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The *sigA* Gene Which Is Borne on the *she* Pathogenicity Island of *Shigella flexneri* 2a Encodes an Exported Cytopathic Protease Involved in Intestinal Fluid Accumulation

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In this study, the *sigA* gene situated on the *she* pathogenicity island of *Shigella flexneri* 2a was cloned and characterized. Sequence analysis showed that *sigA* encodes a 139.6-kDa protein which belongs to the SPATE (serine protease autotransporters of *Enterobacteriaceae*) subfamily of autotransporter proteins. The demonstration that SigA is autonomously secreted from the cell to yield a 103-kDa processed form and possesses a conserved C-terminal domain for export from the cell were consistent with the autotransporter pathway of secretion. Functional analysis showed that SigA is a secreted temperature-regulated serine protease capable of degrading casein. SigA was cytopathic for HEp-2 cells, suggesting that it may be a cell-altering toxin with a role in the pathogenesis of *Shigella* infections. SigA was at least partly responsible for the ability of *S. flexneri* to stimulate fluid accumulation in ligated rabbit ileal loops.

Shigella spp. are gram-negative bacteria that are the etiological agents of bacillary dysentery, a diarrheal disease responsible for the death of more than 500,000 people every year (37). Infections are transmitted via the fecal-oral route and are usually the result of direct person-to-person contact or the consumption of contaminated water or food (12). After ingestion, shigellae invade the colonic epithelium and then spread from cell to cell, resulting in cell destruction and inflammation (35). In the infected host, this leads to a watery diarrhea that often progresses to the bloody, mucoid diarrhea typical of bacillary dysentery (15).

A variety of virulence determinants play important roles in the pathogenesis of shigellosis. Several proteins required for epithelial cell invasion and intercellular spread, including the autotransporter VirG, are encoded by a large virulence plasmid found in all *Shigella* spp. (38). In addition, some *Shigella* spp. produce enterotoxins that probably play a role in the watery diarrhea phase of shigellosis. These include ShET1, an enterotoxin found predominantly in *Shigella flexneri* serotype 2a (15), and ShET2, which is found in more than 80% of *Shigella* strains of diverse serotypes (25). Other determinants include an aerobactin iron uptake system found in *S. flexneri*, *S. sonnei*, and *S. boydii* (45), SepA, which induces both mucosal atrophy and tissue inflammation, indicating that it is involved in tissue invasion by *S. flexneri* 5 (4), and the putative virulence protein Pic (originally ShMu), from *S. flexneri* 2a, which has mucinase and hemagglutinin activities (17; F. R. Noriega, unpublished data).

Both SepA and Pic belong to a rapidly expanding family of autotransporter proteins that share sequence similarity and a common pathway for autonomous secretion (18). This type of autosecretion is characterized by transport into the periplasm, usually via the Sec system, followed by insertion of the transported protein's C-terminal domain, termed the β domain, into the outer membrane. The β domain is thought to form a β-barrel pore through which the remainder of the protein, termed the passenger or α domain, is transported to the exterior of the cell. After transport across the outer membrane, exported proteins either remain tethered to the cell surface or are released from the cell by proteolytic cleavage (18). *Neisseria gonorrhoeae* immunoglobulin A (IgA) protease, the first member of the autotransporter family, is a serine protease that catalyzes its own release from the cell by autoproteolysis (28). However, in some cases autotransporters are released from the cell by the actions of distinct proteases located in the outer membrane (13, 40).

Several autotransporters have established or potential roles in bacterial virulence (18). Their various biological roles and activities include mediating adherence to cell surfaces, proteolysis, cytotoxicity, cell invasion, intracellular motility, and serum resistance (18). Recently, Pet, a member of this subfamily from enteroaggregative *Escherichia coli*, was shown to have cytopathic effects on HEp-2 cells as well as demonstrating enterotoxic activity.

The autotransporter family shows a considerable degree of divergence, and members have been classified into subfamilies based on amino acid sequence. Both SepA and Pic belong to the Tsh subfamily, otherwise known as the SPATE (serine protease autotransporters of *Enterobacteriaceae*) subfamily, and both possess a highly conserved serine protease motif shared by other members of the group (18). The serine protease activity of SepA and some other members of this subfamily has been demonstrated experimentally (5).

In *S. flexneri* 2a, the *pic* (originally *she*) gene, encoding Pic, is associated with a pathogenicity island (PAI) termed the *she* PAI (32). The *she* PAI is distinct from another unstable PAI-like element, encoding multiple antibiotic resistance in *S. flexneri* 2a (31), and the recently described Shi-2 PAI, encoding an aerobactin iron uptake system in a variety of *Shigella* strains

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TABLE 1. Bacterial strains and plasmids used

Bacterial strain or plasmid	Description	Reference or source
E. coli DH5α	F^- *endA1 hsdr 17* (r_K^- m_K^+) *thi-1* λ^- *recA1 gyrA96 relA1* φ80d*lacZ*ΔM15	16
S. flexneri 2a		
YSH6000	Wild-type *S. flexneri* 2a Japanese isolate, Ap^r $Strep^r$ Tet^r Cm^r	39
YSH6200	Derivative of YSH6000 cured of the 230-kb virulence plasmid	33
YSH6000T	Spontaneous Ap^s $Strep^s$ Tet^s Cm^s variant of YSH6000	24
SBA1336	Derivative of YSH6000T harboring a Tc^r cassette within *she*	32
SBA1351	Derivative of YSH6000T harboring a Km^r cassette within *sigA*	This study
SBA1341	Tc^s derivative of SBA1336 exhibiting a 51-kb chromosomal deletion	32
SBA1356	Derivative of SBA1336 harboring an Ω cassette within *sepA*	This study
SBA1361	Derivative of SBA1341 harboring pSBA479	This study
SBA1413	Derivative of SBA1351 harboring pSBA572	This study
Plasmids		
pPBA1100	pUC18-based high-copy-number vector, Km^r Δ*lacZ′*	20
pWSK29	pSC101-based low-copy-number vector, Ap^r Δ*lacZ′*	46
pWSK30	pSC101-based low-copy-number vector, Ap^r Δ*lacZ′*	46
pWSK130	pSC101-based low-copy-number vector, Km^r Δ*lacZ′*	46
pCACTUS	pSC101-based low-copy-number vector with *sacB*, Cm^r	43
pBz	Hybrid of pBluescriptSK+ and pZero 1.1, *ccdB* gene replaces Δ*lacZ′*	S. Doughty, Monash University
pUC4K	Source of Km^r gene from Tn*903*, Ap^r Km^r	44
pPBA1180	2.0-kb Ω fragment consisting of the $Strep^r$/$Spec^r$ gene of the R100.1 plasmid cloned into pUC19	29; M. Hunt, Monash University
pSBA415	19.9-kb *Sal*I fragment of SBA1336 bearing the 3′ truncated *she* gene with an inserted *tetAR*(B) cassette cloned into *Bam*HI site of pWSK29, Ap^r Tc^r	32
pSBA432	3.6-kb *Eco*RI fragment of pSBA415 cloned into *Eco*RI site of pWSK130, Km^r	N. Ngoc, Monash University
pSBA479	5.4-kb *Sal*I/*Hin*dIII fragment of pSBA415 cloned into *Sal*I/*Hin*dIII sites of pPBA1100, Km^r	This study
pSBA493	1.2-kb *Acc*I fragment carrying a Km^r of pUC4K cloned in the unique *Cla*I site in pSBA479	This study
pSBA501	6.7-kb Expand PCR product containing the insertionally disrupted *sigA* gene cloned in pCACTUS T-tailed *Sma*I site	This study
pSBA544	3.3-kb PCR product containing *sepA* cloned into *Bam*HI site of pBz	This study
pSBA549	2.0-kb *Hin*dIII Ω fragment cloned into unique *Hin*dIII site in pSBA544	This study
pSBA550	5.3-kb *Bam*HI fragment (*sepA*-Ω) of pSBA549 cloned in the unique *Bam*HI site in pCACTUS	This study
pSBA572	5.4-kb *Sal*I/*Hin*dIII fragment of pSBA479 cloned into the *Sal*I/*Hin*dIII site in pWSK30	This study

(23, 45). Like several other PAIs, the *she* PAI is an unstable chromosomal locus and spontaneously deletes at a frequency of 10^{-5} to 10^{-6} per cell per generation (32). The extent of the PAI is not known, but *she* PAI deletants appear to have lost a 51-kb region that resides on *Not*I fragment A (32). Very little is known about the genes carried on the *she* PAI and their roles in virulence. Besides the *pic* gene, the *she* PAI carries *set1A* and *set1B*, the genes encoding the two subunits of the ShET1 enterotoxin (15). *set1A* and *set1B* overlap the *pic* gene and are transcribed in an opposite direction to *pic*. In a previous study, we discovered a sequence, lying 3.6 kb downstream of *pic*, which encoded a putative protein with similarity to the carboxy termini of several autotransporter proteins (32). This suggested that as well as Pic, the *she* PAI may encode a second autotransporter protein which was tentatively named SigA. In the present study, we have confirmed that *sigA* encodes a protein belonging to the autotransporter family of proteins. In addition, we report on the regulation of SigA expression in *S. flexneri*, its biological activities, and its role in virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* DH5α and *Shigella* strains were grown in either Luria-Bertani or 2xYT medium at 37°C with aeration. When antibiotic selection was necessary, the growth medium was supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), tetracycline (10 μg/ml), streptomycin (25 μg/ml), or spectinomycin (25 μg/ml).

Recombinant DNA techniques. The preparation of genomic DNA of *S. flexneri* 2a was described previously (2), as was the preparation of plasmid DNA (22). DNA ligations and restriction endonuclease digestions (2) were performed with enzymes supplied by Roche Molecular Biochemicals (Basel, Switzerland) or New England Biolabs (Beverly, Mass.). DNA was introduced into *E. coli* and *S. flexneri* 2a by electrotransformation (41) with a Bio-Rad (Hercules, Calif.) Gene Pulser. Standard methods (34) were used for cloning. PCR products for cloning experiments were amplified using a Expand high-fidelity PCR kit (Roche).

Nucleotide sequencing was performed with a PRISM Ready Reaction Dye Deoxy Terminator cycle sequencing kit (Perkin-Elmer Corp., Norwalk, Conn.), using universal vector primers or synthetic oligonucleotides designed on the basis of preceding sequences. The reactions were analyzed on an Applied Biosystems model 373A DNA sequencing system (Perkin-Elmer). The program Sequencher (GeneCodes Corporation, Ann Arbor, Mich.) was used for routine analysis of DNA sequence information. Similarities and identities between pairwise comparisons of protein sequences were determined with the BlastN, BlastP, or BlastX program (1). Comparisons of multiple sequence alignments were done using the program PileUp (Genetics Computer Group [Madison, Wis.] Wisconsin Package).

Construction of chromosomal mutants. To construct a *sigA* mutant, plasmid pSBA479, which carries *sigA* on a *Sal*I/*Hin*dIII fragment of pSBA415 (32), was cleaved at the unique *Cla*I site within *sigA* and ligated with an *Acc*I fragment carrying the kanamycin resistance (Km^r) cassette from pUC4K to yield plasmid pSBA493. A 6.7-kb DNA fragment containing *sigA-kan* was then amplified by PCR from pSBA493, using vector primers. This PCR fragment was cloned into the *Sma*I-digested, T-tailed site of the vector pCACTUS (44). The resulting construct, pSBA501, was introduced into *S. flexneri* 2a YSH6000T by electroporation, and strains containing *sigA-kan* in the chromosome were selected by growth at 42°C on media containing 5% sucrose and kanamycin. Mutants resulting from double-crossover allelic exchange were identified by screening for chloramphenicol sensitivity. The presence of only an insertionally inactivated copy of *sigA* alone in the chromosome was confirmed by Southern blot analysis (data not shown). One *sigA* mutant strain, SBA1351, was selected for further experiments.

A *sepA*/*pic* double mutant was constructed by amplifying a truncated version of the *sepA* gene (3.3 kb) from *S. flexneri* 2a YSH6000T and cloning it into the *Bam*HI site of pBz to yield pSBA544. A streptomycin-spectinomycin resistance ($Strep^r$/$Spec^r$) Ω cassette was isolated from pPBA1180 by digestion with *Hin*dIII and ligated into an introduced *Hin*dIII site in pSBA544 present at the 5′ end of *sepA* to yield pSBA549. A 5.3-kb *Bam*HI fragment (*sepA*-Ω) was subsequently excised from pSBA549 and cloned into the equivalent site of pCACTUS to produce pSBA550. Plasmid pSBA550 was then introduced into the *pic* strain

TABLE 2. Homologies of SigA from the SPATE family of proteases

Accession no.	% Identity	Protein	No. of amino acids
AF056581	58	Pet	1,295
X97542	57	EspP	1,300
Y13614	57	PssA	1,300
U69128	57	EspC	1,306
U35656	44	Pic (ShMu)	1,372
Z48219	42	SepA	1,366
L27423	39	Tsh	1,377

SBA1336 (33), and the method described above was applied to select for double-crossover mutants resistant to tetracycline, streptomycin, and spectinomycin and sensitive to chloramphenicol. A putative mutant was analyzed by PCR and Southern blot hybridization to confirm allelic exchange and was named SBA1356 (data not shown).

Protein purification and analysis. For the purification of SigA, culture supernatants were recovered by centrifugation at 8,000 × *g* for 10 min from bacteria grown to an optical density at 600 nm of 1.0 and then filter sterilized through 0.22-μm-pore-size filter units (Millipore, Bedford, Mass.). Supernatants were concentrated either 200-fold by ultrafiltration through an Amicon PM50 membrane or 2,000-fold by a combination of ammonium sulfate precipitation (50% saturation) and ultrafiltration using Centriplus-50 concentrators (Amicon, Lexington, Mass.). The retentates were dialyzed three times against phosphate-buffered saline, pH 7.2 (PBS). All manipulations were performed at 4°C. Concentrates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (21) and assayed for proteolytic activity toward casein using an EnzChek protease assay kit (Molecular Probes, Leiden, The Netherlands). The intensity of fluorescence at 520 nm was measured at 37°C in samples excited at 485 nm with a Perkin-Elmer fluorescence spectrophotometer. A unit of activity was defined as an increase of one fluorescence unit over a period of 5 h.

Antisera specific for the 103-kDa secreted form of SigA were produced by immunization of New Zealand White rabbits with polyacrylamide gel slices that had been macerated and mixed with either Freund's incomplete adjuvant or water. The rabbits were boosted three times over the course of 6 months and exsanguinated; sera were collected, stored at −20°C, and used to detect SigA by immunoblotting (8).

Cell culture methods. HEp-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (HyClone, Logan, Utah), 1% nonessential amino acids, 5 mM L-glutamine, penicillin (100 μg/ml), and streptomycin (100 μg/ml). Cells were maintained at 37°C in humidified 5% CO_2–95% air. For experimental use, subconfluent HEp-2 cells were resuspended with EDTA-trypsin, plated in eight-well LabTek slides (VWR, Bridgeport, N.J.), and allowed to grow to 60% confluence. For all experiments, protein preparations were diluted directly in tissue culture medium on the cells to a final concentration of 25 μg/ml. To neutralize the serine protease activity of SigA, the SigA preparations were incubated for 15 min with 2 mM phenylmethylsulfonyl fluoride (PMSF), washed twice with 5 volumes of PBS in a centrifugal filter device with a 100-kDa cutoff to remove residual PMSF, and then added to HEp-2 cells in fresh medium. Equivalent quantities of control concentrates (from vector-containing bacteria) were added to additional wells as controls. After incubation for 6 h at 37°C in humidified 5% CO_2–95% air, the medium was aspirated, and the cells were washed three times with PBS and stained with Giemsa stain as described previously (26).

Fluid accumulation assays. Infection of rabbit ileal loops was performed as described previously (36). In brief, strains of *S. flexneri* were investigated for the ability to induce fluid accumulation in the ileum of New Zealand White rabbits. The ileum was ligated to construct loops approximately 5 cm long, after which 1 ml of PBS containing 10^9 bacteria was injected into the lumen of each loop, using a 25-gauge needle. After 18 h, the rabbits were euthanized, and the amount of fluid accumulation in the loop was measured. Results were expressed as the volume of fluid (milliliters) accumulated per centimeter of ligated intestinal segment. Data were analyzed by Student's *t* test.

Nucleotide sequence accession number. The sequence data reported here have been assigned GenBank accession no. AF200692.

RESULTS

Cloning and sequence analysis of the *sigA* gene. To obtain the entire *sigA* gene, we cloned a 5.4-kb *Sal*I/*Hin*dIII fragment from pSBA415 (32) into pPBA1100 to construct pSBA479. Sequencing of the cloned insert in pSBA479 revealed a predicted open reading frame of 3,858 nucleotides which encodes a putative protein with a deduced molecular mass of 139.6 kDa and a predicted isoelectric point of 8.9. Comparison of the translated sequence of *sigA* with sequences in GenBank determined that SigA exhibited sequence similarity and was comparable in length to a large number of bacterial IgA1 protease-like autotransporter proteins, including Pet (72% similarity and 59% identity) (14), PssA (10), EspP (7), EspC (42), Pic (17; Noriega, unpublished data), SepA (4), and Tsh (30). Sequence identity to these proteins varied from 39 to 58%, while similarity varied from 59 to 72% (Table 2).

In common with this family of autotransporters, the *S. flexneri* 2a SigA protein appears to be divided into three domains comprising an N-terminal signal sequence typical of this subfamily of autotransporters, followed by a putative passenger or α domain and a carboxy-terminal β domain. The degree of similarity to other members of this family was much higher in the C-terminal domain of the protein, consistent with the putative role of this region in protein secretion (18). The polypeptide possessed additional features characteristic of other members of this family. A potential serine protease motif (G^{256}DSGSGS) (18) was found, as well as a putative signal peptidase cleavage site at residues 54 and 55 (27). The conserved serine protease motif consisting of the sequence GD$\underline{S}$G SPLF, where $\underline{S}$ is the active-site serine characteristic of serine proteases, has been identified as part of the catalytic site of the IgA1 proteases (3, 6). The cleavage site of the β domain was predicted to lie between residues N^{1007} and N^{1008} by comparison with other autotransporters.

Secretion of SigA from *E. coli* DH5α. To establish whether the *sigA* open reading frame encoded a secreted protein, supernatants concentrated from logarithmic phase cultures of *E. coli* DH5α/pSBA479 (*sigA*) and the control strain DH5α/pPBA1100 (vector only) were analyzed by SDS-PAGE. Coomassie blue staining indicated that DH5α/pSBA479 expressed and secreted a protein of approximately 103 kDa, while no such protein was secreted from cells with the vector only (Fig. 1). The size of the protein coincided with the molecular weight of the predicted secreted product deduced from the *sigA* nucleotide sequence assuming cleavage of the N-terminal signal peptide and the C-terminal β-barrel pore.

The sequence comparison strongly suggested that the C-terminal domain was required for transport of the protein. Using the TopPredII computer program developed for the prediction of secondary structures adopted by membrane proteins (9), the

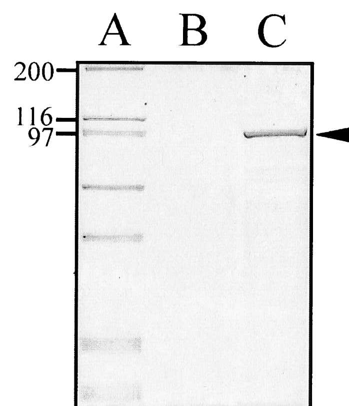

FIG. 1. Analysis of the expression and secretion of SigA in *E. coli* carrying the *sigA* gene. Concentrated culture supernatants were subjected to SDS-PAGE, and the gel was stained with Coomassie blue. Lanes: A, molecular mass markers (indicated in kilodaltons at the left); B, supernatant of DH5α/pPBA1100 (vector only); C, supernatant of DH5α/pSBA479 (*sigA*). The arrowhead indicates the 103-kDa secreted protein encoded by *sigA*.

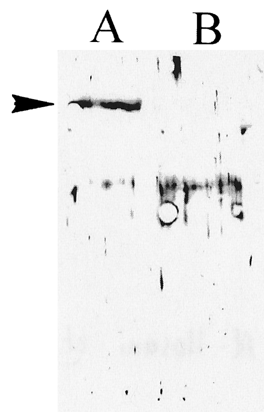

FIG. 2. Temperature regulation of SigA. Supernatants from *S. flexneri* SBA1356 grown at 37°C (lane A) or 21°C (lane B) were immunoblotted and probed with rabbit antiserum raised against SigA. The arrowhead indicates the 103-kDa SigA protein expressed at 37°C.

putative β domain of the SigA polypeptide was found to consist of 14 amphiphilic β strands, strongly suggesting that these strands form a β-barrel pore for the translocation of SigA across the outer membrane. To test this hypothesis, a truncated form of *sigA* lacking the putative β-barrel domain was obtained by deleting the region of *sigA* downstream of nucleotide 2508, giving rise to plasmid pSBA432. A concentrated supernatant of *E. coli* harboring this truncated gene contained no secreted proteins when analyzed by SDS-PAGE (data not shown). These findings support the notion that the C terminus of SigA is indispensable for the secretion of the protein.

Expression of SigA is regulated by temperature in vitro. Since temperature affects the expression of a variety of virulence determinants, including the autotransporter proteins SepA (4), Tsh (30), EspP (7), and Pic (17), we sought to determine whether expression of SigA was also regulated by temperature. In this experiment, *Shigella* strain SBA1356, carrying mutations in *sepA* and *pic* but expressing SigA, was cultured at 37 or 21°C. Protein expression was evaluated by immunoblotting with antibodies specific for SigA. As shown in Fig. 2, SigA was expressed at 37 not 21°C.

Proteolytic activity of SigA. Based on the presence of a protease motif and homology with other proteins which have been demonstrated to act as proteases, we suspected that SigA also possess proteolytic activity. To determine whether SigA was a protease, concentrated supernatant from *E. coli* DH5α/pSBA479 was tested for its ability to degrade a casein-based fluorogenic substrate by using an EnzChek protein assay kit. Protease activity was detected in the supernatant derived from strain DH5α/pSBA479 (Fig. 3, column A), while no protease activity was detected in supernatants of the control *E. coli* DH5α/pPBA1100 (column B). As expected, supernatant from the wild-type *S. flexneri* 2a YSH6000T (column C) also showed proteolytic activity. To test whether this activity was attributable to SigA, we inserted a Km^r cassette into a unique *Cla*I site in the *sigA* gene and introduced it by allelic exchange to construct the *Shigella sigA* mutant SBA1351. No protease activity was observed with this strain (column E), indicating that SigA is the only secreted protease of *S. flexneri* 2a with activity for casein. Moreover, casein-specific proteolytic activity was fully restored in a *she* PAI deletant strain complemented with an intact *sigA* gene carried on pSBA479 (column D). The higher levels observed in the complemented strain SBA1361 appeared to be due to a gene dosage effect.

Biological role of SigA. The properties of many autotransporter proteins suggest roles in bacterial virulence (18). Of the known autotransporters, the enterotoxin Pet from enteroaggregative *E. coli* has the greatest sequence similarity to SigA. To determine whether SigA had similar properties, we tested SigA for cytopathic activity toward HEp-2 cells.

Partially purified SigA protein was obtained by passage of culture supernatants from DH5α/pSBA479 through a 100-kDa-cutoff filter (Materials and Methods). After filtration, the protein was found in the retentate. The concentrated SigA protein preparation was applied to HEp-2 epithelial cells cultured in eight-well chamber slides.

Supernatants containing the SigA protein caused extensive damage to the HEp-2 cells, as characterized by the release of cellular focal adhesion contacts from the glass substratum, rounding of cells, and detachment (Fig. 4B). Similar effects were observed with purified Pet toxin from enteroaggregative *E. coli* (Fig. 4A). However, the level of toxicity appeared to be more pronounced on treatment with Pet. The morphologies of the HEp-2 cells were unaltered by treatment of the cells with concentrated supernatants from DH5α/pPBA1100 (Fig. 4D). In addition, treatment of the SigA protein preparations with PMSF prior to addition of the preparations to the HEp-2 culture medium inhibited the cytopathic activity of the preparations on HEp-2 cells (Fig. 4C).

To test whether SigA had enterotoxic activity, we compared the amount of fluid accumulation induced by a *sigA* mutant (SBA1351) and its parent strain (YSH6000T) in a rabbit ileal loop model of infection. Fluid accumulation induced by the *sigA* mutant was 30% lower than that of the wild-type YSH6000T ($P = 0.004$ by Student's t test, two tailed). Since *sigA* lies upstream of the genes encoding ShET1 enterotoxin (32), it was possible that the decreased fluid accumulation observed was due to polar effects on the expression of the enterotoxin genes. To test this, we compared the induction of fluid accumulation by SBA1351 (*sigA*) and by SBA1351 complemented with pSBA572 ($sigA^+$). A mean fluid accumulation ratio of 0.79 was observed with the mutant as opposed to 1.05 with the complemented strain ($P = 0.016$), indicating that reduced fluid accumulation the phenotype of SBA1351 was due to disruption of *sigA* rather than a polar effect on downstream genes.

DISCUSSION

In this study, we have shown that in addition to Pic, the *she* PAI of *S. flexneri* 2a encodes another protein, SigA, belonging

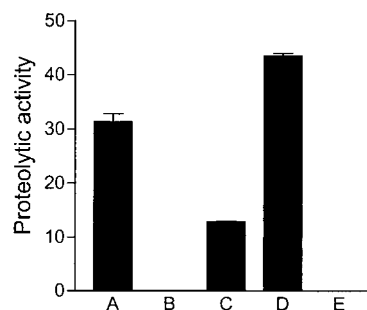

FIG. 3. Protease activity of SigA. Proteolytic activity of concentrated supernatants derived from *E. coli* and *S. flexneri* 2a was measured as described in Materials and Methods. Columns: A, *E. coli* DH5α/pSBA479 (*sigA*); B, DH5α/pPBA1100 (vector only); C, *S. flexneri* 2a YSH6000T (parent); D, SBA1361 (*sigA*); E, SBA1351 (*sigA*::*kan*). Error bars indicate standard errors in assays performed at least in duplicate.

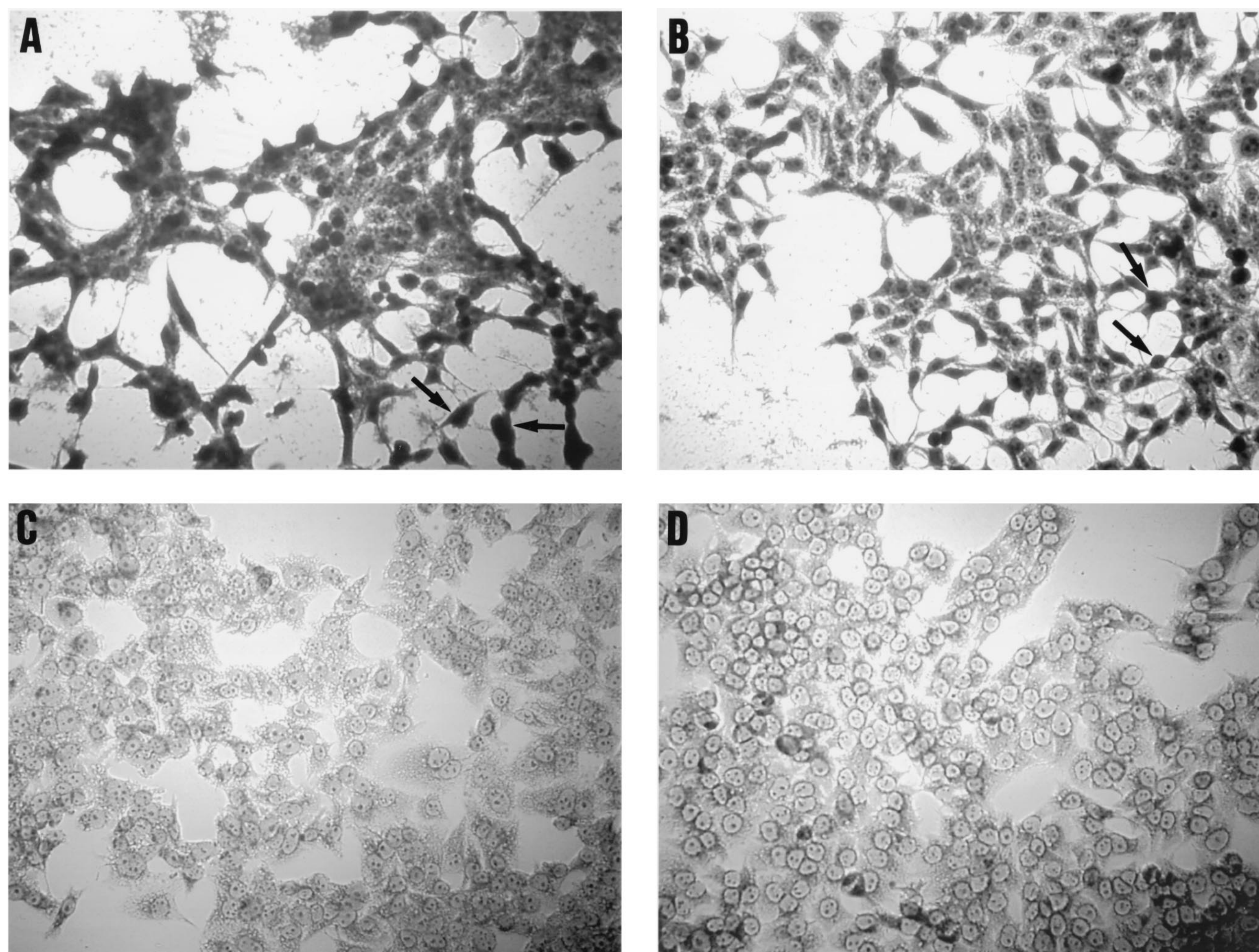

FIG. 4. Effect of SigA protein on epithelial cells. SigA (B) and Pet (A) proteins were added to cell cultures at a final concentration of 25 μg/ml per well and incubated for 3 h. Release from the glass substratum of the cellular focal adhesion points and rounding of cells is indicated by arrows. (C) HEp-2 cells incubated with PMSF (25 μg/ml)-treated SigA preparations. No cell rounding or detachment of focal adhesion contacts is apparent. Appropriate vector control preparations were also added (D; see text).

to the autotransporter family of proteins. The *sigA* gene encodes a protein with a predicted molecular mass of 139.6 kDa that has significant sequence similarity to several autotransporters from a variety of gram-negative bacteria. Like SepA and Pic, two other autotransporters found in *Shigella*, SigA is most closely related to the SPATE subfamily of autotransporters. SigA exhibits several highly conserved features of this family, including a long putative N-terminal signal sequence, a predicted C-terminal β domain, a putative β-domain cleavage site, a serine protease active-site motif in the putative α domain, and the C-terminal amino acid motif YSF, which is necessary for correct localization of autotransporters to the outer membrane (19). Secondary structure predictions also suggested that the putative β domain consists of 14 antiparallel, amphipathic β sheets, a common structural feature of autotransporters.

Many of its structural features suggested that SigA is autonomously secreted from the cell via an autosecretory pathway. This hypothesis was supported by our finding that SigA is secreted from *E. coli* carrying only the cloned *sigA* gene. This finding suggested that no *Shigella*-specific accessory genes are required for the secretion of SigA. Furthermore, the secreted form of SigA is a 103-kDa protein, indicating that the 139-kDa form was proteolytically processed. The molecular mass of the secreted form is consistent with the predicted proteolytic cleavage of the N-terminal signal sequence and the C-terminal β domain at the predicted residues 54-55 and 1007-1008, respectively. Further support for an autosecretory pathway for the secretion of SigA was obtained by deleting the putative β domain. The inability of bacteria to secrete a C-terminally truncated SigA protein showed that the putative β domain was essential for extracellular export.

The presence of a conserved serine protease motif suggested that, like other members of the SPATE subfamily, SigA is a serine protease. This hypothesis was confirmed by the demonstration that SigA had proteolytic activity for a casein-based substrate. By assaying isogenic *sigA* strains, we also demonstrated that SigA is the only secreted protease of *S. flexneri* 2a that has activity toward casein. Although SigA degrades casein in vitro, its natural substrate is not known. We predict that the casein hydrolytic activity of SigA from the cell is dependent on the conserved serine protease active-site motif in SigA.

The close linkage of *sigA* to the genes encoding the ShET1 enterotoxin and the putative virulence protein Pic suggested

that SigA might also be involved in virulence. Since the expression of many bacterial virulence proteins, including the autotransporters SepA (4), Tsh (30), Pic (17), and EspP (7), is thermoregulated, we tested whether the expression of SigA was regulated in the same way. We found that the expression of SigA resembled that of many virulence proteins since high levels of SigA were detectable in the culture supernatant of *Shigella* grown at 37 but not 21°C.

Autotransporters have been implicated in a range of bacterial processes associated with virulence. Given that Pet, the most closely related homolog of SigA, is an enterotoxic and cytopathic protease from enteroaggregative *E. coli* (14), we examined SigA for similar properties. Our results indicate that SigA induces cytopathic effects in HEp-2 cells but not to the same magnitude as Pet.

To test SigA for enterotoxic properties, we examined the ability of *S. flexneri* 2a strains that were isogenic for *sigA* to induce fluid accumulation in a rabbit ileal loop model of infection. A 30% reduction in fluid accumulation was observed for the *sigA* mutant, suggesting that SigA is a putative enterotoxin. However, since the *sigA* mutant was still capable of inducing substantial fluid accumulation, SigA appears to be only one of a number of enterotoxins produced by *S. flexneri* 2a. This is consistent with the presence of the genes for the ShET1 enterotoxin in our test strain. In addition, it is likely that our test strain also produces ShET2, an enterotoxin that is encoded by a DNA sequence which is widely distributed in *Shigella* strains.

It appears likely that the cytopathic and enterotoxic activity of SigA are related to its proteolytic activity, although we have not established this as part of this study. Examples where such a relationship has been established include the Pet enterotoxin from enteroaggregative *E. coli* (26) and the enterotoxin of *Bacteroides fragilis* (11). Although their specific mechanisms of action are unknown, both proteases exert their toxic effects by affecting the eukaryotic cell cytoskeleton. SigA and its closest homolog, Pet, appear to share a number of similar functions, including proteolytic activity, cytopathic effects on cells, and possible enterotoxic activity. However, only the expression of *sigA* is temperature regulated.

In conclusion, we have characterized the novel autotransporter protein SigA, which is encoded by the *she* PAI of *S. flexneri* 2a, and established a possible role for it in virulence. *sigA* is the second autotransporter gene to be found on the *she* PAI and one of three such genes harbored by *S. flexneri* 2a strains which appear to contribute to virulence. Further investigation of the *she* PAI will reveal whether it carries additional virulence determinants and thus contribute to our understanding of the evolution of virulence in *Shigella*.

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