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Disulfide oxidoreductase activity of *Shigella flexneri* is required for release of Ipa proteins and invasion of epithelial cells

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ABSTRACT **Secretion of IpaB, IpaC, and IpaD proteins of *Shigella flexneri*, essential for the invasion of epithelial cells, requires a number of proteins encoded by the *spa* and *mxi* loci on the large plasmid. Introduction of *dsbA*::Tn*5* into *S. flexneri* from *Escherichia coli* K-12 reduced invasiveness, which resulted from a decrease in the capacity to release IpaB, IpaC, and IpaD proteins into the external medium. Examination of the surface-presented Ipa proteins of the *dsbA* mutant, however, revealed Ipa proteins at levels similar to those on wild-type cells. Since the defective phenotype was similar to that of the *spa32* mutant of *S. flexneri* and the Spa32 sequence possessed two Cys residues, the effect of *dsbA* mutation of the folding structure of Spa32 under reducing conditions and on the surface expression of Spa32 was investigated. The results indicated that Spa32 was a disulfide-containing protein whose correctly folded structure was required for its presentation on the outer membrane. Indeed, replacing either one of the two Cys residues in Spa32 with Ser by site-directed mutagenesis reduced its capacity to release Ipa proteins into the external medium and led to the accumulation of Spa32 protein in the periplasm. These results indicated that the DsbA protein performs an essential function during the invasion of mammalian cells, by facilitating transport of the Spa32 protein across the outer membrane.**

Shigella are the causative agents of bacillary dysentery in humans and primates. An early essential step in pathogenesis is the invasion of colonic epithelial cells, followed by bacterial multiplication and spread into adjacent cells. The invasive capacity of *Shigella* is governed by proteins encoded by a subset of three contiguous operons (*ipa*, *mxi*, and *spa*) in a 31-kb DNA sequence on the large 230-kb plasmid (1, 2). The three invasins IpaB, IpaC, and IpaD, encoded by the *ipaBCD* genes in the *ipa* operon, have been shown to play a central role in the invasion of epithelial cells by *Shigella* (3).

Several studies have shown that the IpaB, -C, and -D proteins are secreted onto the bacterial surface and/or into the external medium (2, 4–6), although Ipa sequences do not contain classical signal peptide sequences (7, 8). Furthermore, secretion of Ipa proteins is a prerequisite for invasion, since *Shigella* mutants unable to secrete Ipa proteins are less invasive or noninvasive, even though a normal amount of IpaB, -C, and -D proteins is produced by the bacterium (1, 2, 4–6, 9). These studies have also indicated that transport of Ipa proteins across the inner and outer membranes requires the expression of a set of genes encoded by the *spa* (*s*urface *p*resentation of invasive plasmid *a*ntigens) and *mxi* (*m*embrane e*x*pression of *i*nvasion plasmid antigens) operons, including *spa47*, *spa13*, *spa32*, *spa33*, *spa24*, *spa9*, *spa29*, and *spa40* and *mxiHIJMEDA*. Although the exact roles of the *spa* and *mxi* gene products in the secretion of Ipa proteins are still obscure, recent studies (1, 2, 5, 6, 9) have indicated that some Spa and Mxi proteins, such as Spa32 and MxiJ, are present in the outer membrane and that Spa47, Spa40, and MxiA are located in the inner membrane. Recently, it has been shown that the secretion of IpaB, -C, and -D proteins from *Shigella flexneri* into the medium takes place by a process of surface presentation and then release into the external medium (27). Most of the Spa proteins, such as Spa47, Spa33, Spa24, Spa9, and Spa29, and the Mxi proteins, such as MxiJ and MxiA, are required for the former step (1, 2, 5, 6, 9), and Spa32 is required for the latter step (27).

To obtain insights into the mechanisms underlying Ipa secretion, we have been exploring factors involved in secretion onto the bacterial surface and release into the external medium. Here, we present evidence that the transduction of *dsbA*::Tn*5* from *Escherichia coli* K-12 into *S. flexneri* 2a YSH6000 greatly reduced its invasive capacity. Since the function of *dsbA* (coding for a positive disulfide oxidoreductase) in *E. coli* K-12 has been shown to be involved in the folding of exported proteins and mutations in *dsbA* resulted in a decreased rate of disulfide bond formation for proteins in the periplasm (10–14), we sought protein(s) whose functions were affected by the *dsbA* mutation, leading to changes in the capacity to secrete IpaB, -C, and -D proteins. The results indicated that defective *dsbA* expression in *S. flexneri* reduced its capacity to release cell-surface IpaB, -C, and -D proteins but did not affect the presentation of these proteins on the cell surface. The reduced levels of Ipa proteins released into the external medium were not due to changes in the Ipa proteins themselves but, rather, were caused by inefficient information of the disulfide bond in the Spa32 protein, encoded by *spa32* on the large plasmid, resulting in the absence of Spa32 on the outer membrane. The possible role of Spa32 in the release of Ipa proteins during the invasion process will also be discussed.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Bacterial strains and plasmids used are listed in Table 1.

Detection of IpaB, IpaC, and IpaD Proteins Released from Bacteria into Phosphate-Buffered Saline (PBS). A slight modification of the method of Andrew *et al.* (4) was used. Briefly, bacteria grown to midlogarithmic phase in BHI broth (Difco) at 37°C were harvested and suspended in a PBS to an OD_{600} of 5.0 units. The suspended cells incubated at room temperature for 0, 20, 60, or 120 min were centrifuged, and the supernatant was passed through a 0.22-μm pore-size filter (Millipore). The collected bacteria or the cell-free PBS supernatants were added to wells of 96-well microtiter plates (Costar) and incubated for 2 h at room temperature. The wells were then emptied, and 5% (wt/vol) bovine serum albumin was added. After two washes with PBS/0.05% Tween 20 (PBST), anti-IpaB, -IpaC, or -IpaD antibodies were added to each well and incubated for 2 h at 37°C. After three washes with PBST, horseradish peroxidase-conjugated protein A was added and incubated for 1 h at 37°C. After three washes with PBST, the substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-

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Table 1. Strains and plasmids

Name	Relevant genotype	Ref. or source
E. coli K-12		
SK101	*dsbA*::Tn*5*	15
S. flexneri		
YSH6000	Wild type	7
CS2585	YSH6000T/pMYSH6000 Δ*spa32*	M. W.
CS2586	CS2585/pMAW200 (cloned *spa32*)	This study
CS2589	YSH6000 *dsbA*::Tn*5*	This study
CS2590	CS2589/pMAW205 (cloned *dsbA*)	This study
CS3000	CS2585/pMAW207 (cloned *spa32-19*)	This study
CS3001	CS2585/pMAW208 (cloned *spa32-292*)	This study
Plasmid		
pMW119Tp	A pSC101 derivative encoding trimethoprim(Tp) resistance	T. T.
pCHR312	pBR322Tp containing *ipaBCD*	7
pMAW200	pMW119Tp containing *spa32*	M. W.
pMAW205	pMW119Tp containing *dsbA*	This study
pMAW207	Same as pMAW200 but containing *spa32-19*	This study
pMAW208	Same as pMAW200 but containing *spa32-292*	This study

sulfonic acid) diammonium salt was added. A_{405} was measured by using an ELISA reader model 450 (Bio-Rad).

Generation of Point Mutants by Site-Directed Mutagenesis. A 1310-bp *Eco*RI–*Bgl* II segment of pCHR312 containing the *ipaBCD* genes (7) or a 1539-bp *Eco*RI segment containing the *spa32* gene obtained from the large plasmid of YSH6000 (9) was cloned into pBR322 and the resulting plasmid was used to introduce a point mutation by using the protocol supplied with the U.S.E. mutagenesis kit (Pharmacia). The nucleotide sequences of the mutagenic oligonucleotides were 5′-GAGTAATGGGTTCTGTTGGGAAAATA-3′, 5′-CAAAAGCAATTTTCCAACAAACTACTG-3′, 5′-ACAATAAGCTCATCTACAGATACAGAT-3′, 5′-CAGATAGAAAAATCTGAGAAACTATCT-3′, and 5′-AGTGAAGAAGAATCCTAAGAATTAAAC-3′. These oligonucleotides were used to replace Cys residues with Ser residues at positions 309, 7, and 322 in the IpaB, IpaC, and IpaD proteins (7, 8), respectively, and at positions 19 and 292 in the Spa32 protein (9). Mutagenic bases are underlined and correspond to codons 309 (TGT to TCT), 7 (TGT to TCC), 322 (TGT to TCT), 19 (TGT to TCT), and 292 (TGC to TCC), respectively. The mutated *ipaB*, *ipaC*, and *ipaD* genes were named *ipaB-309*, *ipaC-7*, and *ipaD-322*, respectively, and each of the mutated genes on the 606-bp *Sna*BI–*Hpa* I segment (*ipaB-309*), 1063-bp *Hpa* I–*Sac* I segment (*ipaC-7*), and 720-bp *Eco*RV segment (*ipaD-322*) of the 1310-bp *Eco*RI–*Bgl* II fragment was used to replace those respective restriction segments on pCHR312. The mutated *spa32* genes, named *spa32-19* and *spa32-292*, were cloned into pMW119Tp.

Assay for Epithelial-Cell-Contact-Triggered Ipa Release. The extent of release of IpaB, -C, and -D proteins from bacteria in contact with MK2 cells was determined as follows: Semiconfluent MK2-cell monolayers (2×10^5 cells per ml) in 35-mm plastic tissue culture dishes (Falcon) were inoculated with bacteria (≈100 bacteria per MK2 cell) and centrifuged for 10 min at $700 \times g$. Immediately after the centrifugation, the supernatants were passed through a 0.22-μm pore-size filter (Millipore), and the resulting cell-free tissue culture medium [minimum essential medium (MEM)] was added to wells of a 96-well microtiter plate (Costar). The amounts of IpaB, -C, and -D proteins in the cell-free medium were measured by ELISA with antibodies specific for IpaB, IpaC, or IpaD protein.

Determination of Oxidation State and Localization of Spa32 *in Vivo*. Equal amounts of outer membrane proteins or periplasmic proteins extracted from YSH6000, CS2589 (*dsbA*::Tn*5*), CS2590 (*dsbA*::Tn*5*/*dsbA*$^+$), CS3000 (Δ*spa32*/*spa32-19*), or CS3001 (Δ*spa32*/*spa32-292*) were mixed with SDS sample buffer and boiled for 5 min. For reducing or nonreducing conditions, the SDS sample buffer contained either 4% (vol/vol) 2-mercaptoethanol or no 2-mercaptoethanol, respectively (10). Ten micrograms of protein extract was separated by SDS/PAGE and transferred to a nitrocellulose membrane. Spa32 was detected with an antibody specific for Spa32 protein (M. W., unpublished results).

RESULTS

***dsbA*::Tn*5* Reduces Invasiveness of *S. flexneri*.** To test whether the invasive capacity of *S. flexneri* was affected by a defect in DsbA function, the *dsbA*::Tn*5* mutation of SK101 (15) was introduced into YSH6000 by using P1 phages. A Tn*5* [Kanamycin-resistant (Km^r)] transductant (CS2589) exhibiting a high dithiothreitol sensitivity compared with the wild type, as has been reported for *dsbA* mutants in *E. coli* K-12 (16), was isolated, and its invasive capacity was compared with that of YSH6000 by infecting MK2-cell monolayers. Examination of the Giemsa-stained MK2 cells revealed that the proportion of MK2 cells infected with CS2589 (*dsbA*$^-$) was reduced to 10% that of YSH6000 (Table 2). To confirm that the reduced invasion capacity of CS2589 was caused by P1-phage-mediated introduction of *dsbA*::Tn*5*, the *dsbA* region from YSH6000 was PCR-amplified and the amplified 1219-bp *dsbA* segment was cloned into pTW119Tp (pMAW205), introduced into CS2589 (*dsbA*$^-$) (CS2590), and tested for ability to restore the invasiveness to CS2589. Examination of Giemsa-stained MK2 cells infected with CS2590 (*dsbA*$^+$) showed that the bacteria were restored to an invasive capacity comparable to that of YSH6000 (Table 2).

Effects of *dsbA*::Tn*5* on Ipa Secretion. To investigate effects of *dsbA*::Tn*5* on the secretion of Ipa proteins, we utilized an *in*

Table 2. Percentage of infected cells 2 h after infection

Strain	Genotype or characteristic	Inv, %	Ratio
YSH6000	Wild type	28.1	1.00
CS2589	*dsbA*::Tn*5*	2.1	0.07
CS2590	CS2589/pMAW205 (*dsbA*$^+$)	27.2	0.97
CS2585	Δ*spa32*	0.4	0.01
CS2586	CS2585/pMAW200 (*spa32*$^+$)	27.5	0.98
CS3000	CS2585/pMAW207 (*spa32-19*)	1.2	0.04
CS3001	CS2585/pMAW208 (*spa32-292*)	1.8	0.06
CS2991	N1411 (*ipaB*)/pMAW109 (*ipaB-309*)	28.0	0.99
CS2992	N777 (*ipaC*)/pMAW110 (*ipaC-7*)	27.9	0.99
CS2993	N1249 (*ipaD*)/pMAW111 (*ipaD-322*)	27.7	0.98

Percentage of infected (Inv) cells 2 h after infection is shown. Cells invaded by more than five bacteria were considered to be infected (17).

vitro assay (4) and monitored the changes in Ipa proteins secreted onto the bacterial surface or into the external medium. Accordingly, CS2589 (*dsbA*$^-$), YSH6000, or S325 (*mxiA*$^-$) was suspended in PBS and incubated at room temperature. The levels of IpaB, -C, and -D proteins presented on the surface or released into PBS after 0, 20, 60, and 120 min were then measured by ELISA with antibodies specific for IpaB, IpaC, or IpaD. The results showed that the levels of IpaB, -C, and -D proteins associated with the surface of CS2589 (*dsbA*$^-$) after time 0 were similar to those on YSH6000, those at 120 min were slightly decreased to 90% (IpaB), 91% (IpaC), and 96% (IpaD) of the initial levels, and the Ipa proteins were barely released into PBS (Fig. 1). In contrast, the levels of IpaB, -C, and -D proteins associated with the surface of YSH6000 after 120 min were reduced to 52% (IpaB), 52% (IpaC), and 55% (IpaD) of the initial levels, while Ipa proteins released into PBS increased (Fig. 1). As expected, S325 (*mxiA*$^-$) was unable to secrete the Ipa proteins onto the cell surface and into PBS (data not shown). These results thus confirmed that the *dsbA* mutation did not have a great effect on the secretion of Ipa proteins onto the cell surface but rather markedly affected the subsequent step of release into the external medium.

A Unique Cys Residue in Each Ipa Protein Is Not Essential for Invasive Capacity. The amino acid sequences of the IpaB, IpaC, and IpaD proteins deduced from their nucleotide sequences revealed that each Ipa protein possesses a unique Cys residue (7, 8). As the *dsbA* mutant showed a reduced capacity to release Ipa proteins (Fig. 1) and the released IpaB and IpaC proteins are able to form complexes (18), we wondered whether the Cys residues were involved in release and whether two Ipa Cys residues were capable of forming an intermolecular disulfide bond. To test this hypothesis, pCHR312 encoding the *ipaBCD* genes was utilized (7), and the Cys residues at positions 309 in IpaB, 7 in IpaC, and 322 in IpaD were replaced with Ser residues by using site-directed mutagenesis. Each of the mutated pCHR312 constructs possessing *ipaB-309* (pMAW109), *ipaC-7* (pMAW110), or *ipaD-322* (pMAW111) were introduced into N1411 (*ipaB*::Tn*5*), N777 (*ipaC*::Tn*5*), and N1249 (*ipaD*::Tn*5*), respectively (7), and then tested for changes in invasiveness or the ability to release Ipa proteins into the external medium. The results showed that bacteria possessing any one of the mutated Ipa proteins were still able to invade epithelial cells at a level similar to that of YSH6000 (Table 2) and release Ipa proteins into the external medium (data not shown), indicating that none of the Cys residues in the Ipa proteins directly contributes to their release from the cell surface.

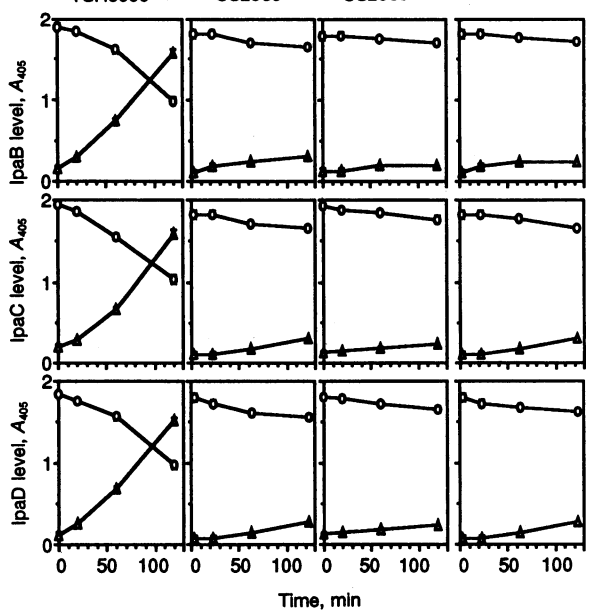

FIG. 1. Release of IpaB, IpaC, and IpaD proteins from YSH6000, CS2589 (*dsbA*$^-$), CS2585 (*spa32*$^-$), and CS3000 (*spa32-19*) cells. The bacteria grown to midlogarithmic phase were suspended in PBS, and levels of Ipa proteins on the cell surface (o) and in the external medium (Δ) were determined by ELISA with antibodies specific for IpaB, IpaC, or IpaD. Each value represents the average of triplicate samples.

DsbA Is Required for Spa32 Function. We recently found that a *spa32* mutant, named CS2585, that possessed an in-frame deletion of the *spa32* gene on the large plasmid of YSH6000, has a reduced capacity to release IpaB, -C, and -D proteins into the culture supernatant but retains the Ipa proteins on the cell surface (27). Since the defect observed in the *dsbA* mutant was similar to that of CS2585 (*spa32*$^-$), we monitored the levels of Ipa proteins secreted onto the cell surface or into the external medium (PBS) with CS2589 (*dsbA*$^-$) and CS2585 (*spa32*$^-$) (Fig. 1). The levels of IpaB, -C, and -D proteins associated with the surface of CS2585 (*spa32*$^-$) after time 0 were similar to those of CS2589 (*dsbA*$^-$), showing only a slight decrease at 120 min, but the Ipa proteins were barely released into the PBS (Fig. 1). Thus, it was likely that the *dsbA* mutation in *S. flexneri* had some effect on Spa32 function, resulting in a reduced capacity to release Ipa proteins and a less-invasive phenotype (Table 2). Examination of the amino acid sequence of Spa32 deduced from the nucleotide sequence revealed that it contained two Cys residues at positions 9 and 292 (9). Furthermore, the Spa32 protein is exposed on the bacterial surface (27). Thus, to determine whether Spa32 was a disulfide-containing protein, the effect of the *dsbA* mutation on the folding of the Spa32 protein was first investigated by observing its mobility by SDS/PAGE under reducing conditions. Periplasmic protein extracts from YSH6000, CS2589 (*dsbA*$^-$), and CS2590 (*dsbA*$^+$) were subjected to SDS/PAGE in the presence or absence of 5% 2-mercaptoethanol, and the Spa32 protein was detected on immunoblots with anti-Spa32 antibody. Different forms of Spa32 protein corresponding to the reduced and oxidized forms were detected in YSH6000 and CS2590 (*dsbA*$^+$) but not in CS2589 (*dsbA*$^-$) (Fig. 2), indicating that a disulfide bond was formed in Spa32.

To further confirm this, we performed site-directed mutagenesis by using a 1539-bp DNA segment containing *spa32* obtained from pMYSH6000 with Cys-19 or Cys-292 replaced with Ser residues. The mutated *spa32* clones, named *spa32-19* and *spa32-292*, possessing Ser-19 and Ser-292, respectively, were ligated to pMW119Tp vector and designated pMAW207 and pMAW208. pMAW207 (*spa32-19*) or pMAW208 (*spa32-292*) was introduced into CS2585 (*spa32*$^-$) (to make CS3000 and CS3001, respectively) and examined for the levels of IpaB, IpaC, and IpaD proteins expressed on the cell surface or released into the external medium. Although CS3000 (*spa32-19*) and CS3001 (*spa32-292*) were able to present Ipa proteins on the cell surface as was YSH6000 or CS2585 (*spa32*$^-$) (data

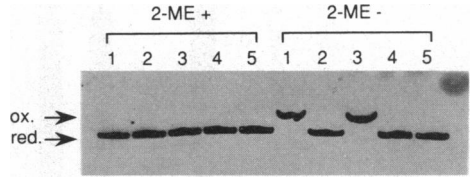

FIG. 2. Effect of the *dsbA*::Tn*5*, *spa32-19*, or *sp32-292* mutation on the oxidation state of Spa32 protein. Lanes: 1, YSH6000; 2, CS2589 (*dsbA*$^-$); 3, CS2590 (*dsbA*$^+$); 4, CS3000 (*spa32-19*); 5, CS3001 (*spa32-292*). Arrows indicate the oxidized (ox.) and reduced (red.) forms of Spa32 proteins detected by immunoblots with anti-Spa32 antibody. 2-ME+ and 2-ME− indicate the presence or absence of 2-mercaptoethanol, respectively.

not shown), only very small amounts of Ipa proteins were released into the culture supernatant (Fig. 1). Furthermore, the invasion capacities of CS3000 (*spa32-19*) and CS3001 (*spa32-292*) were reduced to a low level similar to that of CS2589 (*dsbA*$^-$) (Table 2). These results thus suggested that lack of the disulfide bond formation in the Spa32 protein affected the release of Ipa proteins from the cell surface and resulted in a less-invasive phenotype.

Interestingly, we have recently observed that the release of IpaB, -C, and -D proteins from *S. flexneri* is triggered by contact with MK2 cells. However, CS2585 (spa32$^-$) attached to MK2 cells failed to release Ipa proteins, even though the bacteria were able to secrete Ipa proteins on the cell surface, indicating that the Spa32 protein plays a crucial role in mediating release of the Ipa proteins in *in vivo* conditions (27). In this regard, we tested whether CS2589 (*dsbA*$^-$), CS3000 (*spa32-19*), or CS3001 (*spa32-292*) cells were able to release Ipa proteins upon contact with MK2-cell monolayers. Our results showed that the levels of IpaB, -C, and -D proteins released from attached CS2589 (*dsbA*$^-$), CS3000 (*spa32-19*), and CS3001 (*spa32-292*) cells were greatly reduced, to levels similar to that of CS2585 (*spa32*$^-$) (Fig. 3).

Correctly Folded Spa32 Is Required for Presentation in the Outer Membrane. As mentioned above, Spa32 is a surface-exposed outer membrane protein, and YSH6000, CS2589 (*dsbA*$^-$), CS2590 (*dsbA*$^+$), CS3000 (*spa32-19*), and CS3001 (*spa32-292*) were examined for the effect of a *dsbA* mutation on its surface presentation by whole-cell ELISA with anti-Spa32. The results showed that the level of Spa32 associated with the cell surface of CS2589 (*dsbA*$^-$), CS3000 (*spa32-19*), and CS3001 (*spa32-292*) was reduced to 10% of that of YSH6000 or CS2590 (*dsbA*$^+$) (data not shown). Immunoblot analysis of the Spa32 protein present in the outer membrane or periplasm revealed that, while Spa32 expressed from YSH6000 or CS2590 (*dsbA*$^+$) was present in the outer membrane and in the periplasm (Fig. 4, lanes 1 and 3), the Spa32 protein expressed from CS2589 (*dsbA*$^-$), CS3000 (*spa32-19*), or CS3001 (*spa32-292*) was not present in the outer membrane but accumulated in the periplasm (Fig. 4, lanes 2, 4, and 5), suggesting that the correctly folded protein structure was required for Spa32 to cross the outer membrane.

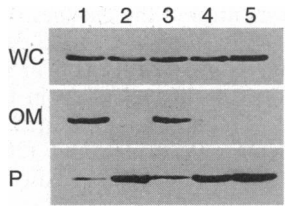

FIG. 4. Effect of *dsbA*::Tn*5*, *spa32-19*, or *spa32-292* mutation on presentation of Spa32 in the outer membrane. Lanes: 1, YSH6000; 2, CS2589 (*dsbA*$^-$); 3, CS2590 (*dsbA*$^+$); 4, CS3000 (*spa32-19*); 5, CS3001 (*spa32-292*). WC, OM, and P indicate whole-cell lysates, outer membrane, and periplasmic protein extracts, respectively (19, 20). The content of 2-keto-3-deoxyoctonate in the outer membrane fractions of YSH6000 and CS2589 was 93.0 and 92.5% of that in whole cells (100%), respectively, whereas the activity of alkaline phosphatase in the periplasm of YSH6000 and CS2589 was 87.6 and 90.0% of that in whole cells (100%), respectively.

DISCUSSION

Recent studies of *dsbA* mutants of *E. coli* K-12 have indicated that, although the *dsbA* gene is nonessential for bacterial growth, the disulfide bonds are crucial to the folding and stability of many secreted proteins (10–16, 21–25). This study has shown that a *dsbA*::Tn*5* mutation in *S. flexneri* 2a reduces its invasion capacity, perhaps as a consequence of the inefficient folded structure of Spa32, a surface-exposed outer membrane protein essential for mediating release of the IpaB, -C, and -D proteins upon contact with epithelial cells.

Examination of a *dsbA*::Tn*5* mutant of YSH6000, CS2589 (*dsbA*$^-$), for its capacity to secrete IpaB, -C, and -D proteins by using an *in vitro* assay (4) revealed that CS2589 suspended in PBS retained Ipa proteins on the cell surface at levels similar to those of YSH6000 at 0 min, which were only slightly reduced after a 120-min incubation. Moreover, the amount of Ipa protein released from CS2589 (*dsbA*$^-$) into PBS was marginal during the incubation compared with the amount released from YSH6000 (Fig. 1). In contrast, ≈50% of the Ipa protein initially associated with the surface of YSH6000 was lost after incubation in PBS for 120 min, accompanied by release of great amounts of Ipa proteins into PBS (Fig. 1). Based on this data, we concluded that the less-invasive capacity of the *dsbA* mutant was not caused by a defect at the step required to place Ipa proteins on the cell surface but, rather, resulted from a problem at the subsequent step, the release of Ipa proteins into the medium.

Although the mechanisms underlying the secretion of IpaB, -C, and -D proteins expressed by *S. flexneri* are still unclear, the Ipa proteins are secreted by a third pathway (type III) (26), which occurs independently of the Sec pathway and requires a large number of accessory proteins encoded by the *spa* or *mxi* genes on the large plasmid (1, 2, 4–6, 9). A recent study by Menard *et al.* (18) has indicated that the released IpaB and IpaC proteins form complexes. In this context, we investigated two possibilities that could lead to the reduced capacity of CS2589 (*dsbA*$^-$) to release Ipa proteins into the external medium. The first possibility was that Ipa proteins formed intermolecular disulfide bonds that were somehow involved in release. Indeed, the amino acid sequences of the IpaB, -C, and -D proteins revealed a unique Cys residue in each Ipa protein (7, 8). To test this, we replaced each Cys with Ser by using

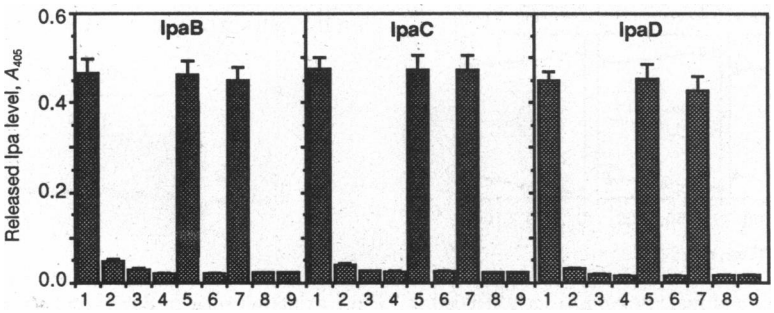

FIG. 3. Epithelial-cell-contact-triggered Ipa release from YSH6000 and the *dsbA* and *spa32* mutants. Ipa proteins released into the tissue culture medium (MEM) by contact with MK2 cells were measured by the cell-free ELISA with antibodies specific for IpaB, IpaC, or IpaD protein. Bars: 1, YSH6000; 2, YSH6000 without MK2 cells; 3, YSH6000 without attachment (no centrifugation); 4, CS2585 (*spa32*$^-$); 5, CS2586 (*spa32*$^+$); 6, CS2589 (*dsbA*$^-$); 7, CS2590 (*dsbA*$^+$); 8, CS3000 (*spa32-19*); 9, CS3001 (*spa32-292*).

site-directed mutagenesis, and the resulting plasmids, possessing the *ipaB-309*, *ipaC-7*, or *ipaC-322* gene, were introduced into the corresponding *ipa* mutant. These strains were then tested for changes in invasiveness or the ability to release Ipa proteins into the external medium. Our results show that bacteria possessing any one of the mutated Ipa proteins were still able to invade epithelial cells at a level similar to that of YSH6000 (Table 2) and to release Ipa proteins into the external medium (data not shown), indicating that none of the Cys residues in the Ipa proteins directly contributes to its releasing activity. We, therefore, searched for disulfide-containing proteins among Spa and Mxi proteins to investigate the second possibility that a correctly folded structure was required for the release of Ipa proteins. The amino acid sequences of Spa47, Spa32, Spa33, Spa29, Spa40, MxiM, MxiE, and MxiA deduced from their nucleotide sequences revealed that each contains at least two Cys residues and the proteins seem to be secreted into the inner or outer membrane of *S. flexneri* (1, 2, 4, 5, 9). However, *spa* mutants such as *spa47*, *spa33*, *spa29*, and *spa40* (2, 9) and *mxi* mutants such as *mxiA* (1) are unable to secrete IpaB, -C, and -D proteins onto the cell surface. In this sense, Spa32 was the most plausible candidate among Spa and Mxi proteins, since Spa32 was located on the bacterial surface as an outer membrane protein, and a *spa32* mutant, CS2585, released reduced amounts of IpaB, -C, and -D proteins into the medium but retained normal amounts on the surface (Fig. 1). Thus, we reasoned that Spa32 was a disulfide-bond-containing protein whose correctly folded protein structure was important for presentation onto the outer membrane and mediation of the release of IpaB, -C, and -D proteins into the external medium. As expected, the Spa32 protein produced from YSH6000, but not from CS2589 (*dsbA*$^-$), did change structure in SDS/PAGE, responding to reduced or oxidized conditions (Fig. 2). The replacement of the Cys residues with Ser at position 19 or 292 in Spa32 by site-directed mutagenesis revealed that bacteria producing the mutated Spa32-19 or Spa32-292 protein had a reduced level of IpaB, -C, and -D protein released into the culture supernatant, as compared with that of YSH6000. Indeed, the level of Spa32 protein expressed on the surface of CS3000 [CS2585 (*spa32*$^-$) carrying pMAW207 (*spa32-19*)], CS3001 [CS2585 (*spa32*$^-$) carrying pMAW208 (*spa32-292*)], and CS2589 (*dsbA*$^-$) was reduced to a low level at 10% of that on YSH6000, as determined by whole-cell ELISA with anti-Spa32 antibody (M. W., unpublished results). Furthermore, immunoblot analysis with anti-Spa32 antibody of the outer membrane and periplasmic fractions revealed that Spa32 expressed from CS2589 (*dsbA*$^-$), CS3000 (*spa32-19*), and CS3001 (*spa32-292*) was not present in the outer membrane but did accumulate in the periplasm (Fig. 4). In agreement with this, the invasiveness of CS3000 (*spa32-19*) and CS3001 (*spa32-292*) and their capacities to release Ipa proteins upon contact with MK2 cells were greatly reduced to a level similar to that of CS2589 (*dsbA*$^-$). These results thus suggested that the correctly folded Spa32 structure formed by the disulfide bond facilitated the presentation of the Spa32 protein to the outer membrane and that the surface-exposed Spa32 protein took part in releasing Ipa proteins, although the exact mechanisms of release of Ipa proteins remain to be elucidated. Clearly, further elucidation of the functional structure and the catalytic domain of Spa32 protein involved in the release of Ipa proteins will be important for understanding the mechanisms underlying epithelial-cell-contact-induced release of Ipa proteins.

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