determined for each sample by the diphenylamine assay (5). The results of one experiment are shown in Table 1 and yielded a mean value of 1.3×10^9 daltons, or 1,950 kbp, for the DNA content per cell.

An examination of the data in Table 1 reveals several features about the growth of *H. influenzae* in culture. (i) Viable counts in log-phase and early-lag-phase cells provide an underestimate of the actual number of cells present as observed under a light microscope. (ii) Cell units joined together in clusters tended to separate on dilution and plating. (iii) For late growth, the number of cell units observed under the microscope and the viable counts were similar. (iv) The DNA content per cell for late growth was reasonably constant and, assuming no other source of error, reflected the maximum size of the *H. influenzae* chromosome. The main purpose for the chemical determination of the DNA content per *H. influenzae* cell was to provide a basis for comparison with the more precise procedure, namely, pulse-field gel electrophoresis of restriction fragments of whole chromosomes.

TABLE 1. Maximum size of the *H. influenzae* chromosome determined by cell count and the diphenylamine assay

OD_{650}[a]	Viable count (mol wt [10^9])	Singles and clusters (cell units [10^9])	Cells (cell units [10^9])	DNA concn (μg/ml)	Gram molecular size (colonies [10^9]) from:	
					Viable count	Cells
0.2	2.3	0.6	3.0	5.0	1.30	1.00
0.4	4.2	2.0	6.0	10.9	1.56	1.10
0.6	6.5	4.0	9.0	20.1	1.86	1.34
0.8	13.0	8.0	13.0	25.0	1.20	1.20
1.0	18.0	14.4	18.0	40.0	1.30	1.30
1.2	23.0	18.0	23.0	45.2	1.20	1.36

[a] OD_{650}, Optical density at 650 nm.

Size of the *H. influenzae* chromosome from restriction enzyme digests. The basic outline for producing restriction enzyme fragments to determine the size of the chromosome was as follows. (i) *H. influenzae* cells were grown in brain heart infusion medium to a density at which cells were still actively growing (2×10^9 to 5×10^9 viable cells per ml). (ii) The cells were immobilized in agarose beads. (iii) The beads were treated with lysozyme and EDTA to disrupt the cell walls and then with sodium dodecyl sulfate to disrupt the membranes. (iv) Proteinase K was added to digest the proteins. (v) Restriction enzymes were used to cut what were assumed to be intact chromosomes. (vi) The restriction enzymes were chosen so that they were likely to make relatively few cuts in the *H. influenzae* chromosome. Since *H. influenzae* has a low G+C content (39%), enzymes were chosen that cut sites that contained only G and C nucleotides. The 8-bp recognition enzymes *Not*I and *Sfi*I did not cut the *H. influenzae* chromosome (data not presented). The 6-bp recognition enzymes *Sma*I, *Apa*I, *Nae*I, and *Sac*II proved to be useful. *Nae*I and *Sac*II produced a number of small fragments that could be ignored without serious consequences, since all of the fragments from *Sma*I and all but the two smallest fragments of *Apa*I were easily identified. The resolution of fragments of DNA with a wide range of sizes was facilitated by the use of combinations of electrophoresis programs (see above). Figure 1 illustrates the results of a typical FIGE of *Apa*I and *Sma*I restriction digests of the *H. influenzae* Rd chromosome. The DNA was separated by FIGE program B to emphasize larger fragments (Fig. 1a), and the DNA was separated by FIGE program A for smaller fragments (Fig. 1b). The sizes corresponding to the positions of the fragments are given in Table 2 and represent average values from six or more experiments. The molecular size of the *H. influenzae* genome can be given unambiguously by adding the sizes of all fragments. The agreement between genome sizes determined separately from *Sma*I and *Apa*I digests was excellent and suggested that 1,980 kbp is the best estimate for the size of the *H. influenzae* Rd genome. Restriction enzyme digests of the DNA from a number of strains derived from the original Rd strain gave the same DNA banding patterns on FIGE.

Physical map of the Rd chromosome. A partial genetic map of the *H. influenzae* chromosome has been available for some time (9, 12); however, the length of the chromosome that was mapped was only a fraction of the length deduced from the DNA content of the lag-phase cell. It therefore appeared to be easier to use a physical approach to obtain a complete map of the *H. influenzae* chromosome. The approach used was to separate restriction enzyme fragments of the chromosome, label them with ^{32}P-labeled deoxynucleoside triphosphates by nick translation, and hybridize the individual labeled fragments to separate fragments of chromosomal DNA digests obtained with different restriction enzymes. A single restriction enzyme fragment should produce a single band of hybridization to DNA digests with the same restriction enzyme; but it may produce one, two, or more bands on hybridization with DNA fragments from another restriction enzyme digest. The presence of two or more bands of hybridization is evidence of overlap and was used to construct a physical map (see Fig. 4). The absence of more than one hybridization band from the hybridization of a restriction enzyme fragment to a set of fragments obtained with the same enzyme suggested that no major duplications were present in the genome to obscure the results of the procedure. Partial results from two experiments are shown in Fig. 2 and 3. The same restriction enzyme digests were run with two programs. Figure 2 illustrates the data obtained with FIGE program B, which was designed to separate the larger fragments of DNA; and Fig. 3 illustrates the data obtained with program A, which was designed to separate the smaller fragments. The smaller fragments were more widely separated in Fig. 3 than they were in Fig. 2 because of compression.

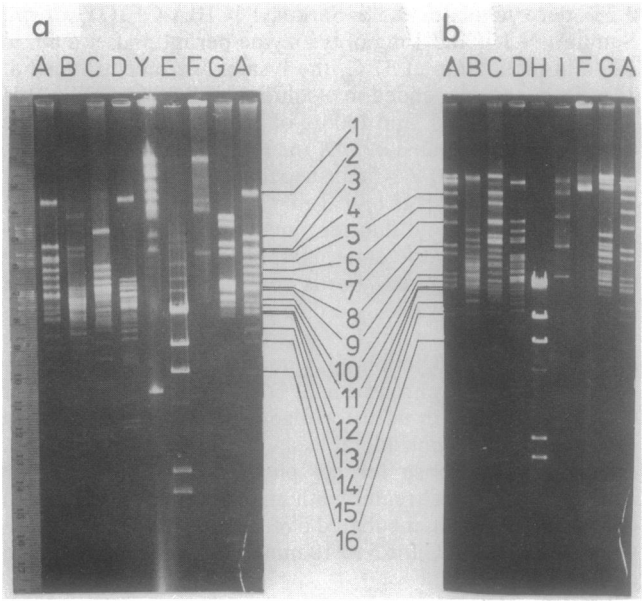

FIG. 1. Restriction enzyme digests of *H. influenzae* Rd DNA. Electrophoresis was done with FIGE programs B (a) and A (b). Lanes A, *Sma*I; lanes B, *Eag*I; lanes C, *Nae*I; lanes D, *Sac*II; lanes F, *Rsr*II; lanes G, *Apa*I; lane E, lambda ladder and lambda DNA digested with *Hin*dIII; lane H, lambda DNA digested with *Hin*dIII; lane I, lambda ladder; lane Y, *Saccharomyces cerevisiae* YPH294 chromosomes. The numbers 1 to 16 represent the different fragments of *Sma*I-digested Rd DNA.

It was not possible to obtain an unambiguous assignment for all fragments with only *Apa*I and *Sma*I digests. *Nae*I fragment 4 provided the overlaps necessary to connect *Sma*I-3 to *Sma*I-7 and *Apa*I-7 to *Apa*I-8 (Fig. 4). *Nae*I-7 aligned *Sma*I-14 to *Sma*I-2 and *Apa*I-1 to *Apa*I-3. It also confirmed the orientation of *Apa*I-9 and *Apa*I-1. The assignments of *Sma*I fragments 7, 8, and 9 (*Apa*I fragments 5, 8, 11, and 12) were derived by introducing *Not*I sites into the two phage S2 integration sites present in *Sma*I fragments 7 and 9 (*Apa*I fragments 11 and 12, respectively) (6a). A *Not*I

TABLE 2. *H. influenzae* DNA restriction fragments determined by FIGE

Fragment no.	Fragment size (kbp)		
	*Apa*I	*Nae*I	*Sma*I
1	330	300	385
2	310	200	280
3	260	180	230
4	230	135	220
5	115	130	200
6	100	120	170
7	98	115	140
8	86	110	105
9	83	110	90
10	70	94	50
11	57	78	35
12	51	70	25
13	45	66	23
14	45	54	14
15	29	38	10
16	29	36	6
17	21	28	
18	18	22	
19	2.7	20	
20	1.8	18	
21	1.6	16	
22		11	
23		8.6	
24		6.2	
25		6.0	
26		3.6	
27		3.4	
28		1.8	
Total	1,983.1	1,980.6	1,983

restriction digest of such a chromosome yielded a fragment of approximately 130 kbp that hybridized strongly to *Sma*I fragments 7, 8, and 12, and *Apa*I fragments 5 and 11 (Fig. 4). This completes the map except for the *Sma*I fragment 11 (*Apa*I-15), which was assigned, by elimination, to a position between *Sma*I-10 and *Sma*I-1 (*Apa*I-10 and *Apa*I-9). The large *Not*I fragment that represents the rest of the chromosome contained *Sma*I fragments 10, 11, and 1 and confirmed that the chromosome was circular.

Assignment of genetic markers to the physical map. Two procedures were used to assign genetic markers to specific restriction enzyme fragments of the *H. influenzae* chromosome. The most direct approach was to extract DNA fragments from the agarose gel after FIGE of chromosomal DNA and to transform competent cells. With this procedure the markers streptomycin, kanamycin, novobiotin, and dalacin resistance were assigned to *Sma*I-1 and naladixic acid resistance was assigned to *Sma*I-6. The markers were also mapped to the corresponding positions of an *Apa*I digest that, in effect, confirmed the order of these fragments in the map. A second approach was used for cloned fragments of *H. influenzae* DNA that could be labeled with ^{32}P-labeled deoxynucleoside triphosphates and hybridized to restriction fragments after FIGE and transfer to a nylon membrane. The results confirmed the assignment of markers by the transformation procedure and demonstrated the feasibility of mapping cloned fragments of *H. influenzae* by selective hybridization. The cloned fragment containing the dalacin resistance marker hybridized to *Sma*I-1; *Apa*I-1, the fragment containing the kanamycin resistance marker, also hybridized to these fragments; and a cloned fragment containing the phage S2 attachment site (6a, 13) hybridized to *Sma*I-7 (or *Apa*I-11) and *Sma*I-9 (or *Apa*I-12). Additional evidence for the presence of these two attachment sites in the *H. influenzae* chromosome will be published elsewhere.

DISCUSSION

One of the major conclusions from this study is the determination of a reasonable size of 1,980 kbp for the *H. influenzae* Rd chromosome. This value obtained by FIGE is not far from the 1,900 kbp obtained by other pulse-gel electrophoresis procedures (7). The precise value awaits the complete nucleotide sequence of the chromosome, which is now within technical reach. The major uncertainty for specifying the size of the chromosome is in the FIGE of large fragments of DNA. The mobility of fragments in FIGE or

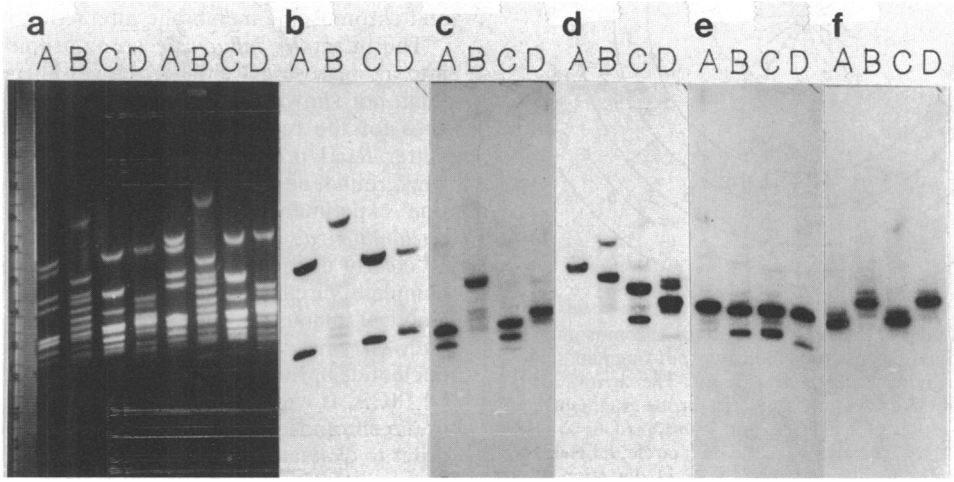

FIG. 2. An example of the banding pattern (a) and hybridization (b to f) with different DNAs used as probes after electrophoresis with program B. FIGE of restriction enzyme digests of Rd DNA was performed. Lanes A, *Apa*I; lanes B, *Sma*I; lanes C, *Apa*I + *Sma*I double digest; lanes D, *Nae*I. (A) Ethidium bromide-stained gel with a duplicate series of digestions. A total of five series were prepared and used for hybridization in panels b to f. The gels were transferred and hybridized with ^{32}P-labeled probes, as follows: *Sma*I fragment 1 (b), *Sac*II fragment 2 (c), *Apa*I fragment 4 (a), *Apa*I fragment 5 (e), and a 1.5-kbp fragment from plasmid pYS (f) (6), which was a portion of *H. influenzae* DNA *Sma*I fragment 7.

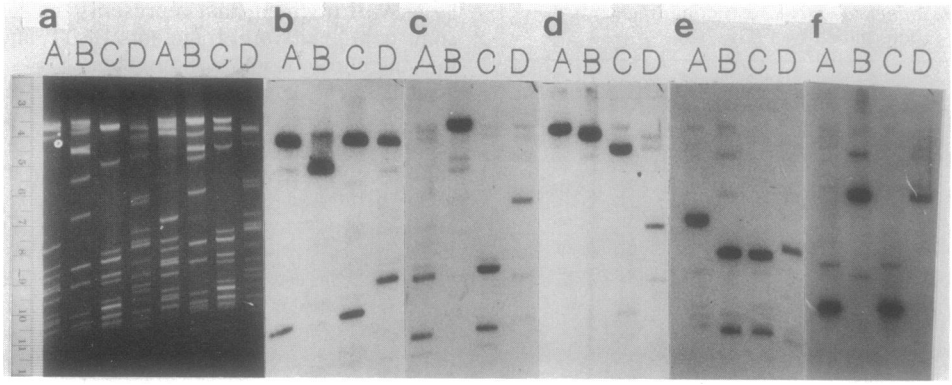

FIG. 3. An example of electrophoresis of the same DNA preparation used in Fig. 2 with FIGE program A. The rest of the legend is the same as that for Fig. 2.

pulse-field gel electrophoresis is not dependent simply on size alone, since variation in field strength can make a larger molecule move faster than a smaller one. Inasmuch as FIGE has not been checked against other pulse-field gel electrophoresis techniques for the mobility of chromosome fragments, a measure of caution is advised in the too rigid acceptance of a value of 1,980 kbp. However, the use of a lambda ladder and lambda restriction enzyme fragments as DNA standards provides some assurance that the values are reasonably accurate. A number of fragments were checked by double digestion, e.g., the *Sma*I fragment 1 cut with *Apa*I yielded two fragments, *Apa*I-1 and *Apa*I-9, that, when added together, give the correct size for *Sma*I-1.

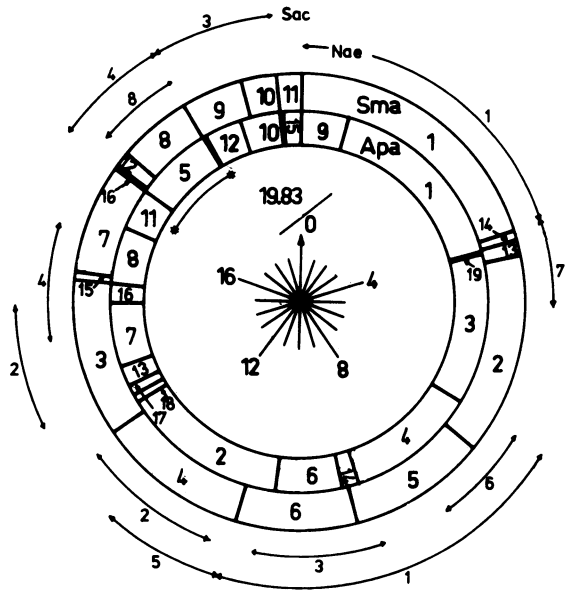

FIG. 4. Physical map of the *H. influenzae* Rd chromosome. The numbers on the complete inner circle indicate *Apa*I fragments; those on the outer circle indicate *Sma*I fragments. The arrows in the outermost circle, labeled Sac, represent the probe fragments prepared by *Sac*II digestion of Rd DNA and hybridized to *Sma*I or *Apa*I digests of Rd DNA. The arrows in the next circle, labeled Nae, represent the probe fragments prepared by *Nae*I digestion of Rd DNA and hybridized to *Sma*I or *Apa*I digests of Rd DNA. The solid line with asterisks at each end designates hybridization of the smaller *Not*I fragment from *Not*I digestion of strain RdA13N to *Sma*I or *Apa*I digests of Rd DNA. The sizes of the chromosome and its restriction enzyme fragments are depicted in the inner figure (in hundreds of kilobase pairs).

Previous chemical determinations of the amount of DNA per *H. influenzae* cell have provided values that varied considerably. Some of these values were as low as 1,200 kbp (2). To be sure that the size of the chromosome obtained from FIGE measurements was within the maximum DNA content per cell, the DNA content per *H. influenzae* cell was reinvestigated. To estimate the size of the chromosome from the chemical determination, it was necessary to assume that the DNA content per individual cell represented the DNA of a single chromosome. The DNA content determined per viable count represents a maximum value for the size of the chromosome and lends credence to the size of the chromosome determined by FIGE. Since the size of DNA fragments separated by FIGE were compared with the sizes of lambda ladders and lambda restriction enzyme fragments, the accuracy of the method was estimated from repetitive samples to be on the order of 5% or less and was certainly less than 10%. If the size of the chromosome determined by FIGE is 1,980 kbp, then most of the cells in the stationary phase contain single chromosomes. This statement should have some significance for the analysis of chromosome replication and chromosome membrane interaction in *H. influenzae*.

The intact *H. influenzae* chromosome does not migrate into an agarose gel either from gel plugs or agarose beads (data not shown), and it does not contain any recognition sites for the restriction enzymes *Not*I and *Sfi*I. The 7-bp cutter *Rsr*II produced four indistinct bands with a heavy background, and the fragments were not good for hybridization experiments. On the other hand, in *Haemophilus parainfluenzae* *Not*I and *Rsr*II produce, respectively, 7 and 10 clearly defined fragments that were used to construct a complete circular restriction map (L. Kauc and S. H. Goodgal, manuscript in preparation). Chromosomes treated with enzymes that recognized 6 bp containing G and C nucleotides produced fragments that were readily separated by FIGE. It would be useful to have restriction sites placed in the chromosome to generate specific cleavage patterns in order to evaluate the interaction of segments of the chromosome with elements of the cell membrane or cell wall. *Not*I or *Sfi*I sites can be generated by attaching *Not*I or *Sfi*I linkers to DNA homologous to DNA in the *H. influenzae* chromosome. Evidence for linking *Sma*I fragments 7, 8, and 9 in the genetic map was obtained by introducing *Not*I sites into *Sma*I fragments 7 and 9.

The map of the chromosome of *H. influenzae* Rd provides a standard for measuring variations in related *Haemophilus* strains and species with different serotypes and virulences. The availability of a standard physical map should provide the basis for manipulating genetic information and assaying the nature, extent, and rate of variability in these organisms.

The physical map that was generated was circular. The ability of a restriction enzyme fragment to hybridize to two or more fragments of a digest with another restriction enzyme provided the basis for constructing the map. In addition, the evidence that a restriction enzyme fragment hybridized only with itself when the same restriction enzyme was used emphasized that the method is not ambiguous and that there are no extensive duplications that interfere with the hybridization analysis.

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