Physical Map of the Genome of *Vibrio cholerae* 569B and Localization of Genetic Markers

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A combined physical and genetic map of the genome of the classical O1 hypertoxinogenic strain 569B of *Vibrio cholerae* has been constructed. The enzymes *Not*I, *Sfi*I and *Ceu*I generated DNA fragments of suitable size distribution that could be resolved by pulsed-field gel electrophoresis. The digests produced 37, 22, and 7 fragments, respectively. The *Ceu*I maps of the genomes of strains 569B and O395, constructed by partial restriction digestion, were identical, and the data are consistent with the concept of circular chromosomes. The genome size of each of the strains was estimated to be about 3.2 Mb. The *Not*I and *Sfi*I digestion profiles of the genomic DNAs of strains 569B and O395 exhibited distinct restriction fragment length polymorphism. The linkages between the 37 *Not*I fragments of the genome of strain 569B were determined by combining three approaches: isolation of linking clones, analysis of partial digestion fragments, and identification of *Not*I fragments in isolated *Ceu*I and *Sfi*I fragments. To align linked fragments precisely, *Not*I-digested genomic DNA was end labeled and separated in the same gel with the *Not*I-digested DNA to be probed with linking clones. This also allowed the identification of smaller restriction fragments that are not visible in ethidium bromide-stained gels. The presence of repetitive DNA sequences in the *V. cholerae* 569B genome has been demonstrated. Twenty cloned homologous and heterologous genes and seven *rrn* operons have been positioned on the physical map. The two copies of the Ctx genetic element in the genome of strain 569B are located about 1,000 kb apart.

Cholera still remains a public health problem in most developing countries. *Vibrio cholerae*, a noninvasive gram-negative bacterium and the causative agent of the disease, is serologically classified as belonging to the O antigenic group (38). Strains belonging to O group 1 (O1) are responsible for cholera. Strains other than O1 are called non-O1. Strains of serotype O1 consist of two biotypes, classical and El Tor. Of the seven pandemics of cholera recorded in recent times, the most recent was caused by *V. cholerae* O1 biotype El Tor (17). The sixth pandemic and probably the fifth (20) were due to classical strains. *V. cholerae* non-O1 strains can cause only sporadic infections and do not have the potential to cause epidemics (29). Only recently, an outbreak of cholera in India and Bangladesh which subsequently spread into several parts of the subcontinent was caused by a novel non-O1 strain, *V. cholerae* O139 Bengal (1, 33). However, several lines of evidence have suggested that strain O139 Bengal very closely resembles biotype El Tor of the O1 serovar (6, 43).

Although a great deal is known about the physiology, biochemistry, and clinical microbiology of *V. cholerae* (20), very little information is available about its chromosomal structure and its genetics. Only recently has transformation of *V. cholerae* cells by plasmid DNA been reported (30). Conjugation is mediated by a factor P (9), which unlike the F factor of *Escherichia coli* cannot integrate into the chromosome and cannot induce Hfr donors. Hence, the mobilization of chromosomal DNA is very limited. An expanded linkage map of the classical strain 162 in which not more than 30 loci have been mapped by conjugal transfer of Tfr donors has been reported (31). The relative distances between these markers are very approximate, and surprisingly, all the mapped genes are clustered in a limited region of the chromosome.

Knowledge of the genome structure is useful in exploring the complicated questions concerning global regulation of metabolisms such as genome rearrangement (18), control and rate of DNA replication-termination (22), and the dynamics and evolutionary aspects of genome organization (34). The alternative to examining the organization of genomes in organisms for which a genetic map is not available is to construct a physical map of the genome which represents the structure of the DNA. With the advent of pulsed-field gel electrophoresis (PFGE), which allows separation of large DNA fragments generated by digesting intact genomic DNA with rare restriction site enzymes, large physical restriction maps can now be assembled (39, 40). This technology has enabled accurate estimation of genome sizes, detection of gross chromosomal aberrations, and qualitative evaluation of intraspecies genetic variations which permit identification of individual isolates of a species by comparing the macrorestriction patterns.

The genome sizes of a number of *V. cholerae* strains have recently been determined by digesting the intact genomic DNA by the enzymes *Not*I and *Sfi*I, which produce a small number of large fragments that could be resolved by PFGE (6, 7, 36). The genome sizes of all classical strains examined were about 3,200 kb, and surprisingly, those of strains of the El Tor biotype were 500 to 600 kb smaller. The *Not*I and *Sfi*I digestion patterns of the genomes of *V. cholerae* strains belonging to different serovars and biotypes showed distinct restriction fragment length polymorphism (RFLP). The present report describes the construction of a physical map of the genome of the classical O1 hypertoxinogenic strain 569B of *V. cholerae* using the enzymes *Ceu*I and *Not*I and a partial *Sfi*I map. Twenty homologous and heterologous genes and seven *rrn* operons have been placed on the physical map. The availability of physical restriction maps will allow us to address the issue of genetic heterogeneity among the *V. cholerae* strains belonging to different serovars and biotypes.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *V. cholerae* classical O1 strains 569B (Inaba) and O395 (Ogawa) used in this study were obtained from the National Institute of Cholera and Enteric Diseases, Calcutta, India. *V. cholerae* cells were grown in a gyratory shaker at 37°C in nutrient broth containing 0.1 M NaCl (pH 8.0) and maintained as described previously (12, 25, 35). *E. coli* cells were grown in Luria-Bertani medium with shaking at 37°C.

Preparation of high-molecular-weight genomic DNA. Intact genomic DNA was prepared as described previously (36). *V. cholerae* cells in the logarithmic phase of growth were suspended in a 10 mM Tris-HCl (pH 7.6) buffer containing 1 M NaCl. Agarose plugs were prepared by mixing equal volumes of the cell suspension and molten 1% low-melting-point agarose (Pharmacia, Uppsala, Sweden). The plugs were suspended in lysis solution containing 6 mM Tris-HCl (pH 7.5), 100 mM EDTA, 1 M NaCl, 0.5% Brij 58, 0.2% deoxycholate, 0.5% Sarkosyl NL-97, 1 mg of lysozyme per ml, and 20 μg of RNase A per ml and incubated at 37°C for 16 h with gentle shaking. After the incubation period, the lysis solution was replaced by a solution containing 0.5 M EDTA (pH 9.0), 1% Sarkosyl, and 1 mg of proteinase K per ml, and incubation continued for 48 h at 50°C, following which the plugs were stored in the same solution at 4°C.

Restriction digestion. The restriction enzymes used were obtained from New England Biolabs. The agarose plugs containing the genomic DNA were treated with 1 mM phenylmethylsulfonyl fluoride and washed three times for 30 min each in at least 10 volumes of TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). The plugs were then sliced to about 1-mm-thick pieces. For digestion with *Not*I or *Sfi*I, one portion of the sliced agarose plugs was immersed into 1× buffer supplied by the manufacturer and incubated in ice for 45 min. The buffer was then replaced by 100 μl of fresh 1× buffer containing 10 μg of bovine serum albumin (BSA) and 10 U of the enzyme, and incubation continued at 37°C for *Not*I and at 50°C for *Sfi*I for 4 to 16 h. For partial digestion with *Not*I, 1 U of the enzyme was used in the reaction mixture and incubation was for 3 h. For *Ceu*I digestion, five or six sliced agarose pieces were incubated in 2× buffer supplied by the manufacturer. After 15 min the buffer was replaced by 100 μl of fresh 1× buffer containing 10 μg of BSA and 4 U of the enzyme, and incubation continued at 37°C for 3 h. For partial digestion with *Ceu*I, six agarose pieces were digested with 1 U of the enzyme in 100 μl of the reaction mixture for 2 h at 37°C.

Isolation of linking clones. To isolate *Not*I linking clones of *V. cholerae* genomic DNA, the method of Zabarovsky et al. (44) was used with some modifications. About 5 μg of 569B genomic DNA was digested to completion with the enzyme *Sau*3A, *Bgl*II, or *Hin*dIII, which cut *V. cholerae* DNA frequently. The digestion products were extracted with phenol-chloroform, ethanol precipitated, and allowed to self-ligate for 16 h in 1 ml of a ligation mixture containing 2.5 U of T4 DNA ligase. The reaction was stopped, and the DNA was precipitated and dissolved in TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). The self-ligated DNA was digested with *Not*I and cloned into a *Not*I-digested, dephosphorylated pBluescript KS+/− vector (Stratagene, La Jolla, Calif.). The linking-clone libraries were maintained in *E. coli* JM109. Recombinant clones were isolated by blue-white selection. Each recombinant represented a linking clone.

Preparation of probe DNA. Plasmid DNA was isolated by the rapid alkaline lysis method (10). DNA was nick translated by using [α-^{32}P]dCTP (Amersham) and DNA polymerase. The reaction was carried out at 16°C for 1 h, and the nick-translated labeled DNA was separated from unincorporated [α-^{32}P]dCTP by passing through a Sephadex G-50 column. For labeling any particular DNA fragment, the fragment was excised from the agarose gel and labeled by random priming using the NEBlot kit (New England Biolabs).

PFGE and Southern hybridization. Electrophoresis was carried out in a Pulsaphor Plus System with a hexagonal electrode array (Pharmacia) as described previously (36). FastLane agarose (FMC, Philadelphia, Pa.) was used when DNA was to be transferred to membranes for Southern blotting, and low-melting-point agarose (Pharmacia) was used when fragments were to be recovered from gels. The enzyme-digested DNA was separated on a 1.0% agarose gel by using 20 mM Tris-acetate (pH 8.3)–0.5 mM EDTA as running buffer at 10 V/cm and various pulse times depending upon experimental conditions. Phage λ multimeric DNA and yeast chromosomal DNA were used as molecular mass markers.

For Southern blot hybridization, the gels were stained with ethidium bromide, irradiated with UV light to nick the DNA, denatured, neutralized, and blotted to nylon membranes (27). Hybridization was carried out at 60°C, and the filters were washed at the desired stringency. The filters were air dried and exposed to X-ray films.

Isolation, redigestion, and end labeling of DNA fragments. Restriction fragments of genomic DNA digests were excised from agarose gels under long-wavelength UV light, and the agarose blocks containing DNA fragments were trimmed to about 1-mm slices and digested with a second enzyme. The double-digested DNA was end labeled by incubating the agarose blocks in a buffer containing Klenow enzyme, [α-^{32}P]dCTP, and unlabeled dGTP and subjected to PFGE followed by autoradiography.

RESULTS

Restriction fragment analysis. Previous analysis of *V. cholerae* genomes using several restriction endonucleases showed
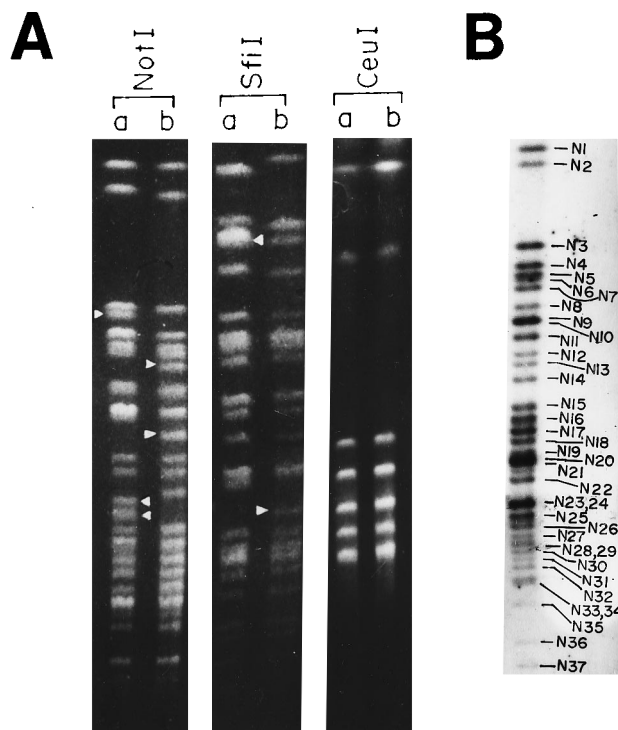

FIG. 1. (A) PFGE separation of *Not*I-, *Sfi*I-, and *Ceu*I-digested genomic DNAs of *V. cholerae* O395 (lanes a) and 569B (lanes b). The enzyme-digested DNA was separated on 1% FastLane agarose (FMC) with pulse time ramping between 5 and 25 s for 22 h at 10 V/cm for *Not*I and *Sfi*I. For the separation of *Ceu*I fragments, pulse time was ramped from 20 to 120 s over 12 h followed by ramping from 60 to 150 s over 12 h at 10 V/cm. The gels were stained with ethidium bromide. Arrowheads, RFLP between O395 and 569B genomic DNAs. (B) Autoradiogram of PFGE-separated *Not*I fragments of *V. cholerae* 569B genomic DNA. DNAs in agarose blocks were digested with the enzyme, end labeled with [α-^{32}P]dCTP, and electrophoresed as described in Materials and Methods. The gel was dried and exposed to X-ray film.

that the enzymes *Not*I and *Sfi*I digest the genomic DNA into a small number of large fragments that could be resolved by PFGE (6, 36) and that these enzymes are, therefore, the most appropriate for the construction of physical maps. Intact genomic DNAs of *V. cholerae* classical strains 569B and O395 in agarose blocks were digested with *Not*I, *Sfi*I or *Ceu*I and subjected to PFGE. The endonuclease *Ceu*I cleaves the DNAs of a number of enteric bacteria at seven sites, and these sites are located in the *rrn* operons (23).

The ethidium bromide-stained gels of PFGE-separated genomic DNA fragments showed that *Not*I digestion and *Sfi*I digestion yielded 26 and 22 fragments, respectively, ranging from 370 to 25 kb for both the 569B strain and the O395 strain (Fig. 1A). Restriction fragments were named on the basis of the enzyme that generated the fragments (N, *Not*I; S, *Sfi*I; C, *Ceu*I) and were numbered on the basis of size in descending order. Fragments N9 and N10, fragments N20 and N21, and fragments N23 and N24 of the genomes of the two strains are of the same size. The genome size of each of the strains, as estimated from the sizes of *Not*I and *Sfi*I fragments, was about 3,200 kb (Table 1), which is about 0.7 times that of the *E. coli* genome. The *Not*I and *Sfi*I digestion profiles of the genomes of strains 569B and O395 exhibited distinct RFLP, the polymorphism being more pronounced when the genomes were digested with the enzyme *Not*I (Fig. 1A, arrowheads).

The smallest fragment that could be clearly identified in stained gels was about 26 kb. In some gels, however, faint

TABLE 1. Sizes of restriction fragments generated by cleavage of *V. cholerae* 569B and O395 chromosomes

Fragment	Size (kb)[a]				
	*Not*I		*Sfi*I		*Ceu*I (3,290)[b]
	569B (3,177)	O395 (3,176)	569B (3,077)	O395 (3,092)	
1	364	364	370	364	1,420
2	324	324	296	296	900
3	210	210	280	280	325
4	189	205	246	246	275
5	180	189	210	210	180
6	175	180	190	190	120
7	166	175	183	183	
8	150	150	175	175	
9	134	134	150	150	
10	134	134	140	140	
11	124	112	128	128	
12	112	106	109	109	
13	106	95	86	107	
14	96	85	83	83	
15	80	80	77	77	
16	74	74	73	73	
17	66	66	66	66	
18	62	62	58	58	
19	55	55	47	47	
20	49	49	44	44	
21	49	49	36	36	
22	42	42	30	30	
23	33	33			
24	33	33			
25	29	29			
26	26	26			
27	15	15			
28	14	14			
29	14	14			
30	13	13			
31	12	12			
32	11	11			
33	10	10			
34	10	10			
35	7	7			
36	5	5			
37	4	4			

[a] Totals are indicated in parentheses.
[b] For both 569B and O395.

bands below 26 kb were frequently observed, suggesting that there might be more smaller fragments than were visible in stained gels. Identification of these smaller fragments was essential for linking the restriction fragments to construct the physical map. To identify the smaller fragments, the restriction fragments were end labeled in agarose inserts before PFGE and the autoradiograms were examined instead of stained gels. In addition to those identified in stained gels, 11 fragments of sizes ranging from 15 to 4 kb were detected for the *Not*I fragments of the *V. cholerae* 569B genome in this way (Fig. 1B).

CeuI cleavage map of the V. cholerae 569B and O395 genomes. *Ceu*I digestion of *V. cholerae* 569B and O395 produced seven fragments ranging from 1,420 to 70 kb (Fig. 1A; Table 1). At a low concentration of the enzyme *Ceu*I, partial digestion resulted in extra bands. Ten partial fragments of the *Ceu*I-digested 569B genome (Table 2) and seven partial fragments of the O395 genome (data not shown) were analyzed to assemble the *Ceu*I physical maps (see Fig. 5). *Ceu*I partial digestion fragment 1 of the 569B genome is equivalent to the sum of the complete digestion fragments C6 and C7 or C5 and C7.

Partial fragment 4 was the sum of fragments C3 and C7. Partial fragment 7 provides the linkage C3-C7-C5. Partial fragment 2, which is the sum of the fragments C5 and C6, along with the partial fragments 3 and 6 confirmed the linkage -C3-C7-C5-C6-. Partial fragment 9 showed that fragments C1 and C3 are adjacent to each other, and fragment 5 yielded the linkages between C4, C6, and C5. Partial fragments 7 and 8 established the linkages between the fragments C2, C4, C6, and C5. Partial fragment 10 can arise only from the combination of fragments C1 and C2. The linkage between the *Ceu*I fragments of the genome of strain 569B is thus -C1-C3-C7-C5-C6-C4-C2-C1-, and the genome is circular (see Fig. 5). From a similar analysis of the seven partial fragments of the *Ceu*I-digested O395 genome (data not shown), the *Ceu*I map was constructed. The *Ceu*I maps of the two classical strains are identical.

NotI cleavage map of the V. cholerae 569B genome. Three approaches were combined to link 37 *Not*I fragments of the *V. cholerae* 569B genome (see Fig. 5). These included (i) isolation of linking clones, (ii) analysis of partial digestion products, and (iii) identification of *Not*I fragments in *Ceu*I and *Sfi*I fragments using the method of fragment excision, redigestion, and end labeling.

To isolate linking clones, the genomic DNA of *V. cholerae* 569B was digested completely with the enzyme *Sau*3A, *Bgl*II, or *Hin*dIII, and the DNA fragments were diluted and allowed to self-ligate as described in Materials and Methods. Among the resulting circular molecules, those containing *Not*I sites were linearized following *Not*I digestion. The linear DNA fragments were cloned at the *Not*I site of the vector pBluescript KS+/−. Each recombinant plasmid represents a linking clone. The three linking-clone libraries generated by digesting genomic DNA with *Sau*3A, *Bgl*II, and *Hin*dIII will be referred to as NLS, NLB, and NLH libraries, respectively. These libraries were screened to identify clones that linked separate *Not*I fragments (Table 3) and were used as probes in hybridization experiments with PFGE-separated chromosomal restriction fragments produced by the enzyme *Not*I to determine the linkages between the different fragments.

One of the major problems encountered in aligning fragments by using linking clones was in the identification of fragments based on size determination using standard molecular weight markers. This often led to erroneous conclusions, particularly for closely migrating fragments. To overcome this difficulty and to unequivocally identify fragments, instead of molecular weight markers the end-labeled fragments of the complete *Not*I digest of the *V. cholerae* 569B genome (Fig. 1B)

TABLE 2. Partial digestion products of the 569B genome generated by the enzyme *Ceu*I

Partial fragment (kb)	Possible composition[a] (kb)
1 (220)	C6 and C7 (190) or C5 and C7 (250)
2 (290)	C6 and C5 (300)
3 (390)	C5, C6, and C7 (370) or C4 and C6 (395)
4 (420)	C3 and C7 (395)
5 (585)	C4, C5, and C6 (575), C3 and C4 (600), or C3, C5, and C7 (575)
6 (745)	C3, C5, C6, and C7 (695) or C3, C4, and C6 (720)
7 (1,320)	C2, C4, C6 (1,295), or C2, C5, C6, and C7 (1,270), or C2, C4, and C5 (1,355)
8 (1,515)	C2, C4, C5, and C6 (1,475)
9 (1,835)	C1 and C3 (1,745)
10 (2,450)	C1 and C2 (2,320)

[a] *Ceu*I fragments.

TABLE 3. Determination of the linkages between *Not*I fragments
of the genome of *V. cholerae* 569B by hybridization
with linking clones

Linking clone(s)	Linkage
NLB 2, 8, 10	N9-N16
NLB 5	N8-N4
NLB 29	N18-N32
NLB 33	N19-N21
NLB 39	N10-N17
NLB 40, 92	N2-N4
NLB 42	N8-N21
NLB 51	N1-N22
NLB 53	N19-N28
NLB 130	N5-N27
NLS 4	N6-N26
NLH 1, 28, 33, 39	N20-N23
NLH 2, 44, 66	N6-N22
NLH 7	N16-N34
NLH 8	N5-N18
NLH 9, 117	N9-N25
NLH 19	N15-N26
NLH 20	N11-N25
NLH 30	N5-N27
NLH 35	N2-N35
NLH 48	N19-N28
NLH 51, 123	N7-N15
NLH 102	N20-N28
NLH 108	N10-N17
NLH 127	N1-N23
NLH 132	N14-N27

TABLE 4. Identification of *Not*I-digested genomic DNA fragments
in isolated *Sfi*I and *Ceu*I restriction fragments

Fragment	*Not*I fragment(s)
C1	N1, N6, N7, N12, N13, N14, N15, N20, N22, N23, N26, N27, N36[a]
C2	N2, N4, N8, N19, N21, N35[a]
C3	N10, N18, N24, N32, N37[a]
C4	N9, N16, N25[a]
C5	N3[b]
C6	N3, N11[b]
S1	N3, N11, N17, N25, N33[c]
S2	N5, N13, N14, N27, N36[c]
S3	N1, N6, N22[c]
S5	N4, N8[b]
S6	N9, N16[b]
S8	N8, N19, N21[b]
S12	N2, N4[b]

[a] Identified by end labeling.
[b] Identified by hybridization.
[c] Identified by both end labeling and hybridization.

were loaded in the same gel next to each lane of the *Not*I-digested genome that was to be probed with linking clones. This procedure allowed identification of fragments directly from the autoradiograms. Some examples of such hybridization experiments using linking clones as probes are shown in Fig. 2. The results presented in Fig. 2 clearly demonstrated that fragments N8 and N21, N6 and N26, N4 and N8, N20 and N28, N11 and N25, and N1 and N22 are linked. Similarly, the other linking clones provided other linkages, and more than 70% of the *Not*I map of the 569B genome was derived from the analysis of linking clones alone.

To determine the linkages between fragments for which linking clones were not obtained from the library search so far, individual PFGE-separated *Sfi*I or *Ceu*I fragments were excised from gels and digested completely with *Not*I and the fragments were end labeled and separated by PFGE. This allowed identification of fragments that are included in a particular *Sfi*I or *Ceu*I fragment. Alternatively, gel-excised *Sfi*I or *Ceu*I fragments were used as probes in Southern blot hybridization of the *Not*I-digested *V. cholerae* 569B genome. These approaches allowed the clubbing of *Not*I fragments that are linked (Table 4). For example, in the Southern blot hybridization experiment, the 370-kb fragment S1 hybridized with the *Not*I fragments N3, N11, N25, N17, N19, and N33 (Fig. 3A). When fragment S1 was digested with the enzyme *Not*I and end labeled, four fragments which comigrated with the *Not*I fragments N3, N11, N25, and N33 were produced (Fig. 3B). Thus, fragments N3, N11, N17, N19, N25, and N33 are possibly linked to each other, and in the linkage the fragments N17 and N19 might be at the two ends. The N11-N25 linkage was confirmed from linking clones (NLH 20; Table 3), and the linkage between N3 and N11 was determined from the analysis of partial fragments (Fig. 3C). Thus, the linkage N25-N11-N3 was established. The linking clones NLH 9 and NLH 117 (Table 3) showed that N25 is linked to N9. Therefore, N33

must be linked to N3 with N17 at the end. Hence, fragment S1 contained the *Not*I fragments linked as N25-N11-N3-N33-N17. In this particular case, in the Southern blot experiment (Fig. 3A) fragment N19 also hybridized strongly. However, neither N19-N25 nor N17-N19 linkages were obtained by any of the approaches adopted to construct the map. On the other hand, from the analysis of the linking clones it has been established that N19 is linked to N27 (Table 3, NLB 53 and NLH 48) and N21 (Table 3, NLB 33). Besides N19, several other weak signals were also observed (Fig. 3A). This was found to be true in Southern blot hybridization experiments when a number of *Sfi*I and *Ceu*I fragments were used as probes. In such cases the sum of the sizes of *Not*I fragments hybridized was much higher than the size of the restriction fragments used as probes. Some of the hybridization signals are presumably due to the presence of repetitive sequences in the 569B genome. This was confirmed when some linking clones were found to hybridize with more than two *Not*I fragments of the *V. cholerae* genome. One such *Not*I linking clone (NLH 115) carrying a 4.2-kb insert which hybridized with seven PFGE-separated *Not*I fragments of the
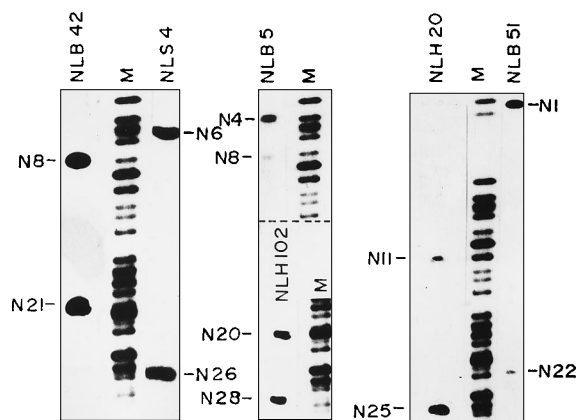
FIG. 2. Determination of linkages between *Not*I fragments of 569B genomic DNA using *Not*I linking clones as probes by Southern hybridization. Nick-translated linking clones NLB 42, NLS 4, NLB 5, NLH 102, NLH 20, and NLB 51 were hybridized with PFGE-separated *Not*I fragments of the 569B genome. *Not*I-digested end-labeled genomic DNA was used as markers (M) for identification of linked fragments. The *Not*I fragments that are linked to each other are marked.
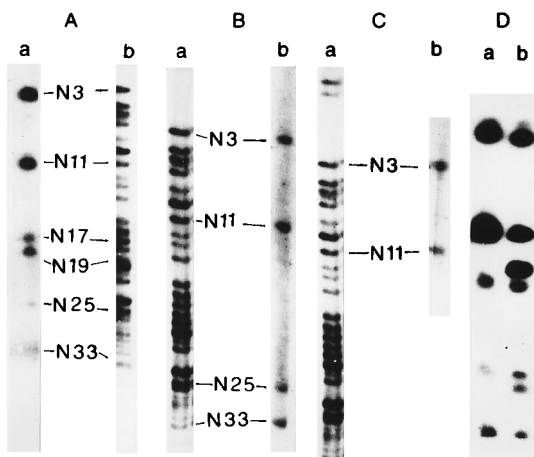
FIG. 3. (A and B) Identification of *Not*I fragments of the *V. cholerae* 569B genome in isolated *Sfi*I fragments. (A) Radiolabeled fragment S1 was used as probe in Southern hybridization with the *Not*I-digested *V. cholerae* 569B genome (lane a). *Not*I-digested, end-labeled 569B genomic DNA (lane b) was used as marker for identification of linked fragments. (B) Fragment S1 was completely digested with the enzyme *Not*I, end labeled, and separated by PFGE (lane b). *Not*I-digested, end-labeled 569B genomic DNA was used as marker (lane a). (C) PFGE analysis of *Not*I partial digestion products. A *Not*I partial digestion fragment was excised from the gel, radiolabeled, and used as probe in Southern hybridization with 569B genomic DNA digested completely with *Not*I. The *Not*I fragments present in the partial digestion products are marked (lane b). Lane a, *Not*I-digested end-labeled 569B genomic DNA. (D) Repetitive DNA sequence in *V. cholerae* O395 (lane a) and 569B (lane b). Southern hybridization of nick-translated linking clone NLH 115 with *Not*I-digested genomic DNA.

the *Ceu*I-digested 569B genome using the linking clones NLH 39 and NLB 33 (Table 3) as probes. Clone NLB 33 hybridized to fragment C2, and clone NLH 39 hybridized to fragment C1 (data not shown). The other similar-sized fragments, N23 and N24, could not be resolved in the present study and can exchange their positions on the physical map.

Positioning of *V. cholerae* genes on the physical map. Twenty cloned genes (Table 5) and seven *rrn* operons have been positioned on the physical map of the *V. cholerae* 569B genome (Fig. 5) by hybridization using homologous and heterologous probes. The gene probes used comprised DNA repair genes, heat shock protein genes, cholera toxin, and some other virulence determinant genes. The *Ceu*I sites in the *V. cholerae* genome were taken as the positions of the *rrn* operons in analogy with those reported for several other enteric pathogens and gram-negative bacteria (23). The genes have been positioned on the physical map on fragments they hybridized with, and the positioning does not represent the exact locations of the genes on the fragment. Genes hybridizing with the same fragment have been positioned on the fragment arbitrarily, and the positioning does not reflect the true order of the genes in the chromosome. The *ctx* gene of *V. cholerae* mapped at two loci about 1,000 kb apart on the physical map. This confirms that the two copies of the Ctx genetic element are widely separated in the classical biotypes of *V. cholerae* (28). Virulence determinant genes *toxR* (43), *tcp* operon (42), and the Ctx genetic elements (32) comprising *zot*, *cep*, *ace*, and *ctxAB* genes are widely separated on the physical map. From the limited number of genes positioned on the physical map, it

569B genome and five *Not*I fragments of the O395 genome was identified (Fig. 3D). Identification of the repetitive DNA sequence in the 4.2-kb insert in clone NLH 115 is in progress.

Mapping of similar-sized fragments. To determine the positions of *Not*I fragments N9 and N10 which appeared as a doublet, on the map, genomic DNA was digested with *Not*I and *Sfi*I and double-digestion products were separated by PFGE. The digestion profile (Fig. 4a) revealed that one of the two fragments N9 and N10 has an *Sfi*I site(s) and the other does not. Linking clones NLB 2, NLH 117, and NLH 108 (Table 3) showed that the doublet N9-N10 has linkages with fragments N16, N17, and N25. When these linking clones were used as probes in Southern blot hybridization of the *Not*I- or *Not*I-*Sfi*I-double-digested 569B genome, clone NLB 2 hybridized with the fragment in which there is no *Sfi*I site (which will hereafter be referred to as N9) and N16 in both *Not*I- and *Not*I-*Sfi*I-double-digested DNAs (Fig. 4b, lanes 1 and 2). Clone NLH 117 also hybridized with fragment N9 in both *Not*I- and *Not*I-*Sfi*I-double-digested DNAs, with fragment N25 in *Not*I-digested DNA, and with a different fragment, X, in the double digest (Fig. 4b, lanes 3 and 4) which appeared following digestion of N25 with *Sfi*I. Thus, fragments N16 and N25 are linked to N9, and the linkage is -N25-N9-N16-. Clone NLH 108, on the other hand, hybridized with the N9-N10 doublet and N17 in *Not*I-digested DNA and with N17 and a different fragment following double digestion (data not shown). Hence, fragment N17 is linked to N10, which has an *Sfi*I site in it.

*Not*I fragments N20 and N21, which are also of similar size, were resolved by digesting excised *Ceu*I fragments with *Not*I to completion. The fragments were end labeled and separated by PFGE. One of the fragments in the N20-N21 doublet is located in fragment C1 (which will be referred to as N20), and the other one is located in fragment C2 (which will be referred to as N21). This was confirmed by Southern blot hybridization of

FIG. 4. (a) PFGE separation of *V. cholerae* 569B genomic DNA digested with *Not*I, *Sfi*I and *Not*I, or *Sfi*I. Electrophoresis was performed as described in the legend to Fig. 1A. (b) Determination of *Not*I fragments linked to N9 and N10 by Southern hybridization of *Not*I and *Sfi*I double-digested 569B genomic DNA using nick-translated linking clones NLB 2 (lanes 1 and 2) and NLH 117 (lanes 3 and 4) as probes. Lanes 1 and 3, *Not*I-digested 569B genomic DNA; lanes 2 and 4, *Not*I- and *Sfi*I-digested 569B genomic DNA. X, fragment derived from N25 following double digestion.

TABLE 5. Probes used for localizing genes on the physical map of the genome of *V. cholerae* 569B

Probe	Gene	Source	Fragment hybridized		Reference
			*Not*I	*Ceu*I	
pCVD15	*ctx* (cholera toxin)	*V. cholerae*	N4, N15	C1, C2	19
pJT470	*mutS* (DNA mismatch repair)	*V. cholerae*	N15	C1	5
PJT250	*mutL* (DNA mismatch repair)	*V. cholerae*	N11	C6	5
pJA360	*mutU* (DNA mismatch repair)	*V. cholerae*	N13	C1	
pRB101	*dam* (adenine methylase)	*V. cholerae*	N9	C4	3
pDCM1	*vsr* (short-patch DNA repair)	*E. coli*	N7	C1	8
pSB180	*sulA* (cell division inhibitor)	*V. cholerae*	N21	C1	11
pSB180	*cdp* (concatemer destabilizer)	*V. cholerae*	N21	C1	11
pKV7	*rpoH* (gene for σ^{32})	*E. coli*	N11	C6	45
pOF12	*groEL* (gene for Hsp60)	*E. coli*	N9	C4	16
pKP31	*dnaK* (gene for Hsp70)	*E. coli*	N22	C1	4
pKY3	*secY* (protein translocation)	*E. coli*	N9	C4	37
Oligomer[a]	*toxR* (virulence gene activator)	*V. cholerae*	N16	C4	43
PCR probe[b]	*tcp* (toxin-coregulated pilus)	*V. cholerae*	N14	C1	20a

[a] 5'-CGGGATCCTACTCACACACTTTGATGGC-3'.
[b] *V. cholerae* classical specific primer sequences 5'-CACGATAAGAAAACCGGTCAAGAG-3' and 5'-ACCAAATGCAACGCCGAATGGAGC-3'. The amplicon size was 618 bp.

appears that there are regions on the *V. cholerae* genome with high gene density. Of the 27 genes positioned, 11 are clustered within a 500-kb (N16-N9-N25-N11-N3; Fig. 5) region.

DISCUSSION

A physical map of the *V. cholerae* 569B genome has been constructed by using PFGE and hybridization techniques, and 26 homologous and heterologous genes and operons were assigned to the physical map. To our knowledge, this is the first combined physical and genetic map of the genome of *V. cholerae*, an organism for which a genetic map is not available. The genomes of strains 569B and O395 are circular, and the size has been estimated to be 3,200 kb, which is about 70% of the

size of the *E. coli* (40) and *Salmonella typhimurium* (24) genomes. The genome sizes of a majority of human pathogens range between 1.6 and 2.6 Mb (21). These organisms are metabolically less active than *E. coli*. The genome size of classical *V. cholerae* is close to that of *Yersinia pestis* (3.5 Mb) (26), and these organisms can be grown in defined media similarly to *E. coli*. The genome size of the biotype El Tor of *V. cholerae* is about 2.5 Mb (36), and these cells are also metabolically active. Thus, the genes controlling the vital metabolic functions are present in *V. cholerae* cells in spite of their genomes being significantly smaller than that of *E. coli*.

A total of 59 restriction sites were mapped by using three restriction endonucleases and by combining results obtained from three different experimental approaches. Linking clones were used to determine the proximity of neighboring PFGE fragments. The linking-clone data were combined with the second method, fragment hybridization, which was used to determine overlapping fragments. *Ceu*I- and *Sfi*I-generated fragments were either hybridized to *Not*I-digested DNA filters or digested completely with *Not*I and end labeled (Table 4). The third method used was analysis of partially digested DNA fragments. The *Ceu*I digestion fragments of the genomes of strains 569B and O395 were assembled from the analysis of partial digestion fragments alone. The *Ceu*I maps of strains 569B and O395 are identical. The *Not*I and *Sfi*I digestion patterns of the genomes of these two strains, however, showed distinct RFLP (Fig. 1A). The enzyme *Ceu*I has seven sites in the genomes of 569B and O395, as reported for *E. coli*, *S. typhimurium*, and several other gram-negative bacteria (23). This enzyme cleaves DNA at no sites other than the *rrn* operons. Thus, it appears that *V. cholerae* also has seven rrn operons. This supports the hypothesis that the locations and number of *rrn* operons in gram-negative bacteria are conserved. *V. cholerae* genes *dam*, *rpoH*, *mutL*, and *groEL* are located close to *rrn* operons as in *E. coli* (2), suggesting that the organization of genes in the neighborhood of *rrn* loci might be maintained in different gram-negative organisms.

Linking clones have been used so far to construct physical maps of *Rhodobacter sphaeroides* (41), a *Synehococcus* sp. (13), and *Neisseria gonorrhoeae* (14). The modified method of constructing linking-clone libraries described in the present report is perhaps the simplest among the existing methods and also allows isolation of a large number of linking clones from a

FIG. 5. Physical and genetic mapping of the *V. cholerae* 569B genome using the enzymes *Not*I, *Ceu*I, and *Sfi*I. The *Sfi*I map is not complete. Restriction fragments are numbered on the basis of size (Table 1). The genetic markers are described in Table 5. The position of the genetic marker in a particular fragment is arbitrary, and the positions of the markers in a single fragment do not reflect their true order in the chromosome. *Ceu*I sites were taken as the positions of the *rrn* operons. The innermost circle represents the scale in megabases.

single experiment. This method is a modification of the approach used to generate a human chromosome 3-specific *Not*I linking-clone library (44). More than 70% of the *Not*I fragments of the *V. cholerae* 569B genome were linked by using linking clones alone. The linking-clone libraries generated during the present study can be used to examine the chromosomal organization of the biotype El Tor and the current epidemic strain *V. cholerae* O139 Bengal, the genome sizes of which are 500 to 600 kb smaller than those of classical strains (6, 36). It appears that the 500- to 600-kb genomic DNA missing in the El Tor biotype is not essential for maintaining the pathogenic and epidemic potentials of this organism, since the strains belonging to the El Tor biotype are as virulent as the classical ones. The linking-clone libraries will be useful for identifying the DNA that is present in the chromosomes of classical strains but absent in the El Tor and O139 strains and in searching for new genes in *V. cholerae*.

Strain 569B is considered unusual from the point of view of regulation of virulence determinants, since this strain is independent of ToxR and ToxS for the expression of the ToxR regulon unlike other toxinogenic *V. cholerae* strains, such as strain O395, in which ToxR controls the virulence regulon (15). It is also assumed that this strain undergoes rapid genetic variation under laboratory conditions. However, in the course of our studies on the construction of the physical map of this strain during the last several years we have never detected any RFLP in the *Not*I or *Sfi*I restriction fragments of the genome of strain 569B.

A number of homologous and heterologous genes have been positioned on the physical map of the *V. cholerae* genome, and with the addition of more genes the utility of the map will be expanded and its resolution will improve. This will lead to more insights into chromosome organization, the mechanisms of gene regulation, and the molecular basis of pathogenicity of this important human enteric pathogen.

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