

# *Vibrio cholerae* O139 Bengal: Combined Physical and Genetic Map and Comparative Analysis with the Genome of *V. cholerae* O1

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**A combined physical and genetic map of the genome of strain SG24 of *Vibrio cholerae* O139 Bengal, a novel non-O1 strain having epidemic potential, has been constructed by using the enzymes *NotI*, *SfiI*, and *CeuI*. The genome of SG24 is circular, and the genome size is about 3.57 Mb. The linkages between 47 *NotI* and 32 *SfiI* fragments of *V. cholerae* SG24 genomic DNA were determined by combining two approaches: (i) identification of fragments produced by enzyme I in fragments produced by enzyme II by the method of fragment excision, redigestion, and end labeling and (ii) use of the linking clone libraries generated from the genome of classical O1 strain 569B. The linkages between nine *CeuI* fragments were determined primarily by analyses of partial fragments of the *CeuI*-digested genome. More than 80 cloned homologous and heterologous genes, including several operons, have been positioned on the physical map. The map of the SG24 genome represents the second map of a *V. cholerae* genome, and a comparison of this map with that of classical O1 strain 569B revealed considerable diversity in DNA restriction sites and allowed identification of hypervariable regions. Several genetic markers, including virulence determinant genes, are in different positions in the SG24 and 569B genomes.**

*Vibrio cholerae*, a noninvasive, gram-negative bacterium, is the causative agent of the diarrheal disease cholera. The specificity of the somatic O antigen of *V. cholerae* resides in the polysaccharide moiety of the lipopolysaccharide present in the outer membrane, which forms the basis of the serological classification of this organism (42). The *V. cholerae* strains causing epidemic cholera have, until recently, been confined to serogroup O1, which consists of two biotypes, classical and El Tor. The classical biotype was responsible for cholera epidemics till 1961, when the El Tor biotype displaced it. *V. cholerae* strains other than O1, which are collectively called non-O1 vibrios, can cause only sporadic infections and are believed to lack the potential to cause epidemics (30). One of the two events, the more alarming one, has dominated the global cholera scenario in the present decade; this was the unprecedented emergence in late 1992 in India of a novel strain of *V. cholerae* which does not agglutinate with O1 polyvalent antiserum but has epidemic and endemic potential, a phenomenon that has never occurred in the recorded history of cholera (1, 13, 36). Strains isolated from different parts of India and Bangladesh during the epidemic were found to be of clonal origin (5, 6) and were classified as new serovar O139, synonym Bengal. The other event was the dramatic and unexpected reappearance of epidemic cholera caused by *V. cholerae* O1 El Tor in South America in January 1991, after a 100-year absence on that continent (21). These two events have necessitated a renewed look into all aspects of the organism that are related to pathogenesis. The epidemic caused by *V. cholerae* O139 persisted for about a year (31, 32) and was again displaced by El Tor. Several lines of evidence have, however, suggested that O139 originated from

the El Tor biotype (4, 6, 10, 13, 43) by the acquisition of a 35-kb DNA segment which replaced most of the O1 antigen-encoding *rfb* gene cluster of the recipient strain (8, 14). Thus, serogroup O139 combines the virulent properties of epidemic strains with the outer appearance of nonepidemic strains.

By using restriction enzymes which have a single site in either the core region or the direct repeat sequence (RS) of the CTX genetic element (27), it was shown that the genomes of most of the O139 strains have two copies of the CTX genetic element in tandem connected by two RSs (6). The chromosomal location of the CTX genetic element in an O139 strain is the same as that reported for El Tor vibrios. The organization of the virulence gene cassettes in different O139 strains showed genetic heterogeneity in the population. While most of the epidemic O139 strains have two copies of the CTX genetic element, in some strains the number of elements has been amplified and in at least one strain a copy of the element has been deleted (6).

The genomes of El Tor strains isolated immediately before and after an O139 outbreak showed extensive restriction fragment length polymorphism (RFLP) among themselves and with the genome of O139 (33, 46). In late 1996, the appearance of a *V. cholerae* O139 strain having altered antibiotic sensitivity compared to that of the O139 previously seen (29) has complicated the epidemiological scenario of *V. cholerae* and has necessitated an examination of possible rearrangements in the genome underlying such rapid changes in phenotypic traits, which are unexpected in well-characterized clonal strains within such a short period. In view of the fact that the genetic basis of *V. cholerae* tropism and pathogenesis is still mostly unknown, comparative genome mapping studies to appraise the extent of genome diversity will be of interest, particularly since the emergence of new variants of this organism having epidemic potential with altered genotypes or phenotypes is turning out to be widespread rather than exceptional (20). The physical map of a classical O1 strain has been constructed (12, 25), and there was previously no second map for comparison of the genomes of *V. cholerae* strains in more detail. It is in this context that the present report describes the construction of a macrorestriction map of the genome of O139 by use of the

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enzymes *NotI*, *SfiI*, and *CeuI*. About 80 homologous and heterologous genes and operons have been positioned on the physical map. A comparison of the *V. cholerae* O139 genome with that of classical O1 revealed several gross differences.

#### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *V. cholerae* classical O1 strain 569B, El Tor O1 strain CO457, and O139 Bengal strain SG24, which were 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 (38). *Escherichia coli* cells were grown in a gyratory shaker at 37°C in Luria-Bertani broth with appropriate antibiotics whenever required.

**Preparation of high-molecular-weight genomic DNA and enzyme digestion.** Agarose plugs of cells in the logarithmic phase of growth were prepared and digested with restriction enzyme *NotI*, *SfiI*, or *CeuI* as described previously (25, 39). For *CeuI* digestion, five or six sliced agarose pieces were incubated in 2× buffer supplied by the manufacturer. After 15 min, the buffer was replaced with 100 μl of fresh 1× buffer containing 10 μg of bovine serum albumin and 4 U of the enzyme, and incubation was continued at 37°C for 3 h. For partial digestion with *CeuI*, six agarose pieces were digested with 1 U of the enzyme in 100 μl of the reaction mixture for 2 h at 37°C.

**PFGE and hybridization experiments.** Pulsed-field gel electrophoresis (PFGE) of enzyme-digested DNA was carried out in a Pulsaphor Plus System with a hexagonal electrode array (Pharmacia, Uppsala, Sweden) as described previously (25, 33, 39). Phage λ multimeric DNA and yeast chromosomal DNA were used as molecular mass markers. Preparation of <sup>32</sup>P-labeled DNA probes and Southern blot hybridization conditions were as described previously (25, 39). End labeling of DNA fragments following *NotI*, *SfiI*, or *CeuI* digestion was done by incubating the agarose blocks in a buffer containing Klenow enzyme and [α-<sup>32</sup>P]dCTP and subjecting them to PFGE followed by autoradiography.

**Isolation, redigestion, and radiolabeling of DNA fragments.** After PFGE, restriction fragments of genomic DNA digests were excised from low-melting-point agarose gels under long-wavelength UV light, and the agarose slice containing the DNA fragment was digested with a second enzyme and labeled as described previously (25).

#### RESULTS AND DISCUSSION

**Restriction fragment analysis and genome size.** Previous studies on the *V. cholerae* genome (6, 25, 39) showed that the enzymes *NotI* and *SfiI* digest the genomic DNA into a small number of large fragments that can be resolved by PFGE. To construct the physical map of the genome of *V. cholerae* O139, intact genomic DNA of strain SG24 in agarose blocks was digested with *NotI*, *SfiI*, and *CeuI* and the resulting fragments were separated by PFGE. The endonuclease *CeuI*, which cleaves the genomic DNAs of all of the organisms examined so far at the 23S rRNA of the *rrn* operon and nowhere else in the genome (22, 26), has nine sites in the genome of *V. cholerae* O1 and O139 (33).

Ethidium bromide-stained gels of PFGE-separated genomic DNA fragments of strain SG24 showed that *NotI*, *SfiI*, and *CeuI* digestion yielded 35, 28, and 8 fragments, respectively (Fig. 1). For comparison, the *NotI*, *SfiI*, and *CeuI* digestion profiles of the genomes of El Tor O1 strain CO457, which displaced O139, and classical O1 strain 569B were also examined. To identify the smaller fragments that are not resolved in stained gels, the restriction fragments were end labeled with [α-<sup>32</sup>P]dCTP in agarose slices before PFGE and autoradiograms were subsequently examined. In addition to those identified in stained gels, 11 *NotI* fragments, 4 *SfiI* fragments, and 1 *CeuI* fragment were detected in the autoradiograms (Fig. 2). The *NotI* and *SfiI* digestion profiles of the genomes of SG24, CO457, and 569B exhibited distinct RFLP. The polymorphism is more pronounced between SG24 and 569B. The *CeuI* digestion profiles of SG24 and El Tor strain CO457 were identical (Fig. 1), which was expected since O139 originated from El Tor. Parenthetically, the *CeuI* digestion profiles of El Tor strain VC44, a strain isolated immediately before the O139 epidemic, and SG24 exhibited RFLP (33).

Restriction fragments were named on the basis of the en-

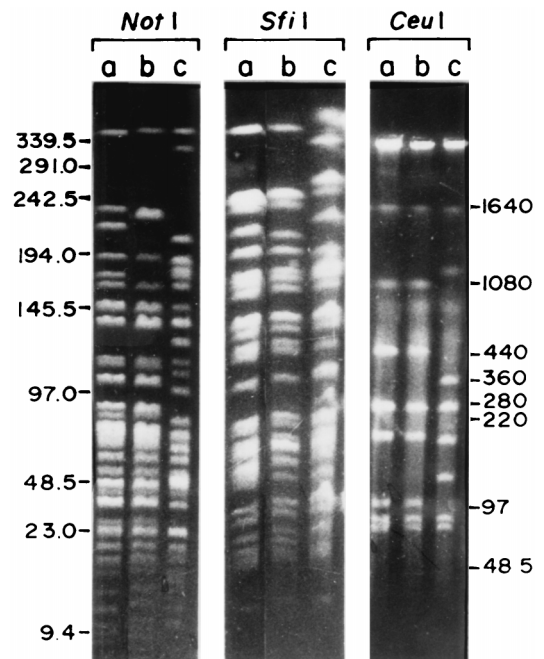


FIG. 1. PFGE separation of *NotI*-, *SfiI*-, and *CeuI*-digested genomic DNAs of *V. cholerae* CO457 (lanes a), SG24 (lanes b), and 569B (lanes c). The enzyme-digested DNA was separated on 1% FastLane Agarose (FMC) with pulse time ramping between 5 and 25 s for 22 h at 10 V/cm and 3°C for *NotI* and *SfiI*. For the separation of *CeuI* fragments, electrophoresis was carried out by using pulse times of 5 (4 h), 10 (4 h), 25 (4 h), and 100 (12 h) s at 10 V/cm and 3°C. The gels were stained with ethidium bromide. Marker molecular sizes, in kilobases, are shown in the margins.

zymes that generated the fragments (N, *NotI*; S, *SfiI*; C, *CeuI*) and were numbered on the basis of size in descending order (Fig. 2). *NotI* fragments N2 and N3, fragments N6 and N7, fragments N8, N9, and N10, fragments N13 and N14, fragments N17 and N18, fragments N21 and N22, fragments N25 and N26 and *SfiI* fragments S2 and S3 and fragments S18 and S19 are the same in size. The genome size of strain SG24 was estimated to be about 3.57 Mb (Table 1), as opposed to the 2.2 Mb reported previously (6). Better resolution of DNA fragments following enzyme digestion and refinement of PFGE methods by altering various parameters allowed us to resolve several previously reported *NotI* fragments as doublets and triplets (Table 1). Thus, the genome size of O139 is close to that of classical O1 strains (25).

***CeuI* cleavage map of the *V. cholerae* O139 genome.** *CeuI* digestion of the *V. cholerae* SG24 genome produced nine fragments ranging from 1,400 to 6 kb (Fig. 2; Table 1). It has been reported that, as in other organisms, *CeuI* has a site only in the *rrn* operon of *V. cholerae* (33). Thus, there are nine *rrn* operons in the *V. cholerae* O139 genome. The *CeuI* map of the SG24 genome was constructed primarily from the analyses of 10 partial fragments of the *CeuI*-digested genome (Table 2). The linkage between the *CeuI* fragments of the genome of *V. cholerae* SG24 is thus C2-C1-C3-C8-C5-C7-C6-C9-C4-C2, and the genome is circular (see Fig. 5). The *CeuI* linkage map of the genome of O139 is different from that of classical O1 strain 569B (see Fig. 6).

***NotI* and *SfiI* cleavage maps of the *V. cholerae* O139 genome.** The linkages between 47 *NotI* and 32 *SfiI* fragments of *V. cholerae* SG24 genomic DNA were determined by combining three approaches. These included (i) identification of fragments produced by enzyme I in fragments produced by enzyme

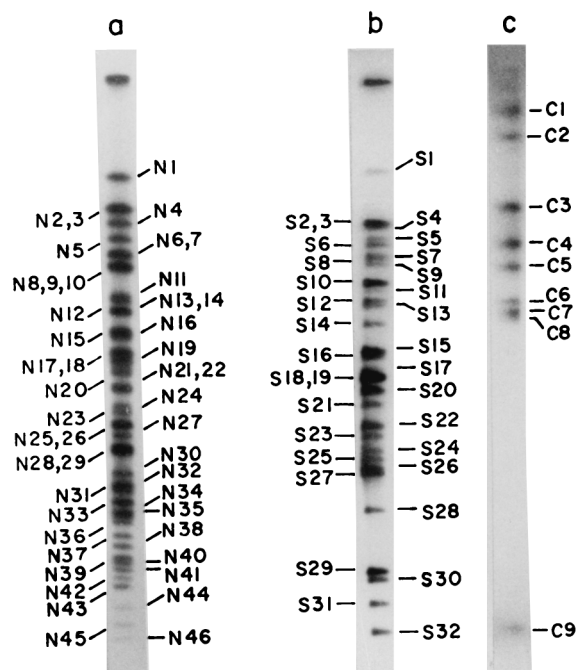


FIG. 2. Autoradiogram of PFGE-separated *NotI* (lane a), *SfiI* (lane b), and *CeuI* (lane c) fragments of genomic DNA of *V. cholerae* O139 strain SG24. DNA in an agarose block was digested with the enzyme, end labeled with [ $\alpha$ - $^{32}$ P]dCTP, and electrophoresed as described in the legend to Fig. 1. The gels were dried and exposed to X-ray film. Restriction fragments were named on the basis of the enzymes that generated the fragments (N, *NotI*; S, *SfiI*; C, *CeuI*) and were numbered on the basis of size in descending order.

II by the method of fragment excision, redigestion, and end labeling, (ii) use of the linking clone libraries generated from the genome of classical O1 strain 569B (26), and (iii) analysis of partial digestion products.

The *CeuI* linkage map was used as the skeleton to determine the linkages between the *NotI* and *SfiI* fragments of the genome of *V. cholerae* SG24. To identify linkages between different *NotI* or *SfiI* fragments, individual PFGE-separated *CeuI* fragments were excised from the gel, digested with *NotI* or *SfiI*, end labeled, and subjected to PFGE (Fig. 3A). Alternatively, the gel-excised *CeuI* fragments were used as probes for Southern blot hybridization of the *NotI*- or *SfiI*-digested genome of *V. cholerae* SG24 (Fig. 3B). Both of these approaches allowed clubbing of *NotI* and *SfiI* fragments that are linked. For example, when gel-excised *CeuI* fragment C3 was digested with the enzyme *NotI*, end labeled, resolved by PFGE, and autoradiographed, nine *NotI* fragments (N17, N45, N21, N16, N32, N40, N39, and two flanking fragments arising from N4 and N19; Fig. 3A, lane a), and seven *SfiI* fragments (S6, S24, S30, S22, S31, and two flanking fragments, S4 and S18; Fig. 3A, lane c) lit up, showing that these fragments are linked. The *NotI* or *SfiI* fragments overlapping the two ends of C3 were determined by hybridizing fragment C3 with the PFGE-resolved *NotI*- or *SfiI*-digested genomic DNA (Fig. 3B, part a). The order in which these fragments are present in the genome was determined by using position-specific probes, such as linking clones (Table 3), *NotI* or *SfiI* fragments that are included in the *CeuI* fragment, and cloned-gene probes (Table 4). Similar analyses were performed with gel-excised *CeuI*, *NotI*, and *SfiI* fragments.

The *NotI* linking clone library generated from the genome of classical O1 strain 569B (25) has been extensively used to determine the linkages between *NotI* fragments. Some representative examples of hybridization experiments using linking

clones as probes are shown in Fig. 4. The linkages between the *NotI* fragments of the 569B genome determined by using linking clones NLH44 and NLB29 (25) are different from those of the *NotI*-digested genome of SG24 (data not shown). Linking clone NLH19 linked *NotI* fragments N15 and N26 of the 569B genome (25), but it hybridized with only *NotI* fragment N15 of the SG24 genome, indicating deletion of the linked fragment (Fig. 4). Besides *NotI* linking clones, several *SfiI* and *CeuI* fragments of the O139 genome having a single *NotI* site served as *NotI* linking clones, and *NotI* and *CeuI* fragments having a single *SfiI* site were used as *SfiI* linking clones (Fig. 4; Table 3).

**Mapping of similar-size fragments.** To identify and position similar-size fragments on the map, a combination of the following approaches was adopted: (i) analysis of PFGE-sepa-

TABLE 1. Sizes of restriction fragments generated by cleavage of the chromosome of *V. cholerae* O139 strain SG24

Fragment no.	Size (kb) <sup>a</sup>		
	<i>NotI</i>	<i>SfiI</i>	<i>CeuI</i>
1	370	380	1,400
2	240	264	1,000
3	240	264	460
4	190	257	245
5	166	220	170
6	150	210	103
7	150	180	87
8	136	175	80
9	136	170	6
10	136	145	
11	116	140	
12	114	135	
13	108	130	
14	108	110	
15	92	90	
16	89	85	
17	84	80	
18	84	75	
19	78	75	
20	72	60	
21	66	55	
22	66	50	
23	58	45	
24	54	40	
25	49	32	
26	49	30	
27	44	28	
28	38	15	
29	38	10	
30	30	9	
31	23	8	
32	22	7	
33	20		
34	18		
35	17		
36	16		
37	14		
38	13		
39	12		
40	11		
41	10		
42	9.5		
43	9		
44	8		
45	7.5		
46	7		
47	2.4		

<sup>a</sup> Totals: *NotI*, 3,570 kb; *SfiI*, 3,574 kb; *CeuI*, 3,551 kb.

TABLE 2. Partial digestion products of the SG24 genome generated by the enzyme *CeuI*

Partial fragment (size [kb])	Possible composition (size [kb])
P1 (113)	C6, C9 (109)
P2 (250)	C5, C8 (250)
P3 (355)	C4, C6, C9 (354)
P4 (465)	C4, C6, C7, C9 (441)
P5 (550)	C3, C8 (540)
P6 (600)	C4, C5, C6, C7, C9 (611)
P7 (680)	C4, C5, C6, C7, C8, C9 (691) or C3, C8, C5 (710)
P8 (1,250)	C2, C4 (1,245)
P9 (1,900)	C1, C3 (1,860)
P10 (2,450)	C1, C2 (2,400)

rated genomic DNA digested with any two of the three enzymes *NotI*, *SfiI*, and *CeuI*, (ii) hybridization of comigrating fragments generated by one enzyme with the genomic DNA digested with a second enzyme, and (iii) use of suitable linking clones or cloned genes. For example, to position *NotI* fragments N2 and N3, N6 and N7, and N13 and N14, which appeared as doublets on the map, genomic DNA was digested with *NotI* and *CeuI* and double digestion products were separated by PFGE. The digestion profile revealed that one of the two fragments N2 and N3, N6 and N7, or N13 and N14 has a *CeuI* site(s) and the other does not (data not shown). One of the fragments in the N2-N3 doublet is located in fragment C2 (which will be referred to as N2), and the other is located in fragment C6 (which will be referred to as N3). This was confirmed by Southern blot hybridization of the *NotI*-digested O139 genome by using fragment C6 (Fig. 3B, part b) or C2 as the probe and by end-labeling experiments. Linking clone NLH44 hybridized with fragment N8 and the doublet N28-N29 (data not shown). When the same probe was used for the Southern hybridization of *NotI*-*SfiI*-double-digested genomic DNA, it hybridized with fragment N8 and another smaller fragment designated X (generated from either N28 or N29), indicating the presence of an *SfiI* site(s). The fragment in the N28-N29 doublet hybridizing with linking clone NLH44 will be referred to as N29. Linking clone NLB51 hybridized with N1 and N28 and with S3. Besides, *dnaK* also hybridized with N28 and S3. Linking clone NLH8 hybridized with C3 and S6 and links fragment N16 and either N21 or N22 (which will be referred to as N21). Linking clone NLB29 links fragments N5 and N22 (Table 3) and also hybridized with C2 and S7. While positioning comigrating *NotI* fragments on the map, it was revealed that N8-N9-N10 is a triplet (Table 1). This was confirmed by digesting the gel-excised triplet with the enzyme *AscI* or by hybridization using fragment C1, C2, or C4 as the probe (data not shown). Doublets N2-N3 and N6-N7 were also digested with *AscI*, which confirmed that they are doublets (data not shown). The other comigrating fragments were similarly positioned on the map. By combining all of the approaches, a multienzyme macrorestriction map of the genome of *V. cholerae* O139 strain SG24 has been constructed (Fig. 5).

**Positioning of genetic markers on the physical map.** More than 80 cloned genes (Table 4) and nine *rnm* operons have been positioned on the physical map of the *V. cholerae* O139 genome by hybridization using homologous and heterologous probes (Fig. 5). The gene probes used comprised O139 antigen-specific genes, the hemolysin gene, DNA repair genes, heat shock protein genes, cholera toxin genes, trimethoprim-sulfamethoxazole resistance genes, and some other virulence determinant genes (Table 4). In the *V. cholerae* genome, the

*CeuI* sites are located only in the *rnm* operons (33) similar to those reported for several other bacteria (22). The genes have been positioned on the physical map on fragments they hybridized with and do not represent the exact locations and orientations 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.

**Comparison of the physical maps of O139 and classical O1 strain 569B.** A comparison of the physical map of the *V. cholerae* O139 genome constructed in the present study with that of classical O1 strain 569B (25) revealed conservation in certain

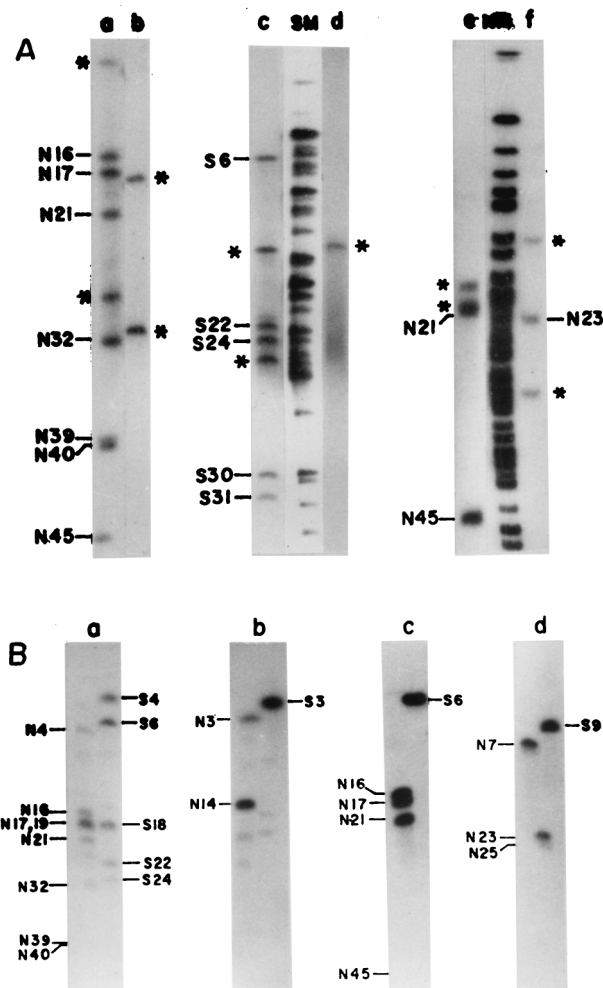


FIG. 3. (A) Identification of *NotI* or *SfiI* fragments of the *V. cholerae* SG24 genome in isolated *CeuI* fragments. *CeuI* fragments C3 (lanes a and c) and C6 (lanes b and d) were completely digested with *NotI* or *SfiI*, end labeled, and separated by PFGE. Identification of *NotI* fragments in isolated *SfiI* fragments. *SfiI* fragments S6 (lane e) and S9 (lane f) were completely digested with the enzyme *NotI*, end labeled, and separated by PFGE. *NotI* (NM)- or *SfiI* (SM)-digested, end-labeled SG24 genomic DNA was used as a marker for identification of linked fragments. Asterisks denote the flanking DNA fragments. (B) Assignment of the flanking *NotI* or *SfiI* fragments (shown in panel A) of *V. cholerae* SG24 genomic DNA in isolated *CeuI* fragments. Radiolabeled *CeuI* fragment C3 (a) or C6 (b) was used as the probe for Southern hybridization with the *NotI*- or *SfiI*-digested genome of *V. cholerae* SG24. For identification of the flanking *NotI* fragments in isolated *SfiI* fragments of the genomic DNA of strain SG24, radiolabeled *SfiI* fragment S6 (c) or S9 (d) was used as the probe for Southern hybridization with the *NotI*- or *SfiI*-digested SG24 genome. Hybridizations were carried out at 60°C. The filters were washed under stringent conditions as described in Materials and Methods.

TABLE 3. Linkages between *NotI* and *SfiI* fragments of the genome of strain SG24 of *V. cholerae* O139 determined by using linking clones or restriction fragments

Clone or restriction fragment	Linkage
NLB2	N12-N27
NLB33	N23-N25
NLB39	N13-N27
NLB42	N7-N23
NLB5	N6-N10
NLB51	N1-N28
NLB92	N2-N5
NLB29	N5-N22
NLB130	N4-N36
NLS5	N18-N20
NLS16	N24-N30
N2 <sup>a</sup>	S5-S12
N5	S5-S7
N7 <sup>a</sup>	S9-S20
N11	S11-S20
C6	N3-N14
C8	N19-N26
NLH35	N10-N42
NLH39	N19-N26
NLH8	N16-N21
NLH30	N4-N39
NLH44	N8-N29
NLH108	N13-N27
NLH117	N9-N33
NLH127	N1-N31
NLH132	N15-N34
S5	N2-N5
S7	N5-N22
S10	N12-N27
S11	N11-N22
S12	N10-N42
S13	N13-N27
S20	N7-N11
S23	N25-N29

<sup>a</sup> Fragment excised from PFGE-separated *NotI* and *CeuI* double-digested genomic DNA.

regions but gross rearrangements in the genome of O139 with respect to the classical O1 genome (Fig. 6). Restriction site variability between the two genomes is another feature emerging from the comparison. A fraction of sites is conserved between the two strains. The genome of *V. cholerae* O139 has gained a number of *NotI* sites. The variable regions in the genomes of both strains might be due to exchange of genetic elements. Extensive RFLP was recorded between the genomes of classical O1, El Tor, and O139 following digestion with *NotI*, *SfiI*, or *CeuI* (Fig. 1) and is more predominant between the O139 and classical O1 genomes. The genome of SG24 has more *NotI* (total, 47) and *SfiI* (total, 32) sites (Table 1) than does the genome of classical O1 strain 569B (25). Although the chromosomal locations of several genes, like *rpoH*, the *tcp* gene cluster, etc., are conserved (Fig. 6), they show RFLP because of loss or creation of restriction sites.

*NotI* linking clones of the genome of the classical O1 strain allowed identification of regions of the O139 genome that might have undergone rearrangements. Several linking clones of the genome of classical O1 strain 569B hybridized with only one fragment of the *NotI*-digested O139 genome (Fig. 4), indicating that either the *NotI* site was lost or one of the linked *NotI* fragments underwent deletion in the O139 genome. While *NotI* linking clone NLH44 linked fragments N6 (175 kb) and N22 (42 kb) in the *NotI*-digested 569B genome, it showed

TABLE 4. Homologous and heterologous genes positioned on the physical map of the genome of strain SG24 of *V. cholerae* O139

Probe	Gene(s) (product or function)	Source	Reference
pCVD15	<i>ctxAB</i> (cholera toxin)	<i>V. cholerae</i>	18
pKB370	<i>mutK</i> (DNA mismatch repair), <i>cspA</i> (cold shock protein), <i>rmt</i> (RNA methyltransferase)	<i>V. cholerae</i>	Unpublished
pJT470	<i>mutS</i> (DNA mismatch repair)	<i>V. cholerae</i>	3
pJA360	<i>mutU</i> (DNA mismatch repair)	<i>V. cholerae</i>	Unpublished
pRB101	<i>dam</i> (adenine methylase)	<i>V. cholerae</i>	2
pGS350	<i>rpoH</i> ( $\sigma^{32}$ )	<i>V. cholerae</i>	40
pOF12	<i>groEL</i> (Hsp60)	<i>E. coli</i>	16
pSC830	<i>dnaK</i> (Hsp70)	<i>V. cholerae</i>	Unpublished
pDB19	<i>secY</i> (protein translocation)	<i>V. cholerae</i>	7
pVM7	<i>toxR</i> (virulence gene activator)	<i>V. cholerae</i>	28
pSC18.1	<i>tcp</i> (toxin-coregulated pilus)	<i>V. cholerae</i>	19
pEB1111	<i>tnpA</i> (transposase, IS1004)	<i>V. cholerae</i>	9
pSXT1	<i>sxt</i> (sulfamethoxazole and trimethoprim resistance)	<i>V. cholerae</i>	44
pKY017	<i>hlyA</i> (hemolysin)	<i>V. cholerae</i>	45
pEB1150	<i>rfb</i> <sub>O139</sub> (O antigen)	<i>V. cholerae</i>	8

linkages between fragments N8 (136 kb) and N29 (38 kb) of the *NotI*-digested SG24 genome. This clone hybridized with the *SfiI*-digested genomes of strains 569B and O139 in fragments S3 (280 kb) and S1 (380 kb), respectively (data not shown). Linking clone NLB29 hybridized with one similar-size *NotI* fragment of both the 569B and SG24 genomes, and the other fragment showed RFLP.

The copy number of direct RS IS1004 is reduced to four in the genome of O139 from eight in that of classical O1 (9). Most of the genetic markers examined exhibited RFLP in the *NotI*- or *SfiI*-digested genomic DNAs of O139 and classical O1. In classical O1 strains, the CTX genetic element is present in two

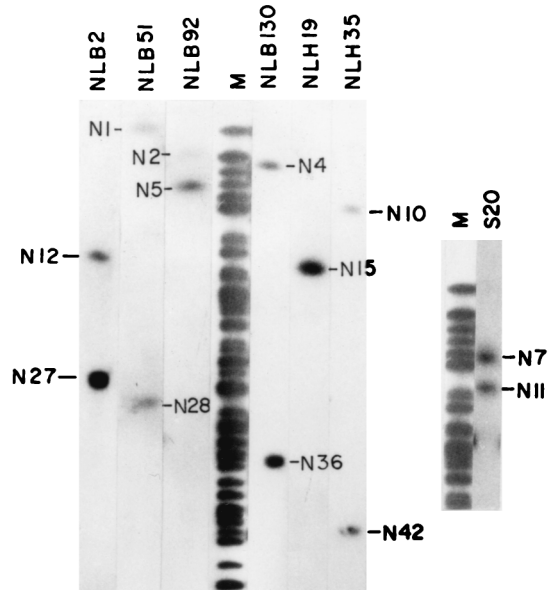


FIG. 4. Determination of linkages between *NotI* fragments of SG24 genomic DNA by using a *NotI* linking clone library of the 569B genome and isolated *SfiI* fragments of the SG24 genome. Nick-translated linking clones NLB2, NLB51, NLB92, NLB130, NLH19, NLH35, and NLH44 and *SfiI* fragment S20 were hybridized with PFGE-separated *NotI* fragments of the SG24 genome. *NotI*-digested and end-labeled genomic DNA was used as molecular size markers (lane M) for identification of linked fragments. The *NotI* fragments that are linked to each other are marked.



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