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vacB, a Novel Chromosomal Gene Required for Expression of Virulence Genes on the Large Plasmid of *Shigella flexneri*

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Shigellae, the causative agents of bacillary dysentery, are capable of adhering to and invading epithelial cells and spreading into adjacent cells. A chromosomal mutant of *Shigella flexneri* 2a YSH6000 with reduced invasive capacity was isolated by Tn*5* insertion mutagenesis. The linkage of the mutant phenotype to the Tn*5* insertion was determined by P1 phage transduction. The site of the Tn*5* insertion was assigned to a *Not*I chromosomal restriction map, confirming that the virulence-associated locus, designated *vacB*, is a new locus on the chromosome. In the *vacB* mutant, production of the four plasmid-encoded virulence antigens, IpaB, -C, and -D and VirG, decreased to a low level compared with that in the wild type. In contrast, levels of transcription of the operons for *virG*, *ipa*, region-3.4, region-5, *virF*, and *virB* on the large plasmid, as determined by Northern dot blotting, were unaffected in the *vacB* mutant. Furthermore, transcriptional activation of the *ipa* operon by exploiting a *tac* promoter could not restore the *vacB* mutant to production of the same levels of the IpaB, -C, and -D proteins as those in the wild type, indicating that the *vacB* locus is involved in expression of the *vir* genes on the large plasmid at the posttranscriptional level. Cloning followed by nucleotide sequencing of the *vacB* region showed it to contain a 2,280-bp open reading frame encoding an 86.9-kDa protein located 669 bp downstream from the 3′ end of the open reading frame for the *purA* gene. Disruption of the *vacB* gene of other serotypes of *Shigella* spp. and enteroinvasive *Escherichia coli* (EIEC) resulted in reduced expression of virulence phenotypes, indicating that the *vacB* gene encodes a novel type of virulence-associated gene required for the full expression of the virulence phenotype of *Shigella* spp. and EIEC.

Shigellae cause bacillary dysentery in humans and monkeys. The essential early steps in infection comprise bacterial adherence and invasion of colonic epithelial cells followed by intracellular bacterial multiplication and spread of invading bacteria to adjacent cells (10). Seven separate virulence-associated DNA segments have been identified on the large 230-kb plasmid of *Shigella flexneri* 2a. In a 31-kb DNA sequence, five contiguous segments, designated *virB*, *ipaBCD* (*ipa*), and region-3, -4, and -5, were characterized. The *virB* gene acts as a positive regulator for the expression of the other four virulence regions (1); the *ipa* operon encodes three invasion-associated antigens, IpaB, -C, and -D; and region-3, -4, and -5 are required for both adherence to epithelial cells and excretion of the Ipa proteins (20a). The *virG* gene, outside the cluster, encodes a 116-kDa immunogenic, surface-exposed outer membrane protein and is essential for the ability of intracellular bacteria to spread into adjacent cells (11, 12). The *virF* gene, encoding a 30-kDa protein, regulates the expression of the *virG* and *virB* genes positively at the transcriptional level (1, 24). Consequently, expression of the virulence (*vir*) genes on the large plasmid is under the control of a dual activation system directed by the *virF* and *virB* genes (1).

Various classes of virulence-associated loci have been identified on the chromosome of *S. flexneri*. Interestingly, some of them appear to be involved in expression of the *vir* genes encoded by the large plasmid. For example, a locus near the *trp* gene designated *virR* was shown to control the temperature-dependent production of the invasion-associated antigens IpaB, -C, and -D (9, 14). The KcpA locus, near the *purE* gene, was shown to mediate intercellular spread of the invading shigellae by controlling expression of a 120-kDa plasmid-encoded antigen which appears to be equivalent to the 116-kDa VirG protein (21). The *envZ-ompR* genes, near the *malA* gene, have also been shown to be involved in the expression of virulence through the regulation of virulence genes on the large plasmid (3). The VacC locus near *phoBR* has been shown to be involved in the expression of the *ipa* operon (20). These findings indicate that the pathogenicity of *S. flexneri* requires various genes dispersed around the chromosome as well as on the large plasmid and that the expression of *vir* genes encoded by the large plasmid is under the control of various regulatory systems.

In this context, we have screened chromosomal Tn*5* insertion mutants for reduced invasive capacity. The chromosomal locus described in this paper, designated *vacB*, has been shown to affect the production of the virulence-associated antigens IpaB, -C, and -D and VirG, as well as the capacity of bacteria to adhere and spread intercellularly. It did not affect the level of transcription of the *vir* genes on the large plasmid, suggesting that the *vacB* gene is involved in expression of the *vir* genes on the large plasmid at the posttranscriptional level. The analysis of the protein products and the nucleotide sequence of the *vacB* region revealed that the *vacB* gene was located near the *purA* gene and encoded an 86.9-kDa protein. Thus, the evidence presented in this study raises the possibility that the *vacB* gene encodes

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference
Strains		
YSH6000	Virulent strain of *S. flexneri* 2a	29
2457T	Virulent strain of *S. flexneri* 2a	6
CS2313	YSH6000 *vacB*::Tn*5*	This study
CS2314	YSH6000 *vacB*::Tn*5* transductant	This study
CS2315	2457T *vacB*::Tn*5* transductant	This study
YSH6200	Derivative of YSH6000 cured of the 230-kb virulence plasmid	24
CS1649-11	Virulent strain of *S. dysenteriae*	This study
CS2419-1	CS1649-11 *vacB*::pTB510	This study
CS1651-21	Virulent strain of *S. flexneri*	This study
CS2419-2	CS1651-21 *vacB*::pTB510	This study
CS1649-1	Virulent strain of *S. boydii*	This study
CS2419-3	CS1649-1 *vacB*::pTB510	This study
CS1651-77	Virulent strain of *S. sonnei*	This study
CS2419-4	CS1651-77 *vacB*::pTB510	This study
CS1503-1	Virulent strain of enteroinvasive *E. coli*	This study
CS2419-5	CS1503-1 *vacB*::pTB510	This study
Plasmids		
pCHR404	pBR322 derivative with a part of Ap^r gene replaced by 1.2-kb Tp^r fragment	This study
pTB101	Expression vector containing the *tac* promoter, $lacI^q$ Ap^r Tp^r	This study
pTB102	pTB101 carrying P_{tac}-*ipa* operon fusion	This study
pTB501	See text	This study
pY228	See text	This study
pTB502	See text	This study
pTB503	Deletion derivative of pTB502	This study
pTB504	Deletion derivative of pTB502	This study
pTB505	Deletion derivative of pTB502	This study
pTB506	Deletion derivative of pTB502	This study
pTB507	Deletion derivative of pTB502	This study
pTB508	pT7-5 carrying *Nae*I-*Sal*I fragment of *vacB* region	This study
pTB509	pT7-6 carrying *Nae*I-*Sal*I fragment of *vacB* region	This study
pGP704	Suicide vector containing *ori* from R6K plasmid	16
pTB510	pGP704 carrying *Stu*I-*Sac*I fragment of *vacB* region	This study

a novel type of regulatory function involved in expression of the full virulence phenotype of *S. flexneri*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. The *vacB*::pTB510 mutation was constructed by insertion of the replication-deficient plasmid pTB510 containing a *Stu*I-*Sac*I fragment from the *vacB* structural gene into the *vacB* coding region on the chromosome. Some of the plasmids were constructed as follows. The chromosome of CS2313 (the *vacB*::Tn*5* mutant) digested with *Sal*I was cloned in pBluescriptII SK^+ (Stratagene, La Jolla, Calif.), and transformants were selected for kanamycin resistance (Km^r). One of the plasmids from the Km^r transformants contained a 7.5-kb *Sal*I fragment, comprising a part of Tn*5* containing the Km^r gene and IS*50*L and a 4.9-kb adjacent chromosomal DNA sequence. This was designated pTB501 (see Fig. 5). pY288 was a clone of the chromosomal DNA library of YSH6200 (35) which hybridized with the 4.9-kb DNA sequence of pTB501. pTB502 was constructed by ligating the 2.2-kb *Eco*RI-*Pst*I segment of pTB501 and the 1.8-kb *Pst*I-*Sal*I segment of pY288 and cloning into the *Eco*RI-*Sal*I sites of pCHR404.

Tn*5* insertion mutagenesis of YSH6000. Random insertion mutagenesis with Tn*5* was performed as described previously with slight modification (20). YSH6000 carrying pCHR81 (30) was streaked out on Mueller-Hinton agar plates containing 12.5 μg of trimethoprim per ml, and each colony which developed at 30°C was inoculated into 1 ml of LN broth (28) containing 50 μg of kanamycin per ml and 10 μg of thymine per ml. After incubation at 30°C overnight, each culture was appropriately diluted, spread on Trypticase soy broth agar (BBL) containing 0.01% Congo red, kanamycin (50 μg/ml), and thymine (10 μg/ml), and incubated at 42°C. At this time Km^r trimethoprim-sensitive (Tp^s) colonies which were negative for Congo red binding (Pcr^-) (13) were isolated, purified once on the same selective plates at 37°C, and kept at −70°C in LN broth containing 50% glycerol.

Virulence phenotype assays. Invasion of rhesus monkey kidney epithelial (MK2) cells (29), adherence to MK2 cells (4), the focus plaque-forming assay (Fpa) (18, 29, 35), and the contact hemolysis activity (Chl) assay (26) have been previously described.

Preparation of DNA, *Not*I restriction digestion in agarose beads, and PFGE. Chromosomal DNA of YSH6000 and CS2313 was prepared as described previously by Smith et al. (31). For *Not*I digestion, the agarose block (100 μl) was incubated at 37°C for 16 h in the presence of 10 U of *Not*I restriction enzyme in a total volume of 250 μl of reaction mixture (10 mM Tris [pH 7.5], 7 mM $MgCl_2$, 150 mM NaCl, 7 mM 2-mercaptoethanol, 0.015% Triton X-100, 100 μg of bovine serum albumin per ml). An orthogonal field alteration gel electrophoresis system (pulsed-field gel electrophoresis [PFGE]) was used. In PFGE, a 1.0% agarose gel in 1× TBE buffer (100 mM Tris, 100 mM boric acid, 2 mM EDTA [pH

8.3]) was used at 10°C. The gels were run at 10 V cm^{-1} for 45 h with pulse times ranging from 10 to 50 s. Lambda concatemers (Clontech Laboratories) were used as standards for DNA fragment length.

DNA sequencing. The sequences of both DNA strands were determined by the chain termination method of Sanger et al. (25), using a Sequenase 7-deaza-dGTP kit (United States Biochemical Corp.), following cloning into pBluescriptII SK^+ and KS^+ (Stratagene).

Western blotting and RNA dot blotting. Bacteria were grown at 37°C in L broth to mid-log phase (about 0.5 optical density unit at 600 nm) and harvested. For Western blotting (immunoblotting), bacteria were washed and lysed in sample buffer for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by boiling for 3 min. For extraction of RNA, bacteria were suspended in lysis buffer for RNA preparation. Western blotting of SDS-PAGE-resolved antigens was performed as described previously (1). RNA dot blot hybridization with ^{32}P-labelled DNA fragments was performed as described previously (19, 33). DNA fragments used as probes for RNA dot hybridization were the same as those described by Tobe et al. (33) except for the *virG* probe, which consisted of an 812-bp *Hin*cII-*Eco*T14I fragment containing the 5′ end of the *virG* gene.

CAT assay. Bacteria grown in L broth at 37°C were harvested, suspended in 0.5 ml of 0.2 M Tris-HCl (pH 7.5), and sonicated (Model W-225R; Branson Sonic Power Co.) for 1 min in short bursts (of about 10 s each). The supernatants were collected by centrifugation at 10,000 × *g* for 5 min at 4°C. The extracts were incubated with [^{14}C]chloramphenicol (Amersham Corp., Arlington Heights, Ill.) to determine chloramphenicol acetyltransferase (CAT) activity as described by Gorman et al. (8).

Gene product analysis. Protein products specific for the cloned fragment were analyzed by the T7 RNA polymerase/promoter system (32) with K38/pGP1-2 (23, 32) harboring the *Nae*I-*Sal*I fragment cloned on pT7-5 or pT7-6.

Nucleotide sequence accession number. *vacB* nucleotide sequence data will appear in the DDBJ, EMBL, and GenBank data bases with accession no. D11024.

RESULTS

Isolation and characterization of a chromosomal mutant defective in invasive ability. In an attempt to determine the involvement of chromosomal genes in expression of the invasive phenotype of *S. flexneri*, we performed transposon mutagenesis and screened for mutants with lower invasive capacity by exploiting the Pcr^+ phenotype, the ability of *S. flexneri* to bind Congo red, known to be closely associated with the invasive phenotype (13). The wild-type *S. flexneri* 2a YSH6000 was mutagenized by Tn*5* insertion, and the Pcr^- clones were identified by plating onto Trypticase soy broth agar plates containing Congo red (see Materials and Methods). The large plasmid was then extracted from each Pcr^- derivative, and its *Sal*I restriction profile was examined. In this manner, only 1 clone, named CS2313, of 217 Pcr^- mutants was found to have no apparent alteration in any of the *Sal*I fragments of the large plasmid (data not shown).

To confirm that the Pcr^- phenotype of CS2313 was linked to the Tn*5* insertion, P1 phage transduction was performed from CS2313 into YSH6000 and 2457T, another wild-type *S. flexneri* 2a strain (5). All of the 24 resulting Km^r transductants of the two *S. flexneri* strains acquired a Pcr^- phenotype. Thus, we tentatively named the locus *vacB*. The other virulence-associated phenotypes were further examined with CS2313 and with two Km^r transductants of YSH6000 and 2457T, designated CS2314 and CS2315, respectively (Table 2). The invasive capacities of the two mutants were determined by two methods, an epithelial cell invasion test with MK2 cells and a Chl assay with sheep erythrocytes (see Materials and Methods), and were shown to be less than 1/10 of that of the wild-type strains. However, the invasive capacities of the three mutants were still significantly higher than that of YSH6200, the large-plasmid-free derivative of YSH6000 (Table 2).

TABLE 2. Expression of virulence-associated phenotypes in the wild type and *vacB* mutants

Strain	Genotype or characteristic	Virulence-associated phenotype			
		Inv[a]	Chl[b]	Pcr[c]	Fpa
YSH6000	Wild type	39.5	1.03	+++	Plaque
CS2313	*vacB*::Tn*5*	3.0	0.07	+	Small plaque
CS2314	*vacB*::Tn*5*	6.6	0.10	+	Small plaque
2457T	Wild type	34.5	1.08	+++	Plaque
CS2315	*vacB*::Tn*5*	3.7	0.09	+	Small plaque
YSH6200	Without 230-kb plasmid	<0.2	0.01	−	Negative

[a] Percentage of infected MK2 cells (11).
[b] Chl is expressed as optical density at 545 nm (26).
[c] Pigmentation on Congo red: +++, red; +, pale red; −, white (29).

The mutants' abilities to spread into adjacent cells were compared with those of wild type strains in the Fpa with confluent monolayers of MK2 cells (Table 2). The mutants had poor spreading ability compared with that of the wild-type strains; even after 3 days of incubation the resulting plaques were tiny, suggesting that the mutation on the chromosome of the *vacB* locus had also affected the ability of the organisms to spread intercellularly.

Effect of the *vacB* mutation on expression of the virulence genes on the large plasmid. Since the ability of *S. flexneri* to invade epithelial cells and to spread into adjacent cells is dependent on the *ipa* genes and the *virG* gene, respectively, on the large plasmid, we investigated the effect of the *vacB* mutation on the expression of the plasmid-encoded virulence genes. The production of the four virulence antigens, IpaB, IpaC, IpaD, and VirG, expressed in CS2314 and CS2315 was compared with that in YSH6000 and 2457T by Western blotting. As shown in Fig. 1, the amounts of IpaB, IpaC, IpaD, and VirG proteins in the mutants were greatly decreased compared with amounts in the wild-type strains. The amounts of IpaB protein in the *vacB* mutants, CS2314 and CS2315, were 28 and 25%, respectively, of that found in the wild type when measured by whole-cell labeling with [^{35}S]methionine followed by immunoprecipitation with antibody against IpaB protein (data not shown). The low levels of Ipa proteins and VirG protein found in the mutants were consistent with their low capacity for invasion and intercellular spread.

Involvement of the *vacB* locus in the posttranscriptional level of expression of virulence genes on the large plasmid. To determine how the *vacB* locus was involved in the expression of the virulence genes on the large plasmid, the levels of transcription from six virulence operons, *virF*, *virB*, *ipa*, region-3.4, region-5, and *virG*, on the large plasmid of YSH6000 and CS2314 were assayed by Northern blot (RNA blot) hybridization. The levels of mRNA for each of the six virulence operons in CS2314 were essentially the same as those in YSH6000 (Fig. 2). To examine the activities of the

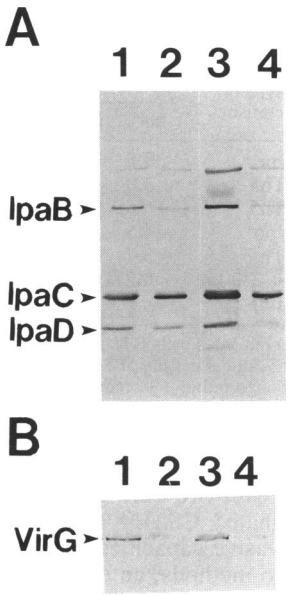

FIG. 1. Expression of IpaB, -C, and -D and VirG proteins in the wild type and the *vacB* mutants of *S. flexneri*. An identical amount of total protein (20 μg) from each strain grown at 37°C was electrophoresed in SDS-polyacrylamide gels and transferred to nitrocellulose membranes. (A) IpaB, -C, and -D proteins were detected by Western blotting with serum from a convalescent shigellosis patient. (B) VirG protein was detected with anti-VirG antibody. Lanes: 1, YSH6000; 2, CS2314; 3, 2457T; 4, CS2315.

promoters of the *ipa*, region-3.4, and region-5 operons, a plasmid carrying an operon fusion constructed with the P1 (ipa), P4 (region-3.4), or P5 (region-5) promoter and the CAT structural gene was introduced into strains 2457T (wild type) and CS2315 (*vacB* mutant), and the CAT activity expressed in each strain was measured. The results are shown in Table 3. The activity for each promoter was not reduced markedly in the mutant compared with that in the wild type. Furthermore, in order to examine the effect of the *vacB* mutation on

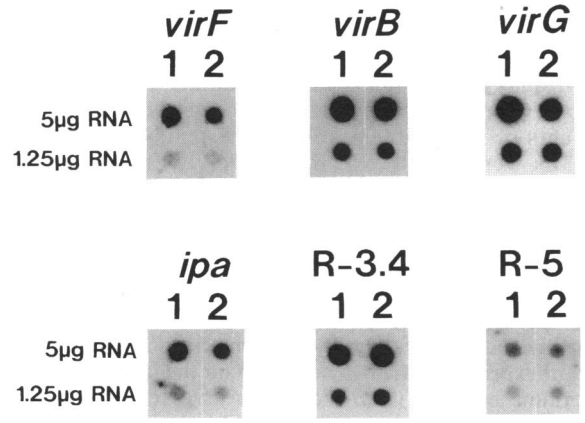

FIG. 2. Transcription of virulence operons on the large plasmid in the wild type and the *vacB* mutants of *S. flexneri*. RNA was extracted from exponentially growing bacteria at 37°C. Aliquots of total RNA (5 and 1.25 μg) were dotted onto nitrocellulose membranes, and mRNAs for *virF*, *virB*, *virG*, *ipa*, region-3.4, and region-5 were detected by hybridization with a ^{32}P-labeled DNA probe specific for each operon. Lanes: 1, YSH6000; 2, CS2314.

TABLE 3. Promoter activities in the wild type and *vacB* mutants

Promoter	Host strain	Promoter activity[a] (U/mg)
P1	2457T (wild type)	237
	CS2315 (*vacB*)	270
P4	2457T (wild type)	553
	CS2315 (*vacB*)	460
P5	2457T (wild type)	63
	CS2315 (*vacB*)	67

[a] Expressed as CAT activity. One unit corresponds to the activity necessary to acetylate 1 nmol of chloramphenicol per 1 min at 37°C. Results represent the mean values for two experiments.

posttranscriptional steps, a plasmid harboring the P_{tac}-*ipa* operon fusion was introduced into the *vacB* mutant. The amounts of Ipa proteins expressed in the *vacB* mutant were still lower than that in the wild type even after induction of transcription of the *ipa* operon (Fig. 3). Thus, these results indicate that the *vacB* locus is not involved in the expression of the transcriptional activators *virF* and *virB* but is involved in regulation at a posttranscriptional level for the *ipa*, region-3.4, and region-5 operons on the large plasmid.

Physical assignment of the *vacB* locus to the *Not*I restriction map of the chromosome of YSH6000. In our previous studies (19, 20), we constructed a *Not*I restriction map of the 4,592-kb chromosome of YSH6000 and assigned each of the nine virulence-associated loci tagged by Tn*5* insertions to 1 of the 19 *Not*I fragments. Hence, we sought to assign *vacB*::Tn*5* in CS2313 to the *Not*I map by using PFGE. As

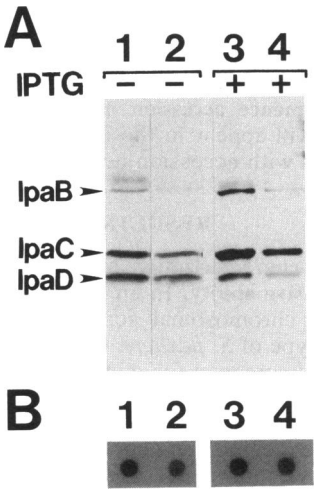

FIG. 3. Effect of transcriptional activation of the *ipa* operon on expression of Ipa proteins in the *vacB* mutants. Bacteria grown at 37°C in LN broth with or without 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were harvested and subjected to Western blot analysis for Ipa proteins (A) and RNA dot hybridization for *ipa* transcripts (B). Strain 2457T containing pTB102 (P_{tac}-*ipa*) (lanes 1 and 3) or CS2315 containing pTB102 (lanes 2 and 4) were grown without (lanes 1 and 2) or with (lanes 3 and 4) IPTG. (A) IpaB, -C, and -D proteins were detected with antibodies raised against synthetic peptides of the IpaB, -C, and -D proteins. Lanes 1 and 2, 20 μg of whole-cell extract was loaded; lanes 3 and 4, 2 μg of extract was loaded. (B) Transcripts from the *ipa* operon were detected by RNA dot hybridization with a ^{32}P-labeled DNA probe specific for the *ipa* operon. Lanes 1 and 2, 5 μg of total RNA was dotted on the membrane; lanes 3 and 4, 0.5 μg of total RNA was dotted.

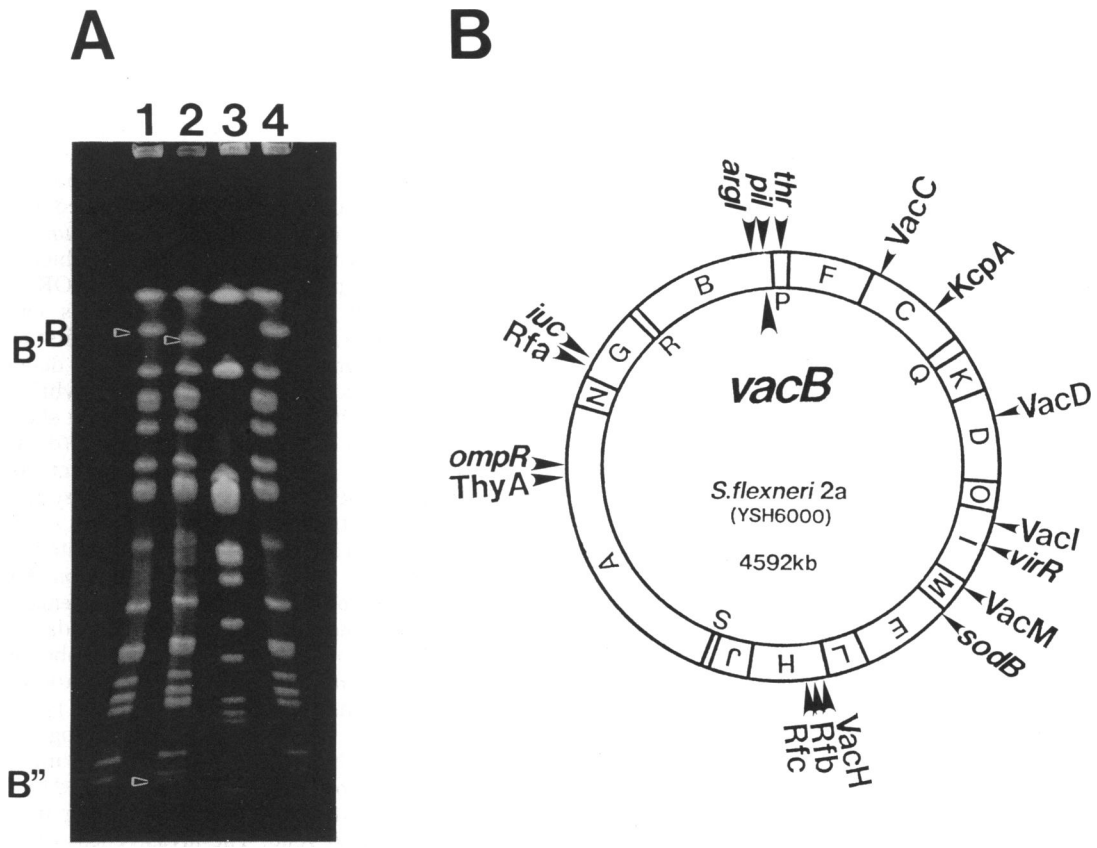

FIG. 4. Direct assignment of *vacB*::Tn*5* to the physical map of the YSH6000 chromosome. (A) PFGE separation of *Not*I-digested chromosomal DNA. Lanes: 1 and 4, YSH6000; 2, CS2313; 3, AB1157 (*E. coli*). Fragment B and the two smaller fragments (B′ and B″) obtained following Tn*5* insertion into fragment B are indicated with arrowheads. (B) Circular map of chromosome with *Not*I restriction fragments and location of *vacB*::Tn*5*. Chromosomal virulence-associated genes reported previously (3, 6, 7, 14, 17, 20) are indicated with external arrowheads. *argI* and *pil* genes located on *Not*I fragment B and the *thr* gene located on *Not*I fragment P are also indicated with external arrowheads. The location of *vacB*::Tn*5* is indicated with an internal arrowhead.

shown in Fig. 4A, the 500-kb *Not*I B fragment was cleaved into 455- and 45-kb segments, demonstrating that it contained the Tn*5* insertion and hence the *vacB* locus. Fragment B was shown to hybridize with three DNA probes, for *glpK* (88.4 min), *argI* (96.6 min), and *pil* (*fim*) (97.6 min), of *Escherichia coli* K-12, and the smaller 45-kb *Not*I segment hybridized with a *Not*I linking probe, S598 (data not shown), hybridizable to *Not*I fragments B and P (19). Thus, the site of the Tn*5* insertion in the *vacB* locus could be localized at 45 kb away from the end adjacent to *Not*I fragment P, whose DNA sequence contains the *thr* gene at 100 min in *E. coli* (2).

Identification and product analysis of the *vacB* region. By cloning a 7.5-kb *Sal*I fragment of CS2313, which consisted of a part of Tn*5* containing the Km^r gene and IS*50*L and its 4.9-kb adjacent chromosomal DNA sequence (pTB501), and using the Tn*5*-flanking DNA as a probe for the *vacB* gene, we screened a previously constructed genomic library of the YSH6200 chromosome (35) for DNA segments which hybridized with the DNA probe. Plasmid pY288 thus isolated contained a 7.6-kb DNA fragment whose restriction map with *Eco*RV and *Pst*I was similar to that of the flanking region of IS*50*L of Tn*5* in *vacB* (Fig. 5). However, the 7.6-kb DNA segment did not restore the Pcr^+ or Inv^+ phenotype to CS2313. Therefore, we extended the left side of the DNA sequence (Fig. 5) by ligating the 2.2-kb *Eco*RI-*Pst*I segment of pTB501 to the *Pst*I-*Sal*I segment of pY288. The resulting

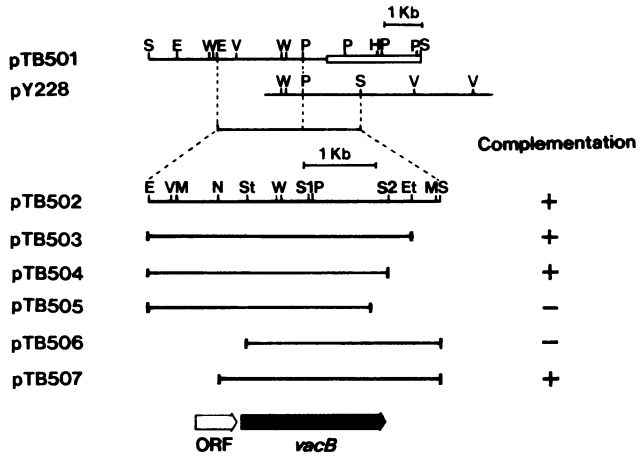

FIG. 5. Cloning and fine mapping of the *vacB* gene. Cloned chromosomal DNA fragments are represented by horizontal lines. The fragment carried on pTB501 was constructed with a part of Tn*5* (open box) and its adjacent chromosomal DNA sequence. VacB function on the cloned fragment was determined by complementation tests for the Pcr^+ phenotype and expression of Ipa proteins (+, restored; −, not restored). The closed box with an arrowhead indicates the structural *vacB* gene, and the open box with an arrowhead indicates the ORF deduced from the nucleotide sequence upstream of the *vacB* gene. S, *Sal*I; E, *Eco*RI; V, *Eco*RV; P, *Pst*I; H, *Hin*dIII; M, *Mlu*I; N, *Nae*I; St, *Stu*I; S1, *Sac*I; S2, *Sac*II; Et, *Eco*T22I.

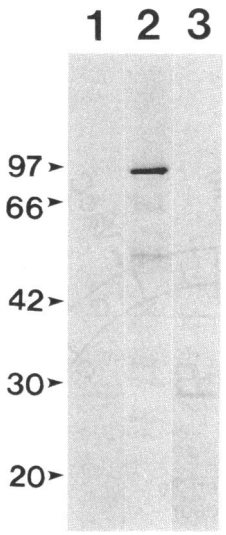

FIG. 6. Protein products from the DNA segment containing the *vacB* region. The *Nae*I-*Sal*I fragment of the *vacB* region was placed downstream of the bacteriophage T7 promoter, and the fragment-specific translation product was labeled with [^{35}S]methionine after induction of transcription by the T7 promoter. Lanes: 1, vector control; 2, pTB508 (transcription from *Nae*I to *Sal*I); 3, pTB509 (transcription from *Sal*I to *Nae*I). The positions of molecular mass markers (kilodaltons) are shown on the left.

4.5-kb DNA segment contained in pTB502 restored the virulence phenotypes to CS2313.

To further localize the *vacB* region, various deletion derivatives constructed from the 4.5-kb DNA segment and designated pTB503, pTB504, pTB505, pTB506, and pTB507 were tested for their ability to complement the *vacB* mutation. As shown in Fig. 5, the DNA sequence required for VacB function was localized to a 2.3-kb *Nae*I-*Sac*II segment. The protein products from this DNA region were analyzed by using the phage T7 RNA polymerase-dependent expression system (Fig. 6). The results showed that a 90-kDa protein was expressed from the *Nae*I-*Sal*I 3.0-kb DNA segment in one orientation to the phage T7 RNA polymerase-directed promoter ϕ10 on the vector pT7-5, in which the 90-kDa protein was translated from the left to the right of the restriction map shown in Fig. 5.

Nucleotide sequence of the *vacB* region. The 2,984-bp *Mlu*I-*Sac*II fragment of pTB502, which contained the *Nae*I-*Sac*II segment encoding VacB function, was sequenced (Fig. 5). Two open reading frames (ORF) from nucleotide 210 to nucleotide 632 (ORF-1) and from nucleotide 674 to nucleotide 2953 (ORF-2) were found. The larger ORF (ORF-2), consisting of 2,280 bp and sufficient to encode the protein observed in the protein analysis (Fig. 6), was found within the 2.3-kb *Nae*I-*Sac*II segment. The deduced sequence of the 760 amino acids indicated a protein of 86.9 kDa, close to the apparent molecular mass of 90 kDa predicted from SDS gels. The other ORF, ORF-1, located upstream of ORF-2, was irrelevant because the *Nae*I site (at nucleotide 363) was within ORF-1, indicating that ORF-2 codes for the *vacB* gene. The Tn*5* insertion in CS2313 was found to disrupt ORF-2 at nucleotide 2413. The hydropathy profile of the amino acid sequence of the VacB protein deduced from the nucleotide sequence revealed that it is slightly hydrophilic with no typical signal sequence at the N terminus (data not shown). Thus, it is likely that the VacB protein is a cytoplasmic polypeptide.

A search of the GenBank and EMBL sequence data bases, using the DNASIS program (Hitachi Software Engineering Co., Tokyo, Japan), revealed a difference of only 2 bp compared with a reported DNA sequence from the *purA* region at 95 min on the *E. coli* K-12 map (2). The 2,984-bp DNA sequence determined in this study contained the last 2 bp at the 3′ end of the ORF for the *purA* gene and the immediate downstream DNA region, which contained an additional 435-bp ORF, corresponding to ORF-1 of the *vacB* region. The 86 N-terminal amino acids encoded by an unknown ORF (34) were identical to the first 86 amino acids of the protein encoded by ORF-2 (*vacB*) deduced from the nucleotide sequence (Fig. 7). Southern hybridization with a DNA probe from the *vacB* coding region showed the presence of the *vacB* gene in *E. coli* K-12 (data not shown). These results, together with the direct assignment of *vacB*::Tn*5* to the *Not*I map of the chromosome of YSH6000, clearly demonstrate that the *vacB* locus also exists in the chromosome of *E. coli* K-12 and is located 669 bp downstream of the 3′ end of the ORF for the *purA* gene.

Involvement of the *vacB* gene in virulence expression in *Shigella* spp. and EIEC. Southern hybridization using the coding region for the *vacB* gene as a probe against *Eco*RI-*Sal*I-digested chromosomal DNA from various serotypes of shigellae and enteroinvasive *E. coli* (EIEC) has revealed that all strains have a DNA fragment homologous to the *vacB* gene (data not shown). To construct *vacB* mutants of shigellae and EIEC, the *vacB* gene was disrupted by integration of the replication-deficient plasmid carrying the internal segment of the *vacB* gene. The invasiveness of the mutant, as measured by Chl assay, decreased to a level about 10 to 20% of that of the parental wild-type strain (Fig. 8). Expression of IpaB, -C, and -D proteins in the mutant was also reduced to a low level (data not shown). These results indicate that the *vacB* gene plays an important role in the expression of virulence of shigellae and EIEC through the regulation of virulence genes on the large plasmid.

DISCUSSION

It is reasonable to expect that expression of the *vir* genes on the large plasmid of *S. flexneri* would be controlled by complex regulatory systems acting at various stages of gene expression, allowing bacterial infection of the human colon to take place efficiently. Indeed, it has been shown that most of the *vir* loci on the chromosome, such as *virR* (14), *kcpA* (21), and *vacC* and *-M* (20), take part in regulating expression of the *vir* genes encoded by the large plasmid.

The *vacB* gene identified on the chromosome in this study would also fall into the same category as the *vir* loci on the chromosome, but it represents a novel type of gene; VacB functions at the posttranscriptional level to enable full production of the Vir proteins encoded by the *virG*, *ipa*, region-3.4, and region-5 operons on the large plasmid. This mechanism is thus a prerequisite for the full expression of the virulence phenotype of *S. flexneri* and EIEC.

In the *vacB* mutant, levels of production of the four virulence antigens, IpaB, IpaC, IpaD, and VirG, all decreased to levels lower than those in the wild type (Fig. 1). In contrast, the levels of transcription of *virF*, *virB*, *virG*, *ipa*, region-3.4, and region-5 in the *vacB* mutant were shown to be essentially the same as those in the wild type (Fig. 2). Furthermore, the activities of the three promoters, P1 for *ipa*, P4 for region-3.4, and P5 for region-5 (33), in the *vacB*

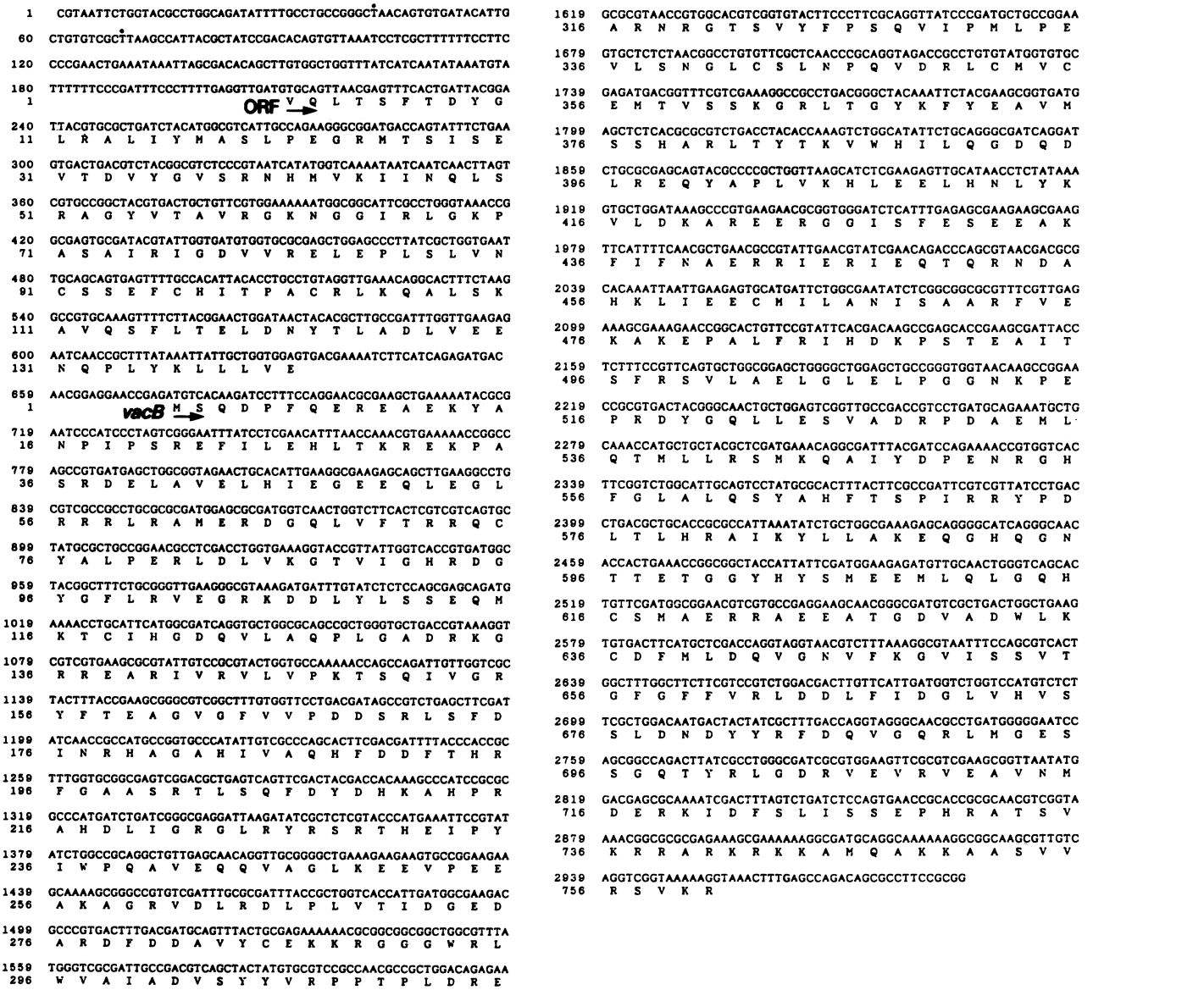

FIG. 7. Nucleotide sequence of the *vacB* region. The sequence of the sense strand is shown as well as the deduced amino acid sequence for two ORFs. Positions 674 to 2953 correspond to the *vacB* gene. The nucleotide sequence from positions 1 to 931 is identical to that of the *purA* region of *E. coli* K-12 (34) except for the base pairs at positions 42 and 69, which are indicated by dots above the sequence.

mutant were the same as those in the wild type regardless of the presence of *vacB*. In a previous study (33) we showed that expression of the *ipa* and region-3.4 and -5 operons were coordinately regulated by the 35-kDa VirB protein at the transcriptional level and that the transcription of the *virB* gene was positively regulated by *virF*. Therefore, it was clearly shown that expression of the two regulatory proteins, VirF and VirB, was not affected by the *vacB* mutation, and it was suggested that the *vacB* gene is involved in the expression of the virulence genes coded by the *ipa*, region-3.4, and region-5 operons at the posttranscriptional level. This was confirmed by the result of the experiment with the P_{tac}-*ipa* operon fusion. Full production of the three proteins induced by transcriptional activation of the *ipa* operon was accomplished only in the presence of VacB function. These results strongly indicated that the *vacB* gene is involved in the expression of *vir* genes at the posttranscriptional level but not at the transcriptional level.

The molecular mechanisms underlying regulation of the *vir* genes by the *vacB* gene are still obscure. Since the half-lives of mRNAs for the *virG*, *ipa*, and region-3.4 operons in the *vacB* mutant, as determined by Northern hybridization or S1 nuclease protection, were the same as those in the wild type (33a), the *vacB* mutation did not seem to affect the stability of mRNA transcribed from these operons. Thus, it is possible that the *vacB* product is necessary for efficient translation of *vir* genes on the large plasmid or stabilization of Vir proteins. The nucleotide sequences of *virG* (11), *ipa* (27), and region-4 (30a), as well as of the other *vir* genes encoded by the large plasmid of *S. flexneri*, have revealed a low GC content and different codon usage compared with those on the chromosome, suggesting that efficiency of

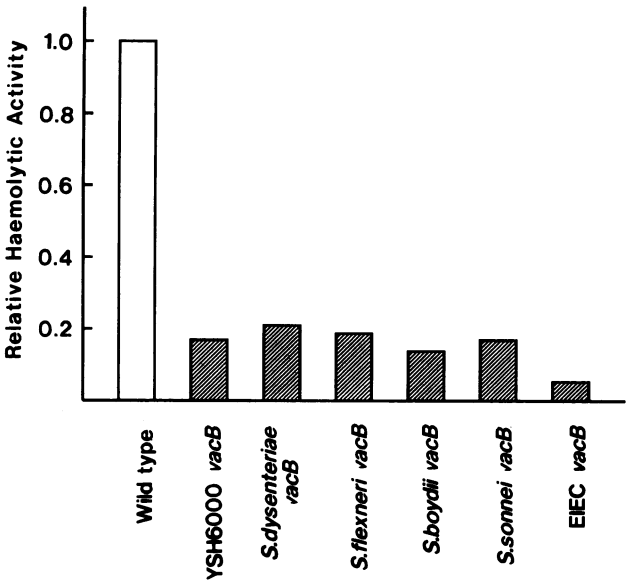

FIG. 8. Invasive activities of the *vacB* derivatives from shigellae and EIEC. Invasive activity was measured by Chl assay as described by Sansonetti et al. (26). The activities of the *vacB* derivatives represent the values relative to that for the parental strain.

translation of the *vir* genes on the plasmid was poor or that their products would be recognized as foreign proteins to be degradated preferentially in *S. flexneri*. Alternatively, the *vacB* protein may be necessary for other steps such as protein assembly or translocation of the Vir proteins across the cytoplasmic membrane, since the VirG protein is known to be located in the outer membrane (11) and IpaB, -C, and -D proteins are excreted outside the bacteria from the periplasmic space (unpublished data).

The location of the *vacB* gene on the chromosome of *S. flexneri* 2a YSH6000 was found to be near *purA* at 95 min on the chromosome of *E. coli* K-12 (2). The direct assignment of the *vacB* gene to the 4,592-kb chromosome of YSH6000 was done by using the measurements of the two segments generated by *Not*I cleavage following Tn*5* insertion into the *vacB* region in *Not*I fragment B. As shown in Results, the site of Tn*5* in the *vacB* region was shown to be 45 kb away from the end adjacent to *Not*I fragment P and was located near the *thr* gene at 100 min on the *E. coli* K-12 chromosome. The location of the *vacB* gene was further confirmed by showing a DNA sequence homologous with the *purA* region (34). The sequence of a previously reported truncated unknown ORF (34) was identical to the DNA sequence for the 5′ end of the ORF encoding the 86.9-kDa VacB protein. Thus, the *vacB* gene could be directly assigned to the chromosome maps of both YSH6000 and *E. coli* K-12, at 669 bp downstream of the *purA* gene.

Hence, the 45-kb segment generated by *Not*I cleavage of fragment B following Tn*5* insertion into *vacB* was expected to hybridize with the two DNA probes for the *argI* (22) and *pil* (15) loci, which are located at 96.6 and 97.6 min, respectively, on the chromosome of *E. coli* K-12 (2). However, Southern hybridization with the two DNA probes showed that they both hybridized with the larger 455-kb *Not*I segment of *Not*I fragment B, indicating that some DNA rearrangement or inversion had occurred, as seen in other DNA regions on the chromosome of YSH6000 (19). In any case, the results presented indicate that the *vacB* gene is a novel chromosomal gene essential for the full virulence phenotype of *S. flexneri*.

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