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Physical Map of the Chromosome of *Neisseria gonorrhoeae* FA1090 with Locations of Genetic Markers, Including *opa* and *pil* Genes

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A physical map of the chromosome of *Neisseria gonorrhoeae* FA1090 has been constructed. Digestion of strain FA1090 DNA with *Nhe*I, *Spe*I, *Bgl*II, or *Pac*I resulted in a limited number of fragments that were resolved by contour-clamped homogeneous electric field electrophoresis. The estimated genome size was 2,219 kb. To construct the map, probes corresponding to single-copy chromosomal sequences were used in Southern blots of digested DNA separated on pulsed-field gels, to determine how the fragments from different digests overlapped. Some of the probes represented identified gonococcal genes, whereas others were anonymous cloned fragments of strain FA1090 DNA. By using this approach, a macrorestriction map of the strain FA1090 chromosome was assembled, and the locations of various genetic markers on the map were determined. Once the map was completed, the repeated gene families encoding Opa and pilin proteins were mapped. The 11 *opa* loci of strain FA1090 were distributed over approximately 60% of the chromosome. The *pil* loci were more clustered and were located in two regions separated by approximately one-fourth of the chromosome.

Neisseria gonorrhoeae, a gram-negative diplococcus, causes the sexually transmitted disease gonorrhea. The intensive effort devoted to studies of gonococcal molecular biology over the past several years has revealed a number of novel aspects to gene structure and regulation in this organism. However, relatively little is known about the organization of genes on the gonococcal chromosome. The only known mechanism of exchange of chromosomal markers is genetic transformation (3). Since only small DNA segments are exchanged via transformation, it has not been possible to use this process to construct a linkage map of the entire chromosome. There is a single linkage group containing several antibiotic resistance loci, as well as genes encoding outer membrane components and some auxotrophic markers, which is thought to constitute 2 to 3% of the chromosome (53). Outside this small region, the gonococcal chromosome is devoid of genetic landmarks.

One of the unusual features of the gonococcal genome is the occurrence of numerous reiterated genes and small sequence elements. Two families of surface proteins that demonstrate phase and antigenic variation of expression have been extensively characterized: the Opa proteins of the outer membrane and pilin proteins (26, 47). The genome contains information encoding different antigenic versions of Opa and pilin proteins, although the structural features of the stored genes differ in the two gene families. Opa proteins are encoded by a family of at least 11 intact structural genes, more than one of which can be expressed at one time (7, 8, 43, 50). For pilin genes, a large number of stored partial gene copies contain information for variant pilin species; changes in the expressed pilin protein occur when different stored information is recombined into an expression locus (15, 35, 48). Repeated sequence elements located outside coding sequences have also been described, although their function is not known (7, 9, 10, 14, 15, 34). With no genetic map, it has been impossible to ask questions about the organization of these sets of repeated genes and sequences on the chromosome.

The advent of pulsed-field electrophoresis techniques for the resolution of large DNA molecules has provided a new approach to analysis of bacterial genomes (33, 36, 37, 39). By using these techniques to separate restriction fragments generated by enzymes with rare recognition sequences, it is possible to construct macrorestriction maps of the chromosomes of bacterial species not previously amenable to genetic mapping. In this study, we have applied these physical mapping approaches to the construction of a macrorestriction map of the chromosome of gonococcal strain FA1090 and have begun to determine the location of single-copy and multicopy genes on the map.

MATERIALS AND METHODS

Bacterial strains. *N. gonorrhoeae* FA1090, a serum-resistant proline-requiring strain isolated from a patient with disseminated gonococcal infection, has been described previously (7, 8). Gonococci were grown in GCB broth or on GCB agar plates (Difco Laboratories) with the supplements of Kellogg et al. (18) at 37°C in a 5% CO_2 atmosphere. *Escherichia coli* strains were grown on LB agar (32).

DNA preparation. Broth cultures of strain FA1090 were grown to mid-log phase; cells were pelleted and resuspended directly in molten (37°C), low-melting-temperature agarose (Incert agarose; FMC Corp.), which was allowed to solidify in small blocks. All subsequent processing of the samples was carried out in the agarose blocks, which were treated with lysozyme, RNase, and proteinase K by procedures described by Smith et al. (39). Blocks were washed in TE buffer (39) at 37°C and subsequently stored in TE buffer at 4°C.

Restriction digestions. Blocks were digested with 5 U of *Bgl*II (5′ AGATCT 3′), *Nhe*I (5′ GCTAGC 3′), or *Spe*I (5′ ACTAGT 3′) (Bethesda Research Laboratories [BRL]) in 120 μl of KGB (52) or with 5 U of *Pac*I (5′ TTAATTAA 3′) (New England BioLabs) in NEBuffer 1 at 37°C for 5 h.

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Digested blocks were melted at 65°C, loaded into a 1% agarose gel (15 by 15 cm) containing either individual wells or one large well extending the width of the gel, allowed to solidify, and subjected to pulsed-field gel electrophoresis.

Electrophoresis. Pulsed-field gel electrophoresis was performed in a contour-clamped homogeneous electric field (CHEF) apparatus with a hexagonal electrode array (6). Electrophoresis was at 90 to 115 V and 12 to 15°C in 0.5× TBE buffer (32) for 40 to 72 h, with pulse times ranging from 2 to 120 s. For pulse times between 5 and 20 s, we used a modified power supply that gave better band separation. This power supply consisted of a 120-V AC input passed through an isolation transformer to a bridge rectifier circuit. No capacitors were used to smooth the output from the rectifier circuit. Hence, the output consisted of a DC sine wave. Voltage was controlled with a stepdown transformer and was set at 100 V, using a volt meter. For pulse times of >20 s, a conventional power supply (EC500; E-C Corp.) gave better resolution.

Most of the restriction fragments could be separated by using two pulse times: 12 s for fragments 20 to 200 kb in length, and 60 s for fragments 250 to 450 kb in length. Additional pulse times were used to obtain maximal separation of particular restriction fragments.

Conventional gels (1% agarose, 1× TAE [32]) were used for visualizing fragments smaller than 10 kb, using cesium chloride-banded chromosomal DNA (43).

DNA transfer and hybridization. After electrophoresis, gels were stained with 1 μg of ethidium bromide per ml, photographed, and exposed to UV irradiation at 254 nm on a Foto UV300 DNA Transilluminator (Fotodyne) for 10 min prior to alkali denaturation and neutralization (38). Gels were transferred with 10× SSC (32) to Optibind or BA-S 85 (Schleicher & Schuell) filters, using a Trans-Vac TE80 vacuum blotter (Hoefer). DNA was cross-linked to the filter with a UV Stratalinker 1800 (Stratagene).

Filters from gels with one continuous sample well were cut into narrow strips. One strip from each gel was hybridized at room temperature with *Sau*3A-digested FA1090 chromosomal DNA labelled with ^{32}P by random priming to mark all of the bands. The autoradiograph of this strip was used as a reference to identify bands on autoradiographs of the remaining strips from that gel after hybridization with individual probes. If there was any uncertainty in identifying a probe-hybridizing band on a strip by comparison to the reference strip, the questionable strip was overprobed with ^{32}P-labelled FA1090 DNA and the two autoradiographs were superimposed and compared.

Oligonucleotide probes were synthesized and purified as described previously (7) and were end-labelled with ^{32}P (32). Oligonucleotides Ngoiga (5′ CGGGGCCGGCTTGACTGGG CGGCCG 3′), Rmp (5′ CGCCGCATTCTACGCGACCTTG GC 3′), and GyrB (5′ CCGGCGGCCTGCACGGCGTGGGC 3′) were derived from the published sequences of the *iga* (30), *rmp* (14), and *gyrB* (41) genes, respectively. The DNA sequences of the remaining oligonucleotides are given in the references cited below. Hybridization and washing temperatures for end-labelled probes varied for different oligonucleotides: 40°C for probe 06 (1); 45°C for probes NC-1 (4), BP3 (2), and RH2 (13); 55°C for probes Az-2 (55), PM007 (13), Rmp, and Ngoiga; 60°C for probe GyrB (41). The sequences and hybridization temperatures for the probes for hypervariable regions of *opa* genes were as described before (8). Hybridizations were in a solution of 4× SSPE, 2× Denhardt's solution (32), 20 mM $NaPP_i$, and 0.2% sodium dodecyl sulfate (SDS). Filters were washed twice for 45 min each in a solution of 1× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5 mM $NaPP_i$, and 0.1% SDS and once for 20 min in a solution of 0.5× SSC, 5 mM $NaPP_i$, and 0.1% SDS and then were autoradiographed.

DNA fragments were labelled with ^{32}P by random priming, using an oligo-labeling kit (Pharmacia). These probes were hybridized at 60°C in a solution of 6× SSC, 0.5% SDS, and 5× Denhardt's solution. Filters were washed once for 5 min in a solution of 2× SSC and 0.5% SDS at room temperature, once for 15 min in a solution of 2× SSC and 0.1% SDS at room temperature, and once for 2 h in a solution of 0.1× SSC and 0.5% SDS at 60°C and then were autoradiographed.

Densitometry. Photographic negatives from stained gels were scanned with an LKB Bromma Ultrascan XL Laser Densitometer. Standard curves were plotted for the size markers (fragment size versus distance migrated), and fragments of unknown size were compared with the standard curve. Molecular weight standards were lambda DNA concatemers (5), *Hin*dIII-digested lambda DNA (BRL), and high-molecular-weight markers (BRL). Only the linear portion of each standard curve was used to determine fragment size.

λgt11 cloning. Restriction fragments were eluted from pulsed-field electrophoresis gels, digested with *Eco*RI (BRL), cloned into λgt11 (57), and packaged into phage particles (Packagene; Promega) that were used to infect *E. coli* Y1090 (Promega). Clear plaques were punched from the agar plate with a Pasteur pipet, and the DNA was eluted in 100 μl of water. Five microliters of eluate was subjected to the polymerase chain reaction (31), using amplimers homologous to the λgt11 sequence: λ forward (5′ GGTGGCGACG ACTCCTGGAGCCCG 3′) and λ reverse (3′ AACCAGACC ACAGTT 5′). The polymerase chain reaction was performed in a TempCycler (Coy), using *Taq*I polymerase (Promega), for 35 cycles with a denaturing temperature of 94°C (1 min), an annealing temperature of 60°C (1 min), and an extension temperature of 72°C (3 min). The polymerase chain reaction products were then radiolabelled by random priming and used to probe Southern blots.

Linking clones. To isolate *Spe*I linking clones, we constructed a library of *Sau*3A-digested FA1090 DNA in pBR322 (32). The library was then digested with *Spe*I and ligated with a kanamycin resistance cassette isolated from pUC4-KIXX (Pharmacia) to which *Spe*I linkers (5′ CACT AGTG 3′) had been added. Clones growing on plates containing kanamycin were those that were linearized by the *Spe*I digestion and ligated to the kanamycin cassette. Before these clones were used as probes, they were digested with *Spe*I to remove the inserted kanamycin cassette and recircularized.

RESULTS

Size of the gonococcal chromosome. The G+C content of gonococcal DNA is 50% (51). To identify restriction enzymes appropriate for analysis of the chromosome of strain FA1090, we screened enzymes predicted to cleave the chromosome into a limited number of fragments. Enzymes *Nhe*I, *Spe*I, and *Xba*I recognize 6-bp sequences containing the tetranucleotide CTAG, which occurs rarely in many bacterial genomes (24). *Nhe*I and *Spe*I cleaved FA1090 DNA into a limited number of fragments that were resolved by electrophoresis in a CHEF apparatus (Fig. 1, Table 1). Digestion with *Xba*I generated several large fragments plus too many smaller fragments for convenient analysis. *Not*I,

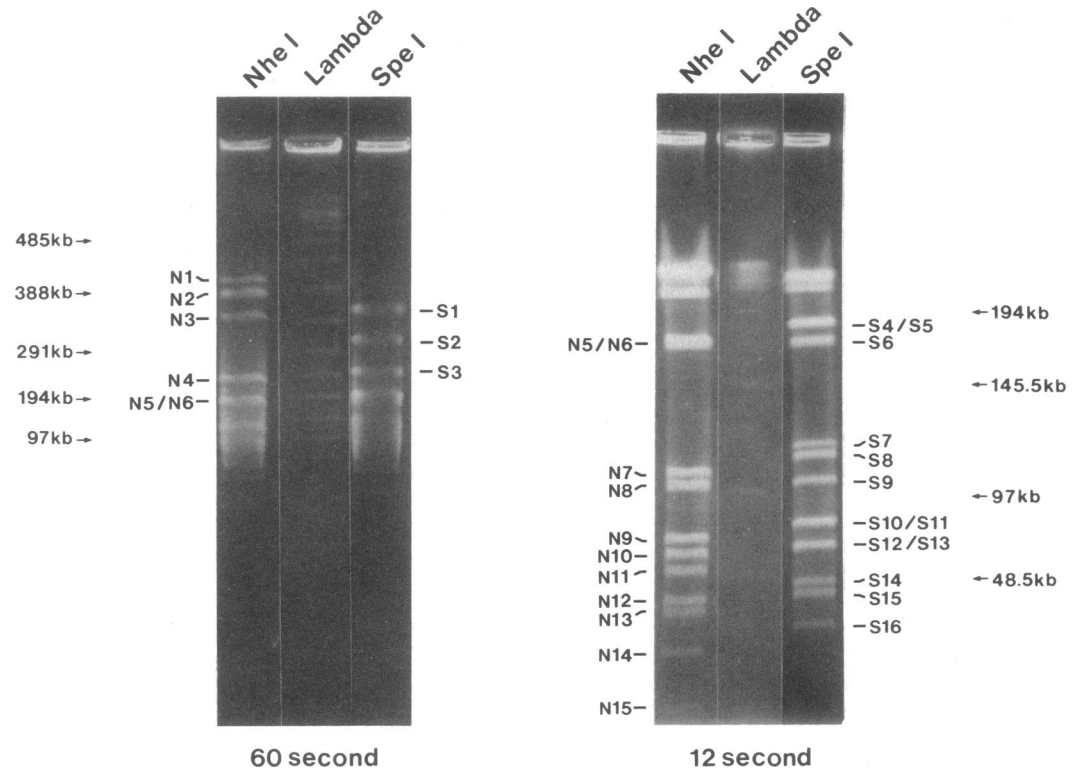

FIG. 1. CHEF electrophoresis of *Nhe*I and *Spe*I digests of strain FA1090 DNA. DNA in agarose blocks was digested with *Nhe*I or *Spe*I and subjected to CHEF electrophoresis at a pulse time of 60 or 12 s. Each panel contains an *Nhe*I digest (left lane), a *Spe*I digest (right lane), and lambda concatemer size standards (center lane) from a single gel. The sizes of the lambda concatemers are indicated at the far left and far right.

*Sfi*I, and *Pac*I, all of which recognize 8-bp sequences, have been useful for physical mapping studies in other organisms. Unfortunately, neither *Not*I nor *Sfi*I digested FA1090 DNA, presumably due to the extensive methylation of gonococcal DNA (11). However, *Pac*I did cleave the DNA into relatively few fragments (Table 1). A fourth enzyme useful for analysis of FA1090 DNA was *Bgl*II, which recognizes a 6-bp sequence. The sum of the fragment sizes from the four digests shown in Table 1 ranged from 2,188 to 2,260 kb, resulting in an estimated genome size of 2,219 kb.

Most gonococcal strains, including FA1090, contain a 4.2-kb cryptic plasmid with two *Nhe*I sites 280 bp apart and no *Spe*I, *Bgl*II, or *Pac*I sites (21). To be certain that none of the presumed chromosomal fragments represented the cryptic plasmid, we used purified plasmid DNA to probe Southern blots of pulsed-field gels. One small *Nhe*I fragment was actually the linearized cryptic plasmid. There was no hybridization to any fragments on blots of *Spe*I-digested FA1090 DNA, presumably because the circular plasmid species ran off the ends of the gels under the electrophoresis conditions used.

Construction of a macrorestriction map. An approach that has been valuable in other studies is to isolate restriction fragments from one digest and use them as probes on Southern blots of fragments resulting from digestion with a second enzyme (36). However, even the smallest restriction fragments we isolated from the pulsed-field gels hybridized to multiple bands, probably due to the many repeated sequence elements present in the gonococcal genome or to contamination of the probe with DNA from other fragments. As an alternative strategy, we used probes corresponding to single-copy chromosomal sequences in Southern blots of digested DNA separated on pulsed-field gels. By determining how the fragments from different digests overlap, they can be ordered into a macrorestriction map of the chromosome.

We probed Southern blots of various digests of FA1090 DNA with a collection of cloned gonococcal genes obtained from other investigators or constructed in our previous studies. For genes that have been sequenced, we made synthetic oligonucleotide probes. All of the clones and oligonucleotides were used to probe *Nhe*I and *Spe*I digests; some were also used on *Pac*I and *Bgl*II digests. CHEF gels with large sample wells extending the width of the gel were transferred to filters, which were cut into narrow strips for hybridization with the different probes. One strip from each gel was probed with radiolabelled FA1090 DNA to allow precise alignment and identification of the hybridizing fragments, as illustrated for several probes hybridizing to large *Bgl*II fragments in Fig. 2. The probes, and the fragments to which they hybridized in different digests, are shown in Table 2.

The available set of probes for specific gonococcal genes was not large enough to allow construction of the complete macrorestriction map. To generate additional probes, restriction fragments were eluted from pulsed-field gels and digested with *Eco*RI and then cloned into λgt11. Although some of the resulting clones contained repeated sequences and hybridized to multiple *Spe*I or *Nhe*I fragments, many hybridized to a single fragment in each digest. The size of the inserts in these clones ranged from approximately 0.1 to 3 kb. The lambda clones and the fragments to which they

TABLE 1. Fragment sizes after digestion of FA1090 DNA with *Bgl*II, *Nhe*I, *Pac*I, or *Spe*I[a]

Fragment	Size (kb) after digestion with:			
	*Bgl*II	*Nhe*I	*Pac*I	*Spe*I
1	398	413	477	367
2	277	392	299	310
3	213	353	221	243
4	182	233	197	182*
5	159	180*	176	182*
6	130	180*	142	167
7	102*	105	125*	118
8	102*	99	125*	112
9	75	71	92	102
10	70	62	70[b]	80*
11	65*	52	59	80*
12	65*	36	42	67*
13	60	30	37	67*
14	50	25	31	44
15	47	18	26	38
16	30	11	24*	24
17	24*		24*	5
18	24*		20	
19	21		16	
20	18		15	
21	16		12	
22	13			
23	10			
24	10			
25	7			
26	7			
27	6			
28	6			
29	6			
30	4			
Total (kb)	2,197	2,260	2,230	2,188

[a] Each value is the average of two to six independent determinations on different gels, except for *Pac*I fragments 12 to 21, *Spe*I fragment 6, and *Bgl*II fragments 5 and 30, for which a single gel was analyzed. The *Nhe*I fragment representing the cryptic plasmid is not included in this list. Fragments marked with an asterisk (*) were identified as doublets by densitometric scanning of negatives of photographs of ethidium bromide-stained gels or by Southern blot analysis of single or double digests. We have not identified electrophoresis conditions that separate these pairs of fragments.

[b] Results of densitometric scanning for this fragment were ambiguous, and it may represent an unresolved doublet.

hybridized are listed in Table 3. On the basis of insert size and lack of cross-hybridization among the different clones, we believe that the listed lambda clones represent distinct chromosomal sites, with few if any duplicates among them.

Analysis of the hybridization of this collection of probes to *Nhe*I and *Spe*I digests of FA1090 DNA allowed us to identify contiguous fragments. For example, several probes showed that fragments S2 and N3 overlap; N3 and S12 were recognized by another set of probes. Thus, fragments S2 and S12 must be adjacent or near each other on the chromosome. Such analysis enabled us to "walk" back and forth between *Nhe*I and *Spe*I fragments, eventually assembling the fragments into three large contigs. To establish linkages among the contigs, results from *Bgl*II and *Pac*I digests were used. For example, one contig had fragments N1 and S14 at one end; a second contig ended with N2 and S3. None of the probes hybridized to N1 and S3 or to N2 and S14. However, probes specific for N1/S14 and for N2/S3 hybridized to fragment B2, thus demonstrating that the two contigs were adjacent. We did not attempt to make complete restriction maps for the *Bgl*II or *Pac*I fragments, since we did not have probes hybridizing to all of the small fragments in those digests. In addition to linking the three *Nhe*I-*Spe*I contigs, the data on hybridization to *Bgl*II and *Pac*I fragments were used to verify the arrangement of *Nhe*I and *Spe*I fragments within the contigs.

Additional information came from using some of the probes in Southern blot analysis of *Nhe*I-*Spe*I double digests to determine whether particular *Nhe*I or *Spe*I fragments contained sites for the other enzyme. Fragment S2 contained an *Nhe*I site, and fragments N5, N7, and N8 each contained a *Spe*I site. The migration of a number of fragments (S5, S7, S9, S10, S11, S15, S16, N6, N9, N11, N12, N13, N15, and N16) was not detectably altered when Southern blots of single and double digests probed with appropriate clones were compared. However, this analysis would probably not reveal the existence of sites located very close to the ends of restriction fragments.

Several bands on the CHEF gels of different digests contained two unresolved fragments. In some cases, densitometric analysis showed the existence of these doublets. For three of the bands (S12/S13, N5/N6, and B7/B8), the doublet was not apparent by densitometry, but was revealed by the pattern of hybridization of various probes to single or double digests. For example, the N5/N6 band was recognized by one set of probes hybridizing to S7 or S2 and to P1, as well as by a second set of probes hybridizing to S1 and P8. Since one fragment cannot be located at two different positions on the map, we concluded that the band contained two unresolved fragments. The list of fragments from the different digests in Table 1 represents the final corrected numbers, after both densitometric and Southern blot analyses.

Linking clones are those containing a site for the enzyme being used in mapping (36, 39). We had hoped to use linking clones in the construction of the map and made a *Spe*I linking clone library. A linking clone with a *Spe*I site should hybridize to two fragments in a *Spe*I digest, showing that those fragments are adjacent in the chromosome. Unfortunately, the majority of the linking clones gave complex multiband hybridization patterns when used to probe *Spe*I and *Nhe*I digests, due to the presence of repetitive sequence elements. However, one of the linking clones (LC#5) was useful in assigning a tentative position for fragment S17, for which there were no other probes. LC#5 hybridized to S17, S6, S2, N4, N3, B1, B8, P2, and an additional small *Pac*I fragment. When analyzed in light of the known overlaps among these fragments, the data were consistent with the presence of two chromosomal copies of sequences contained in the clone. Fragment S17 could be adjacent to S6 or S2. Since the hybridization signals were more intense with S6 and the fragments overlapping it (N4, P2, and B1), we tentatively assigned S17 to the position adjacent to S6, although additional data in the future may necessitate moving it to the position next to S2.

The macrorestriction map constructed from these data is shown in Fig. 3. All of the *Spe*I and *Nhe*I fragments are included, as well as the larger *Pac*I and *Bgl*II fragments. Over 75 probes were analyzed; all but one gave hybridization results with *Spe*I and *Nhe*I digests, and with *Pac*I and *Bgl*II digests when applicable, that were consistent with this map. One of the anonymous lambda clones gave results that were inconsistent with the placement of one fragment on the map; we believe that those results were a consequence of an artifact or rearrangement occurring during cloning, and we did not include that clone in the analysis. The alignment of the *Spe*I and *Nhe*I maps is based on the double digest experiments, which showed whether a fragment from one

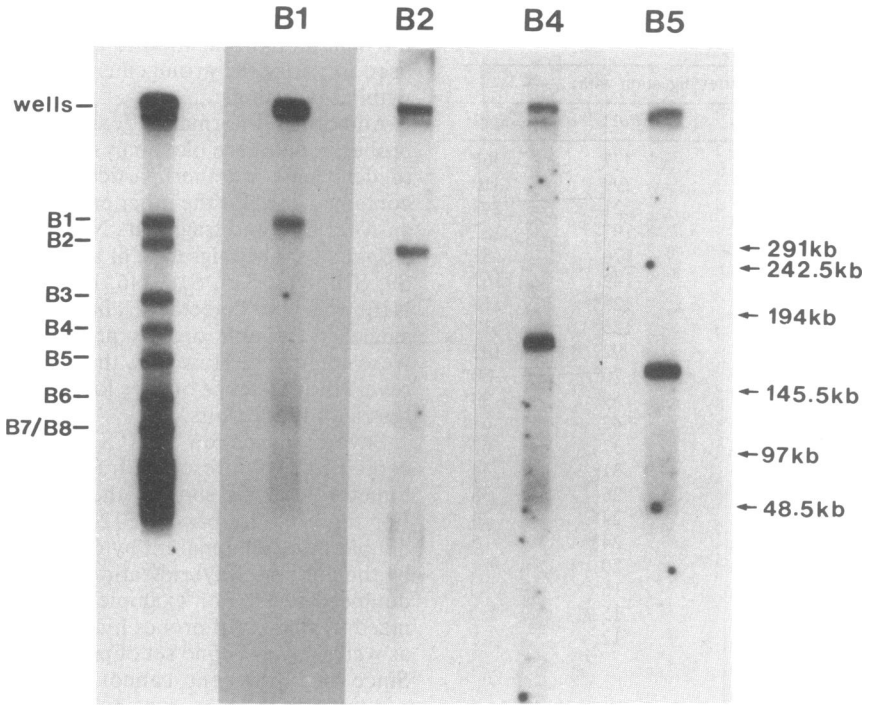

FIG. 2. Southern blots of *Bgl*II-digested FA1090 DNA. A *Bgl*II digest of FA1090 DNA was electrophoresed on a CHEF gel with a single long sample well, with a pulse time of 20 s. DNA fragments were transferred to a filter, which was cut into narrow strips. Individual strips from the same filter were probed with radiolabelled FA1090 DNA (leftmost panel) or with probes hybridizing to single *Bgl*II fragments. The probes were as follows: for B1, λ504; for B2, λ6; for B4, pTME2; for B5, pLV155. Some DNA remained in the sample well, which facilitated alignment of the autoradiographs of different strips. The positions of lambda concatemer size standards are indicated at the far right. At this pulse time, fragments in the 97- to 194-kb size range showed a linear relationship between size and distance migrated. Larger or smaller fragments were compressed together and did not show the same linear migration.

digest was completely contained within a fragment from the other digest. For example, fragments N7 and N8 each contained an *Spe*I site; therefore, fragment S1 (367 kb) must be completely contained within the area defined by N7 + N6 + N8 (396 kb) and could shift only approximately 15 kb in either direction from its current position on the map.

There were four places on the map where we could not determine the relative order of two or three small *Spe*I or *Nhe*I fragments from analysis of the four digests. For example, S11 and S15, contained within large *Nhe*I, *Pac*I, and *Bgl*II fragments, could not be ordered relative to each other. However, we were able to use information from Southern blots of *Xba*I digests to determine their order. As mentioned previously, digestion of FA1090 DNA with *Xba*I resulted in a few large fragments plus a large number of small fragments. Probes recognizing fragments S15 (λ504, λ734) and S6 (λ356, λ515, *pilE*, *pilS6*) all hybridized to the largest *Xba*I fragment (X1), whereas a probe hybridizing to fragment S11 (HV_1-5; described in next section) did not recognize X1, indicating that S15 was closer to S6. Similarly, the relative order of fragments S10 and S13 was determined by analysis of *Xba*I and *Xba*I/*Nhe*I digests. Probes specific for the *recA* gene on fragment S7 and the M.NgoBIII gene on S10 hybridized to the same *Xba*I fragment, which contained an *Nhe*I site within it. The other probes specific for S10 (pTME2 and sc#5) each hybridized to a different small *Xba*I fragment, as did the probe (λ712) specific for fragment S13. Thus, even though we did not identify and enumerate all of the *Xba*I fragments, we could conclude that fragment S10 was adjacent to fragment S7.

There are some limitations to the resolution of the map. We were unable to determine the relative order of two or three small *Nhe*I fragments at two places, even with analysis of *Xba*I digests in addition to *Spe*I, *Pac*I, and *Bgl*II digests. Also, since the *Bgl*II and *Pac*I maps are incomplete, the positions of some of those fragments could shift to one side or the other, within the constraints of the identified overlaps with *Spe*I and *Nhe*I fragments. Finally, the chromosomal region recognized by each probe can be localized to the area of overlap between particular fragments, but the relative order of markers within each of those overlap regions cannot be determined at this time.

Map location of *opa* and *pil* loci. The DNA sequences of *opa* genes, either from one strain or from different gonococcal strains, are similar (7, 8, 29, 43, 50). Each of the genes contains a repeated sequence element (CTCTT) in the region encoding the signal peptide of the protein, and there is near-perfect identity in DNA sequence over approximately 80% of the length of the coding sequence for mature Opa protein. Within that conserved framework, two short hypervariable regions, designated HV_1 and HV_2, differ in length and in DNA sequence in different *opa* gene copies. We previously characterized the *opa* loci of the same variant of strain FA1090 used in this study (7, 8). There are 11 identified *opa* loci, having six versions each of HV_1 and HV_2 among them. Nine of the loci contain genes with unique combinations of HV_1 and HV_2; two loci contain duplications of other *opa* genes (Table 4). We used oligonucleotides specific for the different versions of HV_1 and HV_2 as probes in Southern blots of CHEF gels of FA1090 DNA digested

TABLE 2. Cloned gene probes and fragments to which they hybridized

Probe	Genotype and/or phenotype[a]	Reference/source	Hybridizing fragments			
			*Nhe*I	*Spe*I	*Bgl*II	*Pac*I
pTME4	61K and 58K OMPs	V. Clark	N2	S3	B4	P1
pTME2	29K OMP	V. Clark	N2	S10	B4	P1
pMMB3	M.NgoBIII methylase	D. Stein	N2	S10	B4	P1
pVD308	*recA*	20	N5	S7	<B9[b]	P1
pLES8	*proAB*; proline biosynthesis	42	N5	S2	B7	ND[c]
pCBB49.1	M.R.NgoMI methylase and restriction enzyme	H. Seifert & D. Stein	N5	S2	ND	P1
pFLOB400	OMP with H.8 epitope (from *N. meningitidis*)	54	N3	S2	ND	P1
pFLOB861	*lip*; OM lipoprotein with H.8 epitope	56	N3	S2	ND	ND
Az-2[d]	*laz*; OM lipid-modified azurin with H.8 epitope	55	N3	S2	ND	P1
pRGE172	*argE*; ornithine acetyltransferase	M. Mulks	N3	S12	<B9	P6
pRGF1	*argF*: ornithine transcarbamoylase	23	N3	S4	<B9	P6
pNMBT5701	Dhp; dihydropteroate synthase (from *N. meningitidis*)	22	N12	S4	B6	P4
pBS134	*penA*; penicillin-binding protein 2	40	N6	S1	ND	P8
RMP[d]	*rmp*; reduction-modifiable OMP	14	N6	S1	ND	P8
pGln10	Gln; glutamine synthetase	H. Seifert	N6	S1	ND	P8
pUNCH401	*tbp-1*; 100K transferrin binding protein	C. Cornellisen & P. F. Sparling	N6	S1	ND	ND
pUNCH115	*trf-1*; affects transferrin receptor function	G. Biswas & P. F. Sparling	N6	S1	<B9	<P10[b]
pBS8	M.NgoMIII methylase	D. Stein	N9	S8	B5	P5
pLV155	M. NgoPII methylase	46	N9	S8	B5	P5
NC-1[d]	*por*; OM porin	4	N9	S8	B5	ND
GyrB[d]	*gyrB*; gyrase B subunit	41	N9	S8	B5	ND
pWM3	*sac-4*; serum resistance	25	N9	S8	B5	P5
pABJ01	*pilC*; pilus assembly (two chromosomal copies)	S. Normark & A.-B. Jonsson	N1	S9	ND	P3
			N4	S11	ND	P2
pLV111	M.NgoPI methylase	45	N4	S6	B1	P2
MN32-IC	tyr-tRNAS; tyrosyl tRNA synthetase	B. Spratt	N1	S16	<B10	P3
pTME5	40K OMP	V. Clark	N1	S5	B3	P3
pTME6	*lps*-1; LPS antigen	28	N1	S5	B3	P3
S1/pUC19	*omc*; OMP-MC	49	N1	S5	B3	P3
pWC147	*cnp*; cryptic neisseria protein (two adjacent copies in strain MS11)	H. Seifert	N1	S5	B3	P9
BP3[d]	*fbp*; 37K iron-repressible OMP	2	N1	S5	B3	P9
Ngoiga[d]	*iga*; IgA protease	30	N1	S14	ND	P9
pTME1	*oxiA*; anaerobically repressed OMP	11	N2	S3	B2	P7
pE63	M.NgoDI methylase	D. Stein	N2	S3	ND	ND
pNG1102[e]	*pilE*; hybridizes to pilin expression and storage loci	27	N10*, N4, N6	S6*, S1		
06[d]	*pilE*; pilin expression locus	1	N10	S6	B1	P2
RH2[d,e]	*pilE* + *pilS*; hybridizes to pilin expression and storage loci	13	N10*, N4, N6	S6*, S1	P2*	P8
pM007[d]	*pilS6*; pilin storage locus	13	N10	S6	ND	ND

[a] OMP, outer membrane protein; OM, outer membrane; LPS, lipopolysaccharide; IgA, immunoglobulin A.
[b] The hybridizing fragment was smaller than B9 or P10, but the identity of the fragment was not determined.
[c] ND, not done.
[d] Oligonucleotide probe.
[e] For plasmid pNG1102 and oligonucleotide RH2 (both specific for all *pil* loci), the hybridization signal with fragments marked with an asterisk (*) was stronger than with the remaining fragments.

with *Nhe*I, *Spe*I, or *Pac*I (Table 5). Some of the oligonucleotides hybridized to multiple fragments in a digest, since there are multiple copies of some of the hypervariable regions among the *opa* genes. However, knowing the combination of HV_1 and HV_2 present in each *opa* locus made it possible to match the different hybridizing fragments with the corresponding locus. For example, HV_1-2 is present in two loci: in *opa*A it occurs in combination with HV_2-2; and in *opa*J, with HV_2-4. The HV_1-2 and HV_2-2 probes both hybridized to N1, S16, and P3, showing the location of *opaA*. The remaining fragments recognized by the HV_1-2 probe (N3, S2, and P10) were also recognized by the HV_2-4 probe, showing the location of *opaJ*. Similar analysis allowed placement of all 11 *opa* loci on the map (Fig. 4). The *opa* loci are distributed throughout approximately 60% of the chromosome. There are three locations in which two *opa* loci map to a particular area of fragment overlap, although we do not know how closely linked they are within each of those regions. With one exception (*opaC* and *opaJ*), genes sharing the same versions of HV_1 and/or HV_2 are located in different regions of the chromosome.

The *pil* gene family contains two different types of genes (26, 47). A *pilE* locus contains a complete pilin gene and its promoter. Information encoding alternative antigenic vari-

TABLE 3. Anonymous clones and fragments to which they hybridized

Clone	Hybridizing fragments			
	*Nhe*I	*Spe*I	*Bgl*II	*Pac*I
λ580, λ588, λ608, λ714[a]	N2	S3	B4	P1
sc#5[b]	N2	S10	B4	P1
λ712	N2	S13	B4	P1
λ703	N5	S7	B7	P1
λ609	N5	S2	B7	P1
λ701	N13	S2	ND[c]	P1
λ516	N13	S2	B9	P1
λ371, λ594, λ728, λ733	N3	S2	ND	ND
λ196	N3	S2	B8	P10
λ491, λ489	N3	S12	<B9[d]	P6
λ379	N11	S4	<B9	P6
λ522	N15	S4	B6	ND
λ653	N15	S4	B6	P4
λ670	N16	S4	B6	P4
λ351	N12	S4	B6	P4
λ644, λ740	N7	S1	<B9	P4
λ375	N7	S1	<B9	<P10[d]
λ42	N6	S1	ND	P8
sc#1	N6	S1	ND	ND
λ611	N8	S1	B5	P5
λ601	N8	S8	B5	P5
λ130	N9	S8	B5	P5
λ736	N4	S8	<B9	P2, P5[e]
λ504, λ734	N4	S15	B1	P2
λ361, λ511, λ583	N4	S6	B1	P2
λ356	N10	S6	B1	P2
λ515	N14	S6	B1	P2
λ167	N1	S9	B1	P3
λ732	N1	S16	<B9	P3
λ542	N1	S16	B3	P3
λ711	N1	S14	B2	P9
λ699	N1	S14	B2	ND
λ6, λ37	N2	S3	B2	P7

[a] Clones listed on the same line hybridized to the same restriction fragments, but were shown to be distinct by comparing insert size and by determining that the clones did not cross-hybridize.

[b] Clones sc#1 and sc#5 are anonymous fragments of FA1090 DNA cloned into pBR322.

[c] ND, not done.

[d] The hybridizing fragment was smaller than B9 or P10, but its identity was not determined.

[e] The insert of clone λ736 contains a *Pac*I site; the clone hybridized to two adjacent chromosomal fragments, P2 and P5.

TABLE 4. Strain FA1090 *opa* loci[a]

Locus	Previous locus designation	Previous protein designation	HV_1	HV_2
opaA	1	P.IIa	HV_1-2	HV_2-2
opaB	3	P.IIb	HV_1-4	HV_2-1
opaC	5	P.IIc	HV_1-4	HV_2-4
opaD	2	P.IId	HV_1-1	HV_2-1
opaE	6	P.IIe	HV_1-5	HV_2-5
opaF	7	P.IIf	HV_1-3	HV_2-3
opaG	4	P.IIb	HV_1-4	HV_2-1
opaH	8	P.IIf	HV_1-3	HV_2-3
opaI	9		HV_1-1	HV_2-6
opaJ	10		HV_1-2	HV_2-4
opaK	11		HV_1-6	HV_2-5

The nomenclature of the *opa* genes and the proteins they encode has been revised in accordance with recommendations made at recent international neisseria conferences (17). Previous nomenclature and the information about the HV regions of each gene are from reference 8. In that study, each different version of HV_1 or HV_2 was given an arbitrary number; when a given HV region was found in multiple *opa* genes, the DNA sequence was identical in each one. The DNA sequence of HV_1-6 has not been determined.

ants of pilin is stored in *pilS* loci, each of which contains one or more partial pilin genes. Much of the information about *pil* genes comes from studies of strain MS11, which contains one or two *pilE* loci and seven *pilS* loci (1, 15, 27). The *pilE* loci and three or four of the *pilS* loci are linked within a 50-kb region of the MS11 chromosome, but the relative arrangement of the other *pilS* loci is not known (15, 16).

We used probes that were developed in the studies on strain MS11 (1, 13, 27) to determine the arrangement of the *pil* loci on the FA1090 map. The probes were initially used on Southern blots of conventional gels of *Cla*I-digested DNA from strain FA1090 or MS11 to determine whether they were appropriate for analysis of strain FA1090. A *pilE*-specific oligonucleotide hybridized to a single *Cla*I fragment in both FA1090 and MS11; an oligonucleotide specific for locus *pilS6* of MS11 also hybridized to one *Cla*I fragment in each strain. A *pilE* + *pilS*-specific oligonucleotide and a cloned MS11 *pilE* gene, both of which should detect all *pil* loci, hybridized to six bands in FA1090 and seven bands in MS11 (data not shown). These results showed that the probes could be used for analysis of strain FA1090, and that the number of loci and the conserved sequences in them were similar to strain MS11. The *pil*-specific probes, and the fragments to which they hybridized on blots of pulsed-field gels, are included in Table 2.

The *pil* loci of FA1090 are located in two regions separated by approximately one-fourth of the chromosome (Fig. 4). The *pilE* and *pilS6* loci are within the region defined by fragment N10. Probes specific for all *pil* loci also gave the strongest hybridization signal with N10 and the fragments overlapping it. Although we do not yet know how many of the *pilS* loci of FA1090 are located in each of the two chromosomal regions, it seems likely that most of the *pilS* loci are located near *pilE*, as in strain MS11. Both of the *pil*-containing regions also contain *opa* loci. The proximity of *opaD* and at least one *pilS* locus is consistent with our previous studies showing that a cloned *opa*D gene has *pil*-related sequences immediately upstream of the *opa* coding sequence (7; unpublished results).

DISCUSSION

The gonococcal chromosome is circular; its size of approximately 2,219 kb is one-half the size of the *E. coli* chromosome (37). The genome size determined in this study is larger than the previous estimate of 1,500 kb, which was based on reassociation kinetics (19). It seems likely that the repeated genes and sequences in gonococcal DNA influenced the reassociation studies, resulting in an underestimate of chromosome size. In constructing the macrorestriction map of the FA1090 chromosome, it was essential to use a strategy that avoided the possible complications and ambiguities caused by those repeated genes and sequence elements. The presence of the repeats precluded the use of some approaches that have been valuable in physical map construction for other bacterial species, such as probing one restriction digest with fragments isolated from a second digest. We constructed the map by using Southern blotting experiments to identify overlapping fragments from different restriction digests. The probes used for this purpose hybridized to a single fragment in each restriction digest, thus allowing us to walk back and forth between digests along the chromosome. When the probes were derived from identified

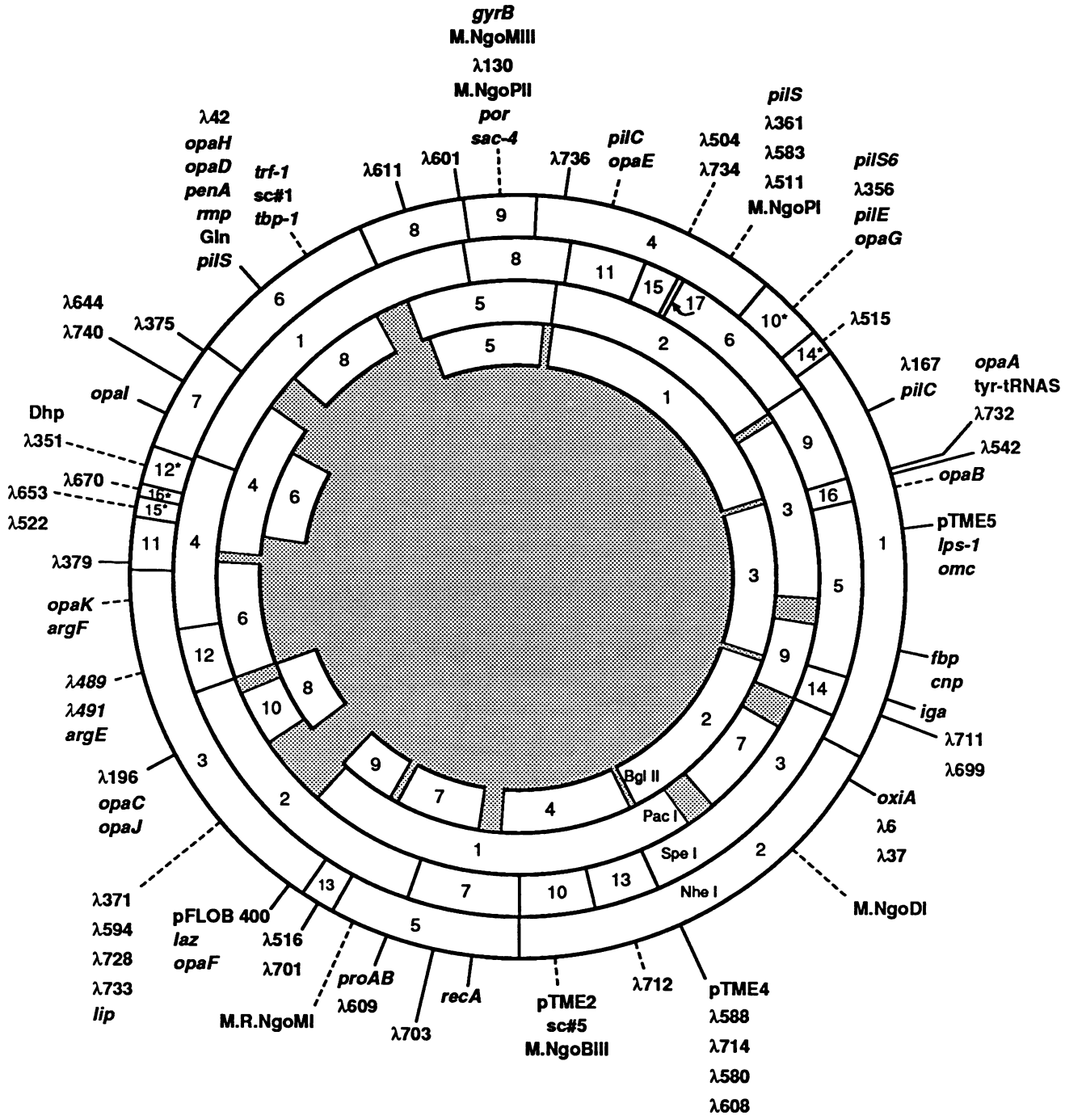

FIG. 3. Macrorestriction map of the strain FA1090 chromosome. If a genetic marker has been assigned a genotype, that designation appears on the map. For markers with no such designation, either the clone name or an abbreviation for the phenotype is shown, as listed in Tables 2 and 3. A group of markers mapping to the same region of fragment overlap is connected to the map by a solid or dashed line. The dashed lines indicate that markers may be located anywhere within the designated region of overlap between an *Nhe*I fragment and a *Spe*I fragment. The solid lines indicate groups of markers whose position within a *Spe*I/*Nhe*I overlap was further localized by the pattern of hybridization to *Bgl*II or *Pac*I fragments. The order of markers within each group cannot be determined. Fragments whose relative order is not known are indicated by asterisks (*).

gonococcal genes, the data were used both to establish fragment overlaps and to determine the location of the genes on the final map. An insufficient number of gonococcal genes have been cloned to make a complete map in this way, so we also used anonymous cloned pieces of FA1090 DNA to identify the relationships between fragments in different digests. Even though we do not know what genes the anonymous clones represent, they provide markers for spe-

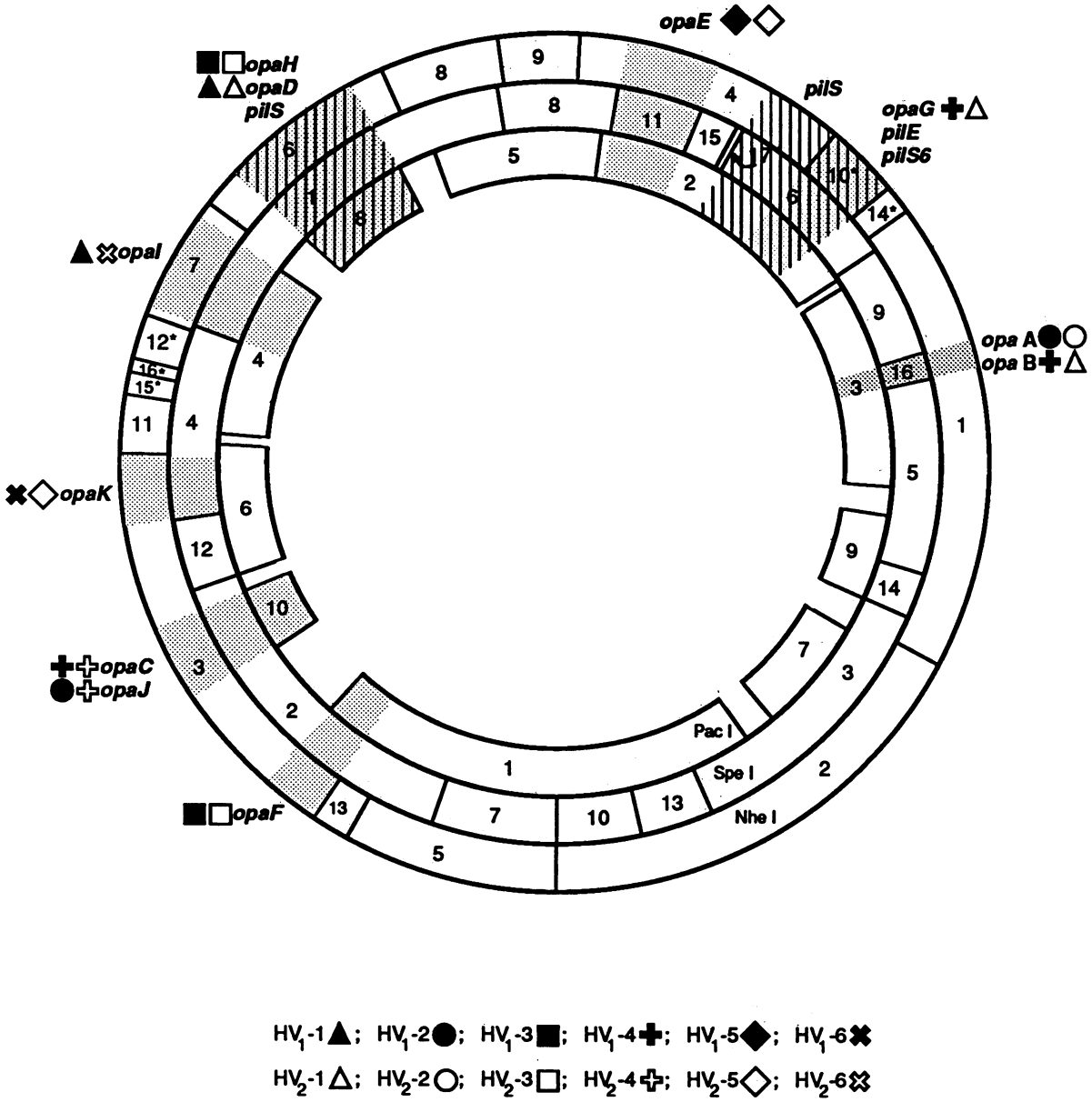

FIG. 4. Location of *opa* and *pil* loci on the physical map of strain FA1090. Only the *Spe*I, *Nhe*I, and *Pac*I maps are shown, since *Bgl*II digests were not used in analysis of these loci. Shaded areas show the regions within which *opa* loci are located, based on the hybridization of probes specific for different HV regions to fragments in each of the three digests. For locus *opaK*, only *Spe*I and *Nhe*I digests were analyzed. The symbols next to the name of each *opa* locus show the versions of HV_1 (closed symbols) and HV_2 (open symbols) present in that locus in this variant of strain FA1090. Striped areas show the regions within which *pil* loci are located.

cific chromosomal locations and will facilitate higher-resolution mapping of the chromosome in the future.

A macrorestriction map is by definition a low-resolution map and cannot give precise information about the location of genetic loci. On the current map, we can localize genes to the region of overlap between particular restriction fragments, but cannot determine the relative order of genes mapping in each region. Higher-resolution genetic or physical mapping will be required to order those groups of markers and determine the distance between them. It is not yet possible to compare the physical map with the single genetic linkage group that has been mapped by transformation, since probes are not available for most of the markers mapping there.

Once the map was constructed, it was possible to examine the distribution of the *pil* and *opa* multigene families on the chromosome. Expression of both Opa and pilin proteins shows phase and antigenic variation, and previous studies have shown that *opa* and *pil* genes can be immediately adjacent to each other (7, 44). The physical map confirms the relative proximity of *opa* and *pil* loci in two places on the FA1090 chromosome. However, the arrangement of the two gene families is very different. Two regions of the chromosome contain *pil* loci. The *pilE* locus and probably most of

TABLE 5. Hybridization of *opa* hypervariable-region oligonucleotide probes

Probe	Hybridizing fragments		
	*Nhe*I	*Spe*I	*Pac*I
HV_1-1	N6, N7	S1	P4, P8
HV_1-2	N1, N3	S2, S16	P3, P10
HV_1-4	N1, N3, N10	S2, S6, S16	ND[a]
HV_1-5	N4	S11	P2
HV_2-1	N1, N6, N10	S1, S6, S16	P2, P3, P8
HV_2-2	N1	S16	P3
HV_2-3	N3, N6	S1, S2	P1, P8
HV_2-4	N3	S2	P10
HV_2-5	N3, N4	S4, S11	ND
HV_2-6	N7	S1	ND

[a] ND, not done.

the *pilS* loci are in one region, with at least one *pilS* locus located in a second region separated from the first by approximately one-fourth of the chromosome. In contrast, the 11 *opa* loci are spread over approximately 60% of the chromosome. There are three locations in which two *opa* loci may be close together, but the remaining loci are distributed singly. Some *opa* genes are partially or wholly duplicated, sharing identical versions of the hypervariable regions HV_1 and/or HV_2 (7, 8, 43, 50). The physical map shows that the duplicated genes and genes sharing HV_1 or HV_2 are generally not adjacent on the chromosome of this variant of strain FA1090, but are widely separated. We have hypothesized that there is a mechanism for reassortment or reshuffling of hypervariable regions among *opa* loci, based on the characteristics of the *opa* genes of strain FA1090 and on the isolation of variants that have undergone such reassortment (7, 8; unpublished data). With the current map as a reference point, it should now be possible to study the "rules" governing such reassortments among different loci, determining which types of events are permissible and which are not.

It is important not to generalize from the map of strain FA1090 to the assumption that all gonococcal strains will have the same arrangement of genetic loci. Gonococci show extensive interstrain variability, and different strains may well show differences in chromosomal organization. This possibility seems especially likely in view of the many reiterated genes and sequences in gonococci, which could be involved in recombination and chromosomal rearrangements. If such rearrangements do occur, then the maps of different variants of a single strain may also differ. Comparison of the maps of multiple strains will be helpful in determining whether chromosomal rearrangements are common and what portions of the chromosome might be involved. The physical map of strain FA1090 provides a tool for future studies on the arrangement of single-copy and multicopy genes and sequence elements and on recombination and possible genomic rearrangement in the gonococcus.

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