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

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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 morgani (PhoC, 80.6%), Salmonella typhi-murium (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 protein 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 Tn5 insertion mutants of YSH6000 possessing phoN-Sf::Tn5 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 SalI 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 IS1, IS3, or IS4 (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-

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C. Ampicillin was added to LN broth at 100 μg/ml as the vector for cloning the gene. S. flexneri Bacteriawereroutinelygrownat37°CinLNbroth(37).Toimposephosphate limitation, Bacteria were 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 A405 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).

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 Laemml (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 (Millipore) by means of TransBlot transfer medium previously described (25). The transferred protein was stained with Coomassie brilliant blue, and the appropriate protein bands 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 Bca best dyeoxy sequencing kit (Takara Shuzo Co.), following cloning into pBluescript-ISK+ 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 Smal-XhoI segment containing the phoN-Sf gene was obtained from the 9.6-kb SalI fragment of pMYSH6000 and cloned in pBluescript-ISK+.

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 SalI fragment clones of pMYSH6000 inserted in pBR322Tp (35) and introduced each of the SalI clones into YSH6200. The screening for the SalI clone able to express the phosphatase activity in YSH6200 showed that one of the SalI fragment clones carrying the 9.6-kb SalI fragment, which was designated the SalI-I fragment (38), gave a positive result. Since a collection of 304 independent pMYSH6000 Tn5 insertion mutants that included Tn5 insertions in the SalI-I fragment had previously been constructed (40), the Tn5 mutants carrying the SalI-I fragment were examined for phosphatase activity. As expected, three of nine Tn5 insertion mutants greatly diminished the phosphatase activity. Indeed, the sites of the three Tn5 insertions were found to be localized in the 4.5-kb Smal-XhoI segment (Fig. 1).

Furthermore, the introduction of various subclones of the SalI-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 BrsEIIf-Cfr 131 segment in the 2.1-kb DNA sequence was determined, since the BrsEIIf-Cfr 131 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 BrsEIIf site (Fig. 2). The protein encoded by the ORF detected. Immunostaining of the nucleotide sequence 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

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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 GNDVTKPDLLYLTND, 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.
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 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.

**DISCUSSION**

In this study, we have identified a gene encoding a nonspecific phosphatase activity on the 230-kb virulence plasmid (pMYSH6000) of S. flexneri 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 identified 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.

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TABLE 1. Effects of various inhibitors on the purified phosphatase

<table>
<thead>
<tr>
<th>Inhibitor* (final concn)</th>
<th>Residual activityb (%)*</th>
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<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>NBS (1 mM)</td>
<td>81.1</td>
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<tr>
<td>NBS (3 mM)</td>
<td>58.1</td>
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<tr>
<td>Benzamidine-HCl (10 mM)</td>
<td>107.4</td>
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<tr>
<td>DTT (1 mM)</td>
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<td>DTT (10 mM)</td>
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<td>SBTI (1 mg/ml)</td>
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<td>o-Phenanthroline (5 mM)</td>
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<td>108.1</td>
</tr>
<tr>
<td>l-Tryptophan (10 mM)</td>
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</tr>
<tr>
<td>L- (+)-Tartaric acid (10 mM)</td>
<td>105.6</td>
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* 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 SalI-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 Tn5 insertion mutations in the SalI-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 Tn5 insertions were subsequently confirmed to be in the phoN-Sf gene (Fig. 1).

Significantly, it was found that the three Tn5 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 Shi-
gella 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.

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 (45), 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, Schoelten 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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