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Identification and Characterization of *phoN-Sf*, a Gene on the Large Plasmid of *Shigella flexneri* 2a Encoding a Nonspecific Phosphatase

KEI-ICHI UCHIYA,[1] MICHIYOSHI TOHSUJI,[1] TOSHIAKI NIKAI,[1] HISAYOSHI SUGIHARA,[1]* AND CHIHIRO SASAKAWA[2]

Department of Microbiology, Faculty of Pharmacy, Meijo University, Yagotoyama 150, Tenpaku-ku, Nagoya 468,[1] and Department of Bacteriology, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108,[2] Japan

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A gene encoding a nonspecific phosphatase, named PhoN-Sf, was identified on the large virulence plasmid (pMYSH6000) of *Shigella flexneri* 2a YSH6000. The phosphatase activity in YSH6000 was observed under high-phosphate conditions. However, it was found that low-phosphate conditions induced a slightly higher level of activity. The nucleotide sequence of the *phoN-Sf* region cloned from pMYSH6000 possessing the *phoN-Sf* gene encoded 249 amino acids with a typical signal sequence at the N terminus. The deduced amino acid sequence of the PhoN-Sf protein revealed significant homology to sequences of nonspecific acid phosphatases of other bacteria, such as *Providencia stuartii* (PhoN, 83.2%), *Morganella morganii* (PhoC, 80.6%), *Salmonella typhimurium* (PhoN, 47.8%), and *Zymomonas mobilis* (PhoC, 34.8%). The PhoN-Sf protein was purified, and its biochemical properties were characterized. The apparent molecular mass of the protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis was calculated to be 27 kDa. The 20 amino acids at the N terminus corresponded to the 20 amino acid residues following the putative signal sequence of PhoN-Sf protein deduced from the nucleotide sequence. The PhoN-Sf activity had a pH optimum of 6.6, and the optimum temperature was 37°C. The enzymatic activity was inhibited by diisopropyl fluorophosphate, *N*-bromosuccinimide, or dithiothreitol but not by EDTA. The subcellular localization of the PhoN-Sf protein in YSH6000 revealed that the protein was found predominantly in the periplasm. Examination of *Shigella* and enteroinvasive *Escherichia coli* strains for PhoN-Sf production by immunoblotting with the PhoN-specific antibody and for the presence of *phoN-Sf* DNA by using a *phoN-Sf* probe indicated that approximately one-half of the strains possessed the *phoN-Sf* gene on the large plasmid and expressed the PhoN-Sf protein. The Tn*5* insertion mutants of YSH6000 possessing *phoN-Sf*::Tn*5* still retained wild-type levels of invasiveness, as well as the subsequent spreading capacity in MK2 epithelial cell monolayers, thus suggesting that the PhoN-Sf activity is not involved in expression of the virulence phenotypes of *Shigella* strains under in vitro conditions.

The genus *Shigella*, whose members are gram-negative bacilli belonging to the family *Enterobacteriaceae*, comprises four species, *Shigella dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*. Dysentery, in addition to being caused by *Shigella* spp., is also caused by a class of pathogenic *Escherichia coli* strains, named enteroinvasive *E. coli* (EIEC), which closely resemble *Shigella* spp. Indeed, *Shigella* spp. and *E. coli* are closely related bacteria which are homologous over 90% of their chromosomal DNA (13). *Shigella* spp. and EIEC possess a 180- to 230-kb virulence plasmid on which major virulence determinants are located. Additionally, over 20 chromosomal virulence-associated genetic loci have been identified (34).

Infection by shigellae begins with the ingestion of bacteria, which, on reaching the colon, invade colonic epithelial cells. The invading bacteria then multiply and spread continuously to adjacent epithelial cells, a process which eventually leads to inflammation and ulceration of the colonic mucosa, resulting in bloody and mucoid diarrhea. The bacilli are excreted by the host and exposed to the natural environment. Thus, *Shigella* life cycles, including the processes leading to disease, are quite complicated, requiring numerous gene functions (34). Indeed, *Shigella* spp. possess complicated regulatory systems for expression of the major virulence determinants encoded by the large plasmid (32, 33), and the bacteria might acquire from other organisms some other genes apart from the virulence genes on the large plasmid or on the chromosome.

To date, over 30 virulence genes on the large plasmid of *S. flexneri* have been identified as the determinants for (i) production of invasins, IpaB, IpaC, and IpaD; (ii) transport functions ensuring the secretion and release of the Ipa proteins; (iii) intra- and intercellular spreading of invading bacteria; (iv) tissue invasion; and (v) regulation of plasmid-encoded virulence genes (34). The genetic regions have also been assigned to the *Sal*I restriction fragment map of the large plasmid of *S. flexneri* (38). These genes include *ipa* (6, 10, 36, 49), *icsB* (2), *mxi* (3, 4), *spa* (39, 50), *virA* (48), *virK* (26), *virG* (19, 23), *virB* (1, 44), *virF* (28, 29), and *sepA* (7). On the large 230-kb plasmid of *S. flexneri*, however, those genetic regions occupy only one-fifth of the total DNA, and the rest of the plasmid DNA remains open for the placement of some other genetic elements. In fact, genetic elements required for maintenance of plasmid replication (24, 42); five copies of *ipaH* genes, encoding the IpaH antigens (15); a gene encoding an ATP diphosphohydrolase activity (8); and some insertion-like elements related to IS*1*, IS*3*, or IS*4* (35, 38, 48) have been found on the large plasmid. In this context, we have been seeking genetic traits presented on the large plasmid (pMYSH6000) of *S. flex-*

* Corresponding author. Mailing address: Department of Microbiology, Faculty of Pharmacy, Meijo University, Yagotoyama 150, Tenpaku-ku, Nagoya 468, Japan. Phone: 81-52-832-1781, ext. 340. Fax: 81-52-834-8780.

neri 2a YSH6000 (38) by exploiting various biochemical reactions that are utilized by YSH6000 but not observed by YSH6200, the large-plasmidless derivative of YSH6000 (29).

In this work, we report a novel-gene-encoded nonspecific phosphatase activity, thereby named PhoN-Sf, encoded by pMYSH6000. Cloning and nucleotide sequencing of the *phoN-Sf* region revealed that the *phoN-Sf* gene existed in the 9.6-kb *Sal*I-I fragment of pMYSH6000 and encoded a 27.2-kDa protein whose deduced amino acid sequence was significantly similar to the other nonspecific acid phosphatases such as PhoN of *Providencia stuartii* and PhoC of *Morganella morganii* (43). The biochemical properties of the purified PhoN-Sf protein were significantly similar to those of the PhoC enzyme. The *phoN-Sf* homologs were distributed among *Shigella* and EIEC strains but were found in only one-half of the strains tested.

MATERIALS AND METHODS

Bacterial strains, plasmid, and culture media. *S. flexneri* 2a YSH6000 (38) and its 230-kb-plasmid-free derivative, YSH6200 (29), were used as positive and negative controls for enzyme assays. A 9.6-kb *Sal*I restriction fragment of pMYSH6000 cloned into pBR322Tp (35) was used as the source for cloning of the *phoN-Sf* gene. S85, M373, and M211 were isolated previously as the *Sal*I-I::Tn*5* insertion mutants of pMYSH6000 (40) and used to determine the sites of Tn*5* in the *phoN-Sf* sequence. *E. coli* JM109 (30) was routinely used for cloning and DNA sequencing. Plasmid pBluescript-IISK$^+$ (Stratagene) was used as the vector for cloning the *phoN-Sf* locus.

Bacteria were routinely grown at 37°C in LN broth (37). To impose phosphate limitation, bacteria were grown in the medium described by Levinthal et al. (20) at 37°C. Ampicillin was added to LN broth at 100 μg/ml.

Phosphatase assay. Extracts were made by sonication and were used as a source for the enzyme preparation. A phosphatase activity was measured at pH 5.0 (0.2 M sodium acetate buffer), pH 7.0 (0.2 M phosphate buffer), and pH 8.5 (0.2 M glycine-HCl buffer) in mixtures containing 20 mM *p*-nitrophenyl phosphate (pNPP) as a substrate. After 15 min at 37°C, the reaction was terminated by the addition of 1 ml of 1 N NaOH. The activity of the enzyme was determined by measuring the A_{410} of liberated *p*-nitrophenol. One unit of activity was defined as the amount of enzyme providing the formation of 1 μmol of *p*-nitrophenol per min (12).

Inhibition assays were performed in 0.2 M sodium acetate buffer (pH 6.6) by using 20 mM pNPP as a substrate. The enzyme was preincubated at 37°C for 20 min with each substance before the assay was started.

Analytical methods for protein. Protein concentration was determined by using a protein assay kit (Bio-Rad), with bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (18). The gels were stained with Coomassie brilliant blue.

Purification of *S. flexneri* phosphatase from a recombinant strain. Cultures of *E. coli* JM109 carrying pKU102-7 (2-liter culture) grown to the mid-log phase were centrifuged, and the bacterial pellet was washed twice with buffer A (30 mM Tris-HCl [pH 7.5] containing 30 mM NaCl), suspended in 20 ml of the same buffer, and then disrupted with an Ultrasonic homogenizer, model GSD-150 (SMT, Co.). Cell debris was removed by centrifugation at 100,000 × *g* for 60 min at 4°C, and the resulting supernatant was used directly for subsequent enzyme purification steps. The crude supernatant containing phosphatase enzyme was applied to a column of DE-52 cellulose (Whatman) and eluted with a linear gradient of NaCl (0.03 to 0.5 M) in buffer A. The pooled active fractions were concentrated and chromatographed on a Cellulofine GC-700 (Seikagaku Kogyo) column equilibrated with buffer A. The elution was carried out with the same buffer. Subsequently, the active fractions were loaded onto a MonoQ HR (Pharmacia) column (5/5) and eluted with a linear gradient of NaCl (0.03 to 0.5 M) in buffer A. The active fractions were concentrated and applied to a Sephadex G-50 (Pharmacia) column equilibrated with 20 mM ammonium bicarbonate buffer (pH 8.0). The preparation was washed, and then the enzyme was eluted with 20 mM ammonium bicarbonate buffer. The purity of the preparation was checked by SDS-PAGE.

Determination of the N-terminal amino acid sequence of PhoN-Sf. Purified PhoN-Sf protein was subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore) by means of a TransBlot transfer medium previously described (25). The transferred protein was stained with Coomassie brilliant blue, and the appropriate proteins were excised. The N-terminal amino acid sequence was determined by automated microsequencing by using Edman degradation reactions on an Applied Biosystem model 477A protein sequencer equipped with an on-line phenylthiohydantoin analyzer.

DNA sequencing. The sequences of both DNA strands were determined by the chain termination method of Sanger et al. (31), by using a Bcabest dideoxy sequencing kit (Takara Shuzo Co.), following cloning into pBluescript-IISK$^+$ and -KS$^+$.

Western blotting (immunoblotting). Protein extracts from each of the subcellular fractions were prepared as described previously (48). The protein extracts were loaded on an SDS–12% PAGE gel, and the electrophoresed protein bands were transferred to a polyvinylidene difluoride membrane (Millipore) by the methods described by Towbin et al. (46). *S. flexneri* PhoN-Sf was detected by immunoblotting with PhoN-Sf-specific antibodies, obtained by immunization of rabbits with the purified PhoN-Sf protein. Immunostaining was done with horseradish peroxidase-conjugated protein A (Boehringer Mannheim), and blots were developed with ECL (enhanced chemiluminescence) reagents (Amersham).

Southern hybridization. A 4.7-kb *Sma*I-*Xho*I segment containing the *phoN-Sf* gene was obtained from the 9.6-kb *Sal*I fragment of pMYSH6000 and cloned in pBluescript-IISK$^+$. The resulting plasmid clone, pKU102, was used as a template for PCR amplification with primers 5′-TGACCTTTACTACCTGACAA-3′ (nucleotides 96 to 115) and 5′-CTGATTGTTAGCGAATTCAT-3′ (nucleotides 741 to 722) from the 5′ end of the *phoN-Sf* gene. The PCR product, which comprised 646 bp, was used as the *phoN-Sf*-specific probe for Southern analysis. *Sal*I-digested chromosomes or plasmid DNAs resolved in a 0.8% agarose gel were transferred to a nylon membrane and hybridized with digoxigenin (DIG)-labeled DNA probe (Boehringer Mannheim). The hybridized bands were detected by using the DIG luminescent detection kit (Boehringer Mannheim) according to the manufacturer's protocol.

Nucleotide sequence accession number. *phoN-Sf* nucleotide sequence data will appear in the DDBJ, EMBL, and GenBank databases with accession number D82966.

RESULTS

Identification of a locus encoding a phosphatase activity. With pNPP as a substrate, phosphatase activity produced by YSH6000 (wild-type *S. flexneri*) or YSH6200 (the 230-kb-plasmidless mutant of YSH6000) grown in LN broth at 37°C was measured at pH 5.0, pH 7.0, and pH 8.5. Phosphatase activity was detected at each pH in YSH6000, but at pH 5.0 one-third of the phosphatase activity was revealed in YSH6200 (data not shown), suggesting that the 230-kb plasmid (pMYSH6000) encoded a phosphatase activity preferentially expressed at pH 7.0 and pH 8.5. Hence, to define the genetic locus responsible for the phosphatase activity, we utilized *Sal*I fragment clones of pMYSH6000 inserted in pBR322Tp (35) and introduced each of the *Sal*I clones into YSH6200. The screening for the *Sal*I clone able to express the phosphatase activity in YSH6200 showed that one of the *Sal*I fragment clones carrying the 9.6-kb *Sal*I fragment, which was designated the *Sal*I-I fragment (38), gave a positive result. Since a collection of 304 independent pMYSH6000 Tn*5* insertion mutants that included Tn*5* insertions in the *Sal*I-I fragment had previously been constructed (40), the Tn*5* mutants with alterations in the *Sal*I-I fragment were examined for phosphatase activity. As expected, three of nine Tn*5* insertion mutants greatly diminished the phosphatase activity. Indeed, the sites of the three Tn*5* insertions were found to be localized in the 4.5-kb *Sma*I-*Xho*I segment (Fig. 1). Furthermore, the introduction of various subclones of the *Sal*I-I fragments or the deletion derivatives into YSH6200 enabled us to localize the DNA region encoding the phosphatase activity within a 2.1-kb DNA sequence (Fig. 1).

Nucleotide sequence of the phosphatase DNA sequence. To identify the genetic determinant encoding the phosphatase activity, the nucleotide sequence of the 1,010-bp *Bst*EII-*Cfr* 13I segment in the 2.1-kb DNA sequence was determined, since the *Bst*EII-*Cfr* 13I segment restored to YSH6200 the production of the phosphatase activity. The results showed that the 1,010-bp sequence contained an open reading frame (ORF) encompassing nucleotides 129 through 875 with the 5′ end proximal to the *Bst*EII site (Fig. 2). The protein encoded by the ORF determined from the nucleotide sequencing was deduced to consist of 249 amino acids with a molecular mass of 27.2 kDa. The 20 amino acid residues constituting the N terminus of the protein possessed a characteristic signal sequence (Fig. 2). Interestingly, the search for amino acid homology with

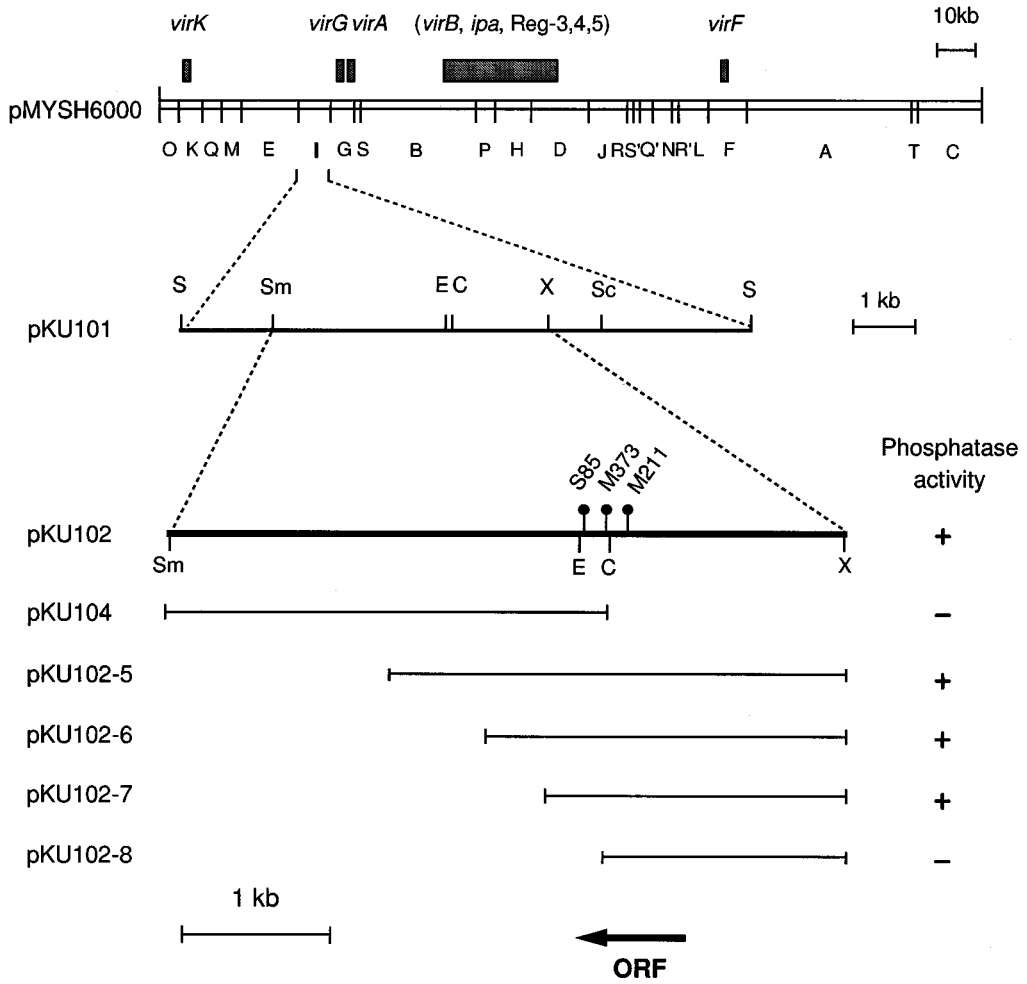

FIG. 1. Identification of *phoN-Sf* locus on pMYSH6000. The top line represents the linearized *Sal*I restriction map of pMYSH6000 (38), and the shaded boxes over the line indicate the positions of known virulence loci. The line below indicates the 9.6-kb *Sal*I-I fragment cloned into pBluescript-IISK$^+$. The thick line under pKU101 shows the 4.6-kb *Sma*I-*Xho*I segment of pKU102, possessing the *phoN-Sf* locus. Closed circles with vertical bars above the line indicate the sites of Tn*5* insertions as determined by DNA sequencing. The bars under pKU102 show the subclone in pBluescript-IISK$^+$ and the deletion derivatives of pKU102. The thick bar (ORF) at the bottom indicates the position of the *phoN-Sf* gene as determined by assaying the phosphatase activity with the deletion derivatives of pKU102 or by nucleotide sequencing. Abbreviations: C, *Cla*I; E, *Eco*RI; S, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; X, *Xho*I.

other proteins revealed this protein to be similar to PhoN of *P. stuartii* (83.2%), PhoC of *M. morganii* (80.6%), PhoN of *Salmonella typhimurium* (47.8%), and PhoC of *Zymomonas mobilis* (34.8%) (Fig. 3). The most critical feature found among the phosphatase domains was the presence of the highly conserved GSYPSGHT motif located between residues 162 and 169 of the phosphatase protein of YSH6000. The phosphatase protein encoded by pMYSH6000 was tentatively designated PhoN-Sf.

Identification and characterization of PhoN-Sf protein. In order to characterize the enzymatic activity of the PhoN-Sf protein, sonic extracts of *E. coli* K-12 JM109 carrying pKU102-7 (cloned *phoN-Sf*) (Fig. 1) were purified as described in Materials and Methods. The purified protein appeared as a single band with a molecular size of approximately 27 kDa on SDS-PAGE (Fig. 4). To confirm the identity of the purified protein as the PhoN-Sf protein, the first 20 amino acids at the N terminus were sequenced by automated Edman degradation. The results showed that the amino acid sequence was SIPP GNDVTTKPDLYYLTND, which corresponded to the deduced 20 amino acids at the N terminus of the putative mature PhoN-Sf protein (Fig. 2).

The purified PhoN-Sf protein was investigated for pH-dependent pNPP hydrolysis activity. Among different pH conditions ranging from at pH 2.0 to pH 12.0 (9), the optimum phosphatase activity was observed at pH 6.6 and was achieved at 37°C. The enzymatic activity was stable up to 40°C for 15 min in a phosphate buffer (see Materials and Methods) and retained 80% activity at 50°C. However, at temperatures over 50°C the activity declined rapidly, and activity was lost completely at 70°C. The enzymatic activity showed a broad substrate specificity. The phosphatase activities for various substrates relative to the activity for pNPP (1.0) were 0.5 (β-glycerophosphate), 0.85 (glucose-6-phosphate), 0.7 (AMP), and 0.86 (UMP). With pNPP as the substrate, the inhibitory effects of a number of compounds on PhoN-Sf activity were examined. As shown in Table 1, the PhoN-Sf activity was decreased to 6.4% of the original activity (100%) when mixtures were treated with diisopropylphosphate an inhibitor for serine enzyme. The enzyme activity was also decreased to 8.4% of the original activity in the presence of 10 mM dithiothreitol and to 58.1% in the presence of 3 mM *N*-bromosuccinimide (Table 1), suggesting that serine and tryptophan residues and disulfide bonds are involved in PhoN-Sf activity.

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     BstEII
  1  GGTAACCTTTGTTTTAGCATCTTCTGATTTATTTAAATTATGTGATAGCACACACAATT

 60  TTATTATCCAGTACTTTATGCTCTTAATAAAATAATTATAAATATGTATCCTCTATAAGG
                                                          SD
120  AGATGTTATATGAAAAGACAGCTTTTTACTCTTAGTATTGTCGGGGTATTTTCTTTAAAT
  1            M  K  R  Q  L  F  T  L  S  I  V  G  V  F  S  L  N

180  ACCTTTGCATCAATTCCTCCGGGAAATGATGTGACAACAAAACCTGACCTTTACTACCTG
 18   T  F  A  S  I  P  P  G  N  D  V  T  T  K  P  D  L  Y  Y  L

240  ACAAACGATAATGCTATTGACAGTCTGGCATTATTACCGCCTCCACCACAAATCGGAAGT
 38   T  N  D  N  A  I  D  S  L  A  L  L  P  P  P  P  Q  I  G  S

300  ATTGCCTTCCTGAATGACCAGGCCATGTATGAAAAGGGGCGTTTATTGCGAAATACTGAA
 58   I  A  F  L  N  D  Q  A  M  Y  E  K  G  R  L  L  R  N  T  E

360  AGAGGGAAGCTGGCAGCTGAGGATGCCAATCTCAGTAGTGGTGGCGTGGCAAATGTATTT
 78   R  G  K  L  A  A  E  D  A  N  L  S  S  G  G  V  A  N  V  F

420  TCTGCTGCTTTTGGTTCTCCGATAACGGCTAAAGATTCACCAGAATTGCATAAGTTACTG
 98   S  A  A  F  G  S  P  I  T  A  K  D  S  P  E  L  H  K  L  L

480  ACAAATATGATTGAGGATGCCGGAGACCTGGCAACGCGTTCAGCAAAAGAATATTATATG
118   T  N  M  I  E  D  A  G  D  L  A  T  R  S  A  K  E  Y  Y  M

540  CGTATTCGACCTTTTGCTTTTTATGGTGTTTCTACGTGTAATACAAAAGAACAGGATACA
138   R  I  R  P  F  A  F  Y  G  V  S  T  C  N  T  K  E  Q  D  T

600  TTATCCAGAAATGGCTCTTATCCATCAGGTCATACATCGATTGGTTGGGCAACAGCACTT
158   L  S  R  N  G  S  Y  P  S  G  H  T  S  I  G  W  A  T  A  L

660  GTATTGTCCGAAATAAATCCGGCACGCCAGGATACCATTCTGAAACGGGGTTATGAACTG
178   V  L  S  E  I  N  P  A  R  Q  D  T  I  L  K  R  G  Y  E  L

720  GGGGACAGCAGGGTTATTTGCGGTTATCACTGGCAAAGTGATGTTGACGCAGCACGTATT
198   G  D  S  R  V  I  C  G  Y  H  W  Q  S  D  V  D  A  A  R  I

780  GTTGGCTCTGCTATTGTAGCAACACTGCACTCAAATCCTGTGTTCCAGGCACAATTACAA
218   V  G  S  A  I  V  A  T  L  H  S  N  P  V  F  Q  A  Q  L  Q

840  AAAGCGAAAGATGAATTCGCTAACAATCAGAAAAAATAATGCTTTTAGCCAGTACGCCTT
238   K  A  K  D  E  F  A  N  N  Q  K  K  *

900  ATAACCCCGACCTTCAGGGCTAATGCCAGTCAGTTAAGCAATCTGACTGGCTCTTTTTCA
                                                        Cfr13I
960  GGGCTGTGGGGTATTTCCAGGGCCTCTCCTTTACCACTCTCGGAAAGGCCC
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FIG. 2. Nucleotide sequence of the *Bst*EII-*Cfr* 13I fragment containing the *phoN-Sf* gene. The sequence of the sense strand, as well as the deduced amino acid sequence for the ORF at positions 129 to 875, corresponding to the PhoN-Sf protein, is shown. The putative Shine-Dalgarno (SD) sequence is shown by a double line. The putative signal sequence of the PhoN-Sf protein (amino acids 1 to 20) is underlined. The sites of the three Tn*5* insertions, M211, M373, and S85 (see Fig. 1) (40), were at 448 (M211), 513 (M373), and 713 (S85) bp from the 5′ end of the *phoN-Sf* gene. The asterisk indicates a stop codon.

Subcellular localization of PhoN-Sf. To further assess the role of the PhoN-Sf protein in YSH6000, its subcellular localization was investigated by immunoblotting with a PhoN-Sf-specific antibody (see Materials and Methods). The whole-cell, cytoplasm, periplasm, outer membrane, and culture supernatant fractions prepared from YSH6000 were subjected to SDS-PAGE. A band corresponding to the 27-kDa PhoN-Sf protein was present in lanes containing the whole-cell, cytoplasm, and periplasm but not in those containing the outer membrane or the culture supernatant (Fig. 5). These results, together with the presence of the signal sequence at the N terminus of PhoN-Sf protein (Fig. 2), indicate that PhoN-Sf is a typical periplasmic enzyme.

PhoN-Sf expression in *Shigella* strains and EIEC. To investigate whether the ability to express PhoN-Sf protein was a particular trait of *S. flexneri* YSH6000, natural isolates of *Shigella* and EIEC were grown under low-phosphate conditions (see Materials and Methods) and PhoN-Sf production was examined by immunoblotting with the PhoN-Sf-specific antibody. The results showed that 8 of 18 *S. dysenteriae*, 2 of 9 *S. flexneri*, 12 of 18 *S. boydii*, 13 of 22 *S. sonnei*, and 1 of 10 EIEC isolates were PhoN-Sf positive. The large plasmid DNA, but not the chromosomal DNA, derived from the PhoN-Sf-positive strains was shown to hybridize with the *phoN-Sf* probe.

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S. flexneri     : MKRQLFTLSIVGVFSLNTFASIPPGNDVTTKPDLYYLTNDNAIDSLALLPPPPQIGSIAF  60
P. stuartii     : MKKLLAVFCAGAFVSTSVFAAIPPGNDVTTKPDLYYLKNSQAIDSLALLPPPPEVGSILF  60
M. morganii     : MKKNIIAGCLFSLFSLSALAAIPAGNDATTKPDLYYLKNEQAIDSLKLLPPPPEVGSIQF  60
S. typhimurium  : MKSRYLVFFLPLIVAKYTSAETVQPFHSPEESVN----------SQFYLPPPPGNDDPAY  50
Z. mobilis      : MIKVPRFICMIALTSGVLASGLSQSVSAHTEKSEPSSTYHFHSDPLLYLAPPPTSGSPLQ  60
                  *.          .  . .. ... ..... . ..... .*.***  ... .

S. flexneri     : LNDQAMYEKGRLLRNTERGKLAAEDANLSSGGVANVFSAAFGSPITAKDSPELHKLLTNM 120
P. stuartii     : LNDQAMYEKGRLLRNTERGEQAAKDADLAAGGVANAFSEAFGYPITEKDAPEIHKLLTNM 120
M. morganii     : LNDQAMYEKGRMLRNTERGKQAQADADLAAGGVATAFSGAFGYPITEKDSPELYKLLTNM 120
S. typhimurium  : RYDKEAYFKGYAIKGSPRWKQAAEDADVSVENIARIFSPVVGAKINPKDTPETWNMLKNL 110
Z. mobilis      : AHDDQTFNSTRQLKGSTRWALATQDADLHLASVLKDYACAAGMNLDIAQLPHLANLIKRA 120
                  ..*..... .....*.. *. ** .  ....  .. ..* ... .. *.. ......

S. flexneri     : IEDAGDLATRSAKEYYMRIRPFAFYGVSTCNTKEQDTLSRNGSYPSGHTSIGWATALVLS 180
P. stuartii     : IEDAGDLATRSAKEKYMRIRPFAFYGVATCNTKDQDKLSKNGSYPSGHTAIGWASALVLS 180
M. morganii     : IEDAGDLATRSAKEHYMRIRPFAFYGTETCNTKDQKKLSTNGSYPSGHTSIGWATALVLA 180
S. typhimurium  : LTMGGYYATASAKKYYMRTRPFVLFNHSTCRPEDENTLRKNGSYPSGHTAYGTLLALVLS 170
Z. mobilis      : LRTEYDDIGR-AKNNWNRKRPFVDTDQPICTEKDREGLGKQGSYPSGHTTIGWSVALILA 179
                  ..........**. ..*.***....  .*... .  *. .******** .*.. **.*.

S. flexneri     : EINPARQDTILKRGYELGDSRVICGYHWQSDVDAARIVGSAIVATLHSNPVFQAQLQKAK 240
P. stuartii     : EINPENQDKILKRGYELGQSRVICGYHWQSDVDAARIVASGAVATLHSNPEFQKQLQKAK 240
M. morganii     : EVNPANQDAILERGYQLGQSRVICGYHWQSDVDAARIVGSAAVATLHSDPAFQAQLAKAK 240
S. typhimurium  : EARPERAQELARRGWEFGQSRVICGAHWQSDVDAGRYVGAVEFARLQTIPAFQKSLAKVR 230
Z. mobilis      : ELIPDHAANILQRGQIFGTSRIVCGAHWFSDVQAGYIMASGEIAALHGDADFRRDMELAR 239
                  * .*  .. .. **...* **..**.**.***.*.....  .*.*.. . *. .. ...

S. flexneri     : DEFANNQKK                                                  249
P. stuartii     : DEFAKLKK                                                   248
M. morganii     : QEFAQKSQK                                                  249
S. typhimurium  : EELNDKNNLLSKEDHPKLNY                                       250
Z. mobilis      : KELEKARTSAHTPDDLLCKIEQSAR                                  264
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FIG. 3. Comparison of the deduced amino acid sequences of *S. flexneri* PhoN-Sf with those of *P. stuartii* PhoN (EMBL accession number X64820), *M. morganii* PhoC (43), *S. typhimurium* PhoN (14, 17), and *Z. mobilis* PhoC (27). Amino acid residues identical among the predicted gene products are marked by asterisks, and conservative amino acid substitutions are marked by dots. Gaps are indicated by hyphens. The numbers indicate amino acid residue positions.

DISCUSSION

In this study, we have identified a gene encoding a nonspecific phosphatase activity on the 230-kb virulence plasmid (pMYSH6000) of *S. flexneri* YSH6000. The genetic determinant, named *phoN-Sf*, was localized to a 2.1-kb DNA segment

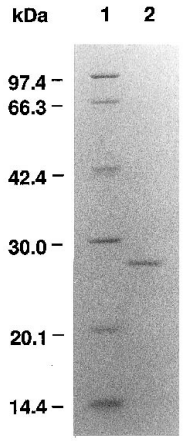

FIG. 4. SDS-PAGE of purified PhoN-Sf protein. The purified protein was loaded on an SDS-PAGE gel (12% acrylamide) and stained with Coomassie brilliant blue. Lane 1, molecular mass markers; lane 2, the purified phosphatase protein.

TABLE 1. Effects of various inhibitors on the purified phosphatase

Inhibitor[a] (final concn)	Residual activity[b] (%)
None	100
NBS (1 mM)	81.1
NBS (3 mM)	58.1
Benzamidine-HCl (10 mM)	107.4
DTT (1 mM)	40.9
DTT (10 mM)	8.4
SBTI (1 mg/ml)	103.6
o-Phenanthroline (5 mM)	100.7
EDTA (20 mM)	115.3
DFP (5 mM)	88.9
DFP (10 mM)	6.4
Cysteine (10 mM)	105.9
L-Phenylalanine (10 mM)	108.1
L-Tryptophan (10 mM)	114.5
L-(+)-Tartaric acid (10 mM)	105.6

[a] NBS, *N*-bromosuccinimide; DTT, dithiothreitol; SBTI, soybean trypsin inhibitor; DFP, diisopropyl fluorophosphate.

[b] Residual activities were calculated from the amount of *p*-nitrophenyl formed. The value for the reaction with no addition was set at 100%. The data are the averages for three experiments. The reaction was performed as described in Materials and Methods.

in the *Sal*I-I fragment on pMYSH6000 (38) that was placed ~6 kb downstream of the *virG* gene (Fig. 1). The nucleotide sequence of the *phoN-Sf* locus revealed that the gene encoded a 27.2-kDa protein, and the PhoN-Sf protein occurred predominantly in the periplasm.

Previously, nine strains with Tn*5* insertion mutations in the *Sal*I-I fragment of pMYSH6000 (40) were isolated, and in this study it was found that three of them (S85, M373, and M211) had diminished levels of PhoN-Sf production in comparison with that of the wild type. The sites of the three Tn*5* insertions were subsequently confirmed to be in the *phoN-Sf* gene (Fig. 1). Significantly, it was found that the three Tn*5* insertion mutants were capable of invading MK2 epithelial cells and forming plaques on epithelial cell monolayers in the focus-plaque-forming assay at levels similar to that of the wild type, YSH6000 (data not shown). Furthermore, when various *Shigella* and EIEC strains were tested for PhoN-Sf production or the presence of *phoN-Sf* DNA as determined by immunoblotting with the PhoN-Sf-specific antibody or by Southern blotting with the *phoN-Sf*-specific probe, 53% of the strains were phosphatase negative. On the basis of these results, it would appear that the PhoN-Sf function is not essential for expression of the virulence phenotypes of *Shigella* strains under in vitro conditions.

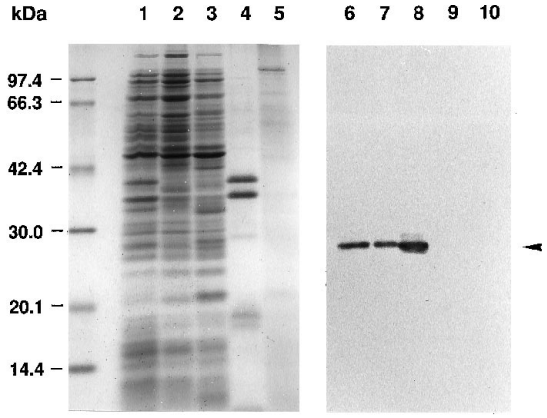

FIG. 5. Subcellular localization of PhoN-Sf protein in YSH6000. Detection of PhoN-Sf was performed by immunoblotting with the PhoN-Sf-specific antibody. Lanes 1 and 6, whole-cell protein extracts; lanes 2 and 7, cytoplasmic protein extracts; lanes 3 and 8, periplasmic protein extracts; lanes 4 and 9, outer membrane protein; lanes 5 and 10, culture supernatant protein extracts. Lanes 1 through 5 represent SDS-PAGE and staining with Coomassie brilliant blue, while lanes 6 through 10 are immunoblottings. The arrowhead indicates the PhoN-Sf protein. Molecular masses are indicated on the left.

The PhoN-Sf protein was purified to homogeneity, and its biochemical properties were characterized. When phosphatase activity was assayed by using pNPP as the substrate, the purified enzyme revealed a pH optimum of 6.6. In general, phosphatases are divided into two major groups according to pH optima. For example, alkaline phosphatase exhibits a pH optimum above 8.0, while acid phosphatase expresses its activity at an optimum pH of 5.0 or lower. In this regard, PhoN-Sf may be classified into neither of the phosphatase groups, rather belonging to a neutral phosphatase family, although this type of phosphatase has not yet been reported to have been purified from members of the family *Enterobacteriaceae.*

Interestingly, the amino acid sequence of the PhoN-Sf protein revealed significant homology to sequences of PhoN of *P. stuartii* (83.2%) and PhoC of *M. morganii* (80.6%) and some homology to those of PhoN of *S. typhimurium* (47.8%) and PhoC of *Z. mobilis* (34.8%) (Fig. 3). Although the enzymatic properties of PhoN of *P. stuartii* have not been reported, PhoC of *M. morganii* was shown to be expressed as a high-level phosphate-irrepressible acid phosphatase activity, termed HPAP (43), which was also observed in the PhoN-Sf activity expressed in YSH6000 (data not shown). Indeed, PhoN-Sf and PhoC of *M. morganii* shared several properties: (i) both are secreted in the periplasm, (ii) both possess nonspecific phosphatase activity, and (iii) both have enzymatic activity resistant to EDTA, phosphate fluoride, and tartrate. Consequently, the overall properties of PhoN-Sf and PhoC are similar to each other, or to those of other nonspecific acid phosphatases, such as PhoN of *S. typhimurium* (14, 17), although PhoN-Sf and PhoC are apparently more active on 5′ nucleotides than on 3′ nucleotides (47) and are not inhibited by fluoride ions; those properties are not noted with the PhoN activity in *S. typhimurium* (14, 17).

Although the PhoN-Sf protein and *phoN-Sf* mRNA are sufficiently expressed in YSH6000 under high-phosphate conditions such as those obtained with LN broth (47), under low-phosphate conditions (20) the levels of PhoN-Sf production, as well as *phoN-Sf* expression, are slightly (approximately twofold) increased (data not shown). In contrast, in *E. coli* phosphatase activity such as alkaline phosphatase (PhoA) and production of the outer membrane protein PhoE or proteins involved in the active transport of phosphate and *sn*-glycerol-3-phosphate have been shown to be greatly induced upon phosphate limitation (5, 11, 16, 45). The set of *pho* genes of *E. coli* are expressed as the *pho* regulons, whose expression is under the control of the *phoB-phoR* genes (21, 22). In this control mechanism, the phosphorylated *phoB* product acts as the direct transcriptional controller by interacting with the *pho* box in the promoter region of the various genes that belong to the *pho* regulons. Recently, Scholten et al. (41) reported that *S. flexneri* contains a functional PhoB-PhoR regulatory system, since when the *E. coli phoA* gene was introduced into *S. flexneri*, PhoA production was induced by phosphate limitation. However, introduction of *phoE* on a plasmid did not lead to the expression of PhoE. Examination of the *phoB* gene by nucleotide sequencing revealed that the deduced PhoB sequence contained two amino acid changes from the *E. coli* PhoB sequence, one of which was involved in the PhoB activ-

ity, indicating that *S. flexneri* PhoB does not recognize the *phoE* promoter region (41). In this regard, the *phoN-Sf* promoter region may also be less responsive to PhoB activity, since PhoN-Sf activity in YSH6000 revealed the HPAP phenotype (see above) and PhoN-Sf expression and its mRNA synthesis were not remarkably affected by the introduction of *phoB-phoR* double mutations in YSH6000 (data not shown). Indeed, a putative *pho* box motif was not found in the *phoN-Sf* promoter region (Fig. 2). These results may suggest that the *phoN-Sf* gene, or the DNA region, on the large plasmid of *Shigella* spp. is derived from some member of the family *Enterobacteriaceae*, such as the genera *Providencia* and *Morganella*, which are taxonomically close to each other (43).

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