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dksA Is Required for Intercellular Spread of *Shigella flexneri* via an RpoS-Independent Mechanism

SCOTT A. MOGULL, LAURA J. RUNYEN-JANECKY, MEI HONG,† AND SHELLEY M. PAYNE*

Section of Molecular Genetics and Microbiology and Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, Texas 78712-1095

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Pathogenesis of *Shigella flexneri* is dependent on the ability of the bacterium to invade and spread within epithelial cells. In this study, we identified *dksA* as a gene necessary for intercellular spread in, but not invasion of, cultured cells. The *S. flexneri dksA* mutant exhibited sensitivity to acid and oxidative stress, in part due to an effect of DksA on production of RpoS. However, an *S. flexneri rpoS* mutant formed plaques on tissue culture monolayers, thus excluding DksA regulation of RpoS as the mechanism responsible for the inability of the *dksA* mutant to spread intercellularly. Intracellular analysis of the *dksA* mutant indicates that it survived and divided within the Henle cell cytoplasm, but the *dksA* mutant cells were elongated, and some exhibited filamentation in the intracellular environment. Some of the *S. flexneri dksA* mutant cells showed aberrant localization of virulence protein IcsA, which may inhibit spread between epithelial cells.

Shigella flexneri colonizes the colon and causes bacterial dysentery in humans (13, 27, 37). These bacteria are highly infectious, with as few as 10 cells being sufficient to cause disease in healthy adults (6). This low infectious dose is partially due to a significant resistance to acidic conditions, which permits survival during transit through the acidic conditions encountered in the human stomach (12, 38). In the colon, *Shigella* cells cross the epithelial layer, attach to the basal surfaces of epithelial cells in a receptor-specific process, and induce their own internalization (29). Following internalization, *Shigella* cells are temporarily located in a phagocytic vacuole. Lysis of this vacuole occurs within minutes, and *Shigella* cells initiate replication and cell to cell spread. This process is accompanied by a complex pattern of protein induction and suppression (16). Cell-to-cell spread requires that *Shigella* protein IcsA (VirG) be targeted to the old pole of the bacterium, where it induces assembly of F-actin by the epithelial cell (2, 25, 26, 39). The process by which *S. flexneri* localizes IcsA to a polar location is unknown, but the unipolar localization of IcsA when expressed in *Escherichia coli* indicates that *Shigella* virulence plasmid proteins are not involved in this process in *E. coli* (36). Polymerization of actin at one pole is responsible for propelling the bacterium through the host cytoplasm and into a protrusion of a double-membrane barrier between two host cells. *Shigella* lyses this membrane barrier, and, after being released into the new epithelial cell, the bacteria repeat the process of multiplication and spread. Progressive spread of these bacteria leads to degradation of the epithelium and inflammation, resulting in symptoms of disease. An in vitro model utilizing tissue culture monolayers that mimics the process of *S. flexneri* invasion, intercellular multiplication, and spread has been developed (14, 22, 31). Bacteria that form plaques on these tissue culture monolayers are capable of performing these intracellular processes. Using this model, we examined Tn*phoA* mutants that were deficient in intracellular multiplication or intercellular spread. One gene, *dksA*, was identified here to be required for the intercellular spread of *S. flexneri*.

* Corresponding author. Mailing address: Section of Molecular Genetics and Microbiology, The University of Texas, Austin, TX 78712-1095. Phone: (512) 471-9258. Fax: (512) 471-7088. E-mail: payne@mail.utexas.edu.

† Present address: DoubleTwist Inc., Oakland, CA 94612.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used here are listed with their sources in Table 1. All strains were stored at −80°C in tryptic soy broth plus 20% glycerol. *S. flexneri* strains were grown on Congo red agar to screen colonies that bind Congo red (33). *E. coli* strains were grown in Luria broth (LB) or on Luria agar (L agar). Intracellular salts medium (ISM) was used to mimic the intracellular conditions (16). All strains were routinely cultured at 37°C unless otherwise noted. Antibiotics were used as appropriate at the following concentrations: carbenicillin, 250 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 50 μg/ml; tetracycline, 12.5 μg/ml.

Isolation and identification of the *dksA* mutation. Tn*phoA* mutagenesis of wild-type *S. flexneri* 2a (SA100) Cm^r derivative SA511 was performed as described previously (19). Mutants that were invasive but unable to form plaques on HeLa cell monolayers were isolated, and the sites of the insertions were determined by inverse PCR as described below.

The chromosomal DNA from the Tn*phoA* insertion mutants was digested with *Taq*I restriction endonuclease, which has a site 403 bp downstream from the 5′ end of Tn*phoA*. DNA fragments were circularized by ligation with T4 DNA ligase. The ligation reaction mixtures were purified using the QIAquick PCR purification kit (Qiagen, Valencia, Calif.). PCR was conducted on the ligation reaction mixture using *Taq* polymerase (Qiagen) in the reaction buffer supplied by the manufacturer and supplemented with 250 μM deoxynucleoside triphosphates and 1 μM concentrations of primers Tn*phoA*-1 (5′CAAAACGGGAAAGGTTCCG) and Tn*phoA*-2 (5′GCTCTGCGTGATTCTCTTAGCG). The reaction conditions were 30 cycles of 94°C for 1 min, 51°C for 1 min, and 72°C for 1 min. The DNA sequences of the inverse PCR products were determined with an ABI Prism 377 DNA sequencer (Perkin-Elmer Co., Applied Biosystems Division).

Cloning of the *dksA* gene. The *dksA* gene of *S. flexneri* SA100 was amplified by PCR using chromosomal DNA and *Pfu* polymerase (Stratagene Cloning Systems, La Jolla, Calif.) in the reaction buffer supplied by the manufacturer and supplemented with 250 μM deoxynucleoside triphosphate and 1 μM concentrations of primers *dksA*-1 (5′AGAACGCAGCCGTATTGAC) and *dksA*-2 (5′CAGAGAGCCAAAATGAAGC). The reaction conditions were 30 cycles of 95°C for 45 s, 55.9°C for 45 s, and 72°C for 200 s. This PCR fragment was cloned into low-copy-number vector pWKS30 to generate pSAM1.

Construction of bacterial mutants. The *dksA* mutation from SA2287 was moved into wild-type SA100 by P1 transduction (28) to generate SA5287. The presence of the Tn*phoA* insertion in *dksA* was confirmed by PCR. *S. flexneri rpoS*

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Reference or source
Strains		
DH5α	*E. coli* cloning strain	
SA100	*S. flexneri* wild-type serotype 2a	34
SA511	SA100 Cm^r	19
SA710	SA100 *rpoS*::*kan*	This work
SA2287	SA511 *dksA*::Tn*phoA*	This work
SA5287	SA100 *dksA*::Tn*phoA*	This work
ZK1000	*E. coli* W3110 Δ*lacU169 tna-2 katF* (*rpoS*)::kan	3
Plasmids		
pDEB2	*katF* (*rpoS*) in pUC19	3
pLR56	*Y. enterocolitica invA* in pUC19	L. Runyen-Janecky
pSAM1	SA100 *dksA* in pWKS30	This work
pUC-GFP	*gfp* in pUC19	S. Seliger
pWKS30	Low-copy-number cloning vector	45

mutant SA710 was constructed in SA100 by P1 transduction of the *rpoS*::*kan* allele from *E. coli* ZK1000 (3). The presence of the *kan* insertion in *rpoS* was confirmed by PCR.

Environmental stress resistance assays. Resistance to either acid or the oxidizing agent cumene hydroperoxide (CHP) (Sigma Chemical Co.) was determined by a modification of the method of Waterman and Small (46). Bacterial cultures containing the appropriate antibiotics were incubated in LB for 19 h with aeration at 37°C. Bacteria were then exposed to either acid or CHP as follows. In the acid resistance assay, acidic medium was prepared by adjusting LB to pH 2.5 with HCl and filter sterilization. Overnight bacterial cultures were diluted 1:50 into LB, pH 2.5. In the CHP resistance assay, the CHP stock (approximately 80%) was diluted 1:10 in dimethyl sulfoxide. One milliliter of each overnight culture was removed, and 3.8 μl of 8% CHP was added to yield a final concentration of approximately 2 mM CHP. Immediately following exposure to either acid or CHP, cultures were briefly vortexed and an aliquot was removed, diluted in saline, and plated on L agar to determine the initial CFU per milliliter. The remaining culture was incubated without aeration at 37°C. At each time point, an aliquot of the sample was removed, diluted, and plated as described above. L plates were incubated overnight at 37°C, and values of CFU per milliliter were calculated the following day. Percent survival is reported as CFU per milliliter at each time point divided by CFU per milliliter at time zero.

Tissue culture, cell invasion, and plaque assays. Henle 407 cell monolayers were used in all experiments and were cultured in Earle's minimal essential medium plus 2 mM glutamine plus 10% fetal calf serum (Life Technologies, Grand Island, N.Y.) and incubated in a 5% CO_2 atmosphere at 37°C. Invasion assays to examine the ability of *S. flexneri* to invade and multiply within Henle cells were performed as described by Hale and Formal (14) and as modified by Hong et al. (19). Plaque assays were conducted to assay the intercellular spread of *S. flexneri* as described by Oaks et al. (31) and as modified by Hong et al. (19).

Phagocytic vacuole escape assay. Henle cells were grown on glass coverslips according to the invasion assay procedure (19). Approximately 3 h prior to invasion, the medium was removed and replaced with serum-free medium containing 0.015 mg of 5-(and 6)-carboxytetramethylrhodamine succinimidyl ester (Molecular Probes, Eugene, Oreg.)/ml to label Henle cell membrane proteins. Cells were incubated at 37°C for 1 h, washed four times with phosphate-buffered saline (PBS) to remove excess rhodamine probes, and incubated for 1 h in fresh serum-containing medium to conjugate unbound rhodamine probes to serum proteins. The Henle cells were then washed twice with PBS, and fresh medium was added. Bacterial invasion was conducted as described previously (19) using bacteria expressing green fluorescent protein (GFP) encoded by plasmid pUC-GFP. At 1 h postinvasion, the glass coverslips were washed four times with PBS, fixed for 10 min in 4% (wt/vol) paraformaldehyde in PBS, washed twice with water, and sealed inverted on a glass slide. Fluorescent images were visualized by excitation at either 488 or 568 nm on a Leica TCS 4D confocal laser scanning microscope. Images show the excitation from only one wavelength to ensure that the fluorescence signal results from only one signal. Each image was examined by optically sectioning through the eukaryotic cell membrane and cytoplasm to confirm that the bacteria were located intracellularly.

In vitro growth rate. In vitro growth rate was determined by diluting overnight LB cultures to approximately 10^6 CFU/ml in 25 ml of LB or ISM and incubating them with aeration at 37°C. At each time point, CFU were determined by diluting and plating.

IcsA localization assay. IcsA (VirG) localization was determined by modification of the indirect immunofluorescence procedure of van den Bosch et al. (44). For localization of IcsA in vitro, 1 ml of bacteria grown to late logarithmic phase in LB was fixed in 4% paraformaldehyde for 10 min; this was followed by two washes with PBS. The bacteria were resuspended in 100 μl of PBS, and rabbit anti-IcsA antiserum 35 (obtained from Edwin Oaks) was added at a 1:50 dilution. The samples were incubated at room temperature for 1 h, centrifuged, and washed two times with PBS. Samples were resuspended in 100 μl of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (ICN Pharmaceuticals, Inc., Aurora, Ohio) at a 1:100 dilution. After 1 h of incubation at room temperature, the samples were centrifuged, washed two times with PBS, and resuspended in 20 μl of PBS. One microliter of each sample was air dried on a glass slide and mounted in 90% glycerol–10% PBS containing 1 mg of *p*-phenylenediamine/ml (pH 8.0).

For localization of IcsA in vivo, invasion assays were done as described above with Henle cells grown on glass coverslips. After 3 h, samples were fixed in 1% paraformaldehyde for 15 min, washed two times with PBS, treated with PBS containing 50 mM NH_4Cl for 5 min, and washed two times with PBS. Henle cells were permeabilized with 0.2% Triton X-100 for 10 min and then washed once with PBS for 5 min. Coverslips were incubated cell side down on 50 μl of anti-IcsA antisera diluted 1:50 in PBS containing 1 mg of bovine serum albumin(BSA)/ml for 1 h at room temperature and washed three times for 5 min each with PBS. Coverslips were incubated cell side down on 50 μl of FITC-conjugated goat anti-rabbit antisera diluted 1:100 in PBS–1 mg of BSA/ml for 1 h at room temperature, washed three times for 5 min each with PBS, and mounted in 90% glycerol–10% PBS containing 1 mg of *p*-phenylenediamine/ml (pH 8.0).

Samples were visualized on a Leica TCS 4D confocal laser scanning microscope equipped with a 488-nm laser, FITC detection filters, and differential interference contrast for phase-contrast imaging.

Nucleotide sequence accession number. The DNA sequence of the *S. flexneri dksA* gene has been submitted to the GenBank database under accession no. AF323722.

RESULTS

Isolation of an *S. flexneri dksA* mutant defective in plaque formation. A library of *S. flexneri* Tn*phoA* mutants was previously constructed to identify mutants that were defective in intracellular multiplication or cell-to-cell spread by screening for mutants that were invasive but unable to form plaques on tissue culture monolayers (19). Although Tn*phoA* mutagenesis was used primarily to identify mutations in periplasmic or membrane proteins by screening for $PhoA^+$ colonies, some mutants with little or no activity were isolated for further study. DNA sequencing of inverse PCR products revealed that one mutant that had low alkaline phosphatase activity, SA2287, contained a Tn*phoA* insertion in *dksA*, a gene originally identified in *E. coli* as a suppressor of a *dnaK* mutation. The deduced amino acid sequence of *S. flexneri* DksA has 98% amino acid identity to the predicted *E. coli* and *Salmonella enterica* serovar Typhimurium sequences.

The initial *dksA*::Tn*phoA* mutation was transduced with phage P1 into a wild-type SA100 background, generating SA5287. This mutant exhibited the same invasive but plaque-deficient phenotype as SA2287 (Table 2). Transformation of SA5287 with plasmid pSAM1, containing the wild-type *S. flexneri dksA* gene, restored plaque formation in the *dksA* mutant (Table 2). These results indicated that the *dksA* mutation was responsible for the plaque-deficient phenotype and that DksA is required for intracellular multiplication or cell-to-cell spread, but not for invasion of cultured cells, by *S. flexneri*.

Plaque assay of the *rpoS* mutant. DksA has been reported to be required for the optimal expression of sigma factor RpoS (47), which regulates the stress and starvation response. There-

TABLE 2. Comparison of wild-type and mutant *S. flexneri* in Henle cell monolayers

Strain	Phenotype	Result of assay for:	
		Invasion[a]	Plaque formation[b]
SA100	Wild type	+	+
SA2287	DksA$^-$	+	−
SA5287	DksA$^-$	+	−
SA5287/pSAM1	DksA$^+$	+	+
SA710	RpoS$^-$	+	+

[a] +, at least 30% of the Henle cells contained three or more intracellular bacteria in the invasion assay.
[b] +, formation of wild-type-size plaques in Henle cell monolayers; −, pinpoint or no plaques.

fore, we postulated that *S. flexneri* may require DksA for survival of possible environmental stresses experienced in the intracellular environment. To determine whether the defect in plaque formation in the *dksA* mutant was due to reduced expression of RpoS, we constructed an *rpoS* mutant by P1 transduction from an *E. coli rpoS*::*kan* mutant and tested it for the ability to form plaques. *S. flexneri rpoS* mutant SA710 formed plaques on tissue culture monolayers (Table 2). Thus, the failure of the *dksA* mutant to spread on cultured cell monolayers is independent of RpoS.

Acid sensitivity of the *dksA* and *rpoS* mutants. Although the *rpoS* plaque assay indicated that DksA regulation of RpoS is not required for virulence in Henle cells, this regulation may be important for survival of *S. flexneri* during passage through the host stomach. *S. flexneri* is highly resistant to acidic conditions and is capable of surviving at pH 2.5 for several hours (12, 38). This ability has been attributed to expression of RpoS-dependent genes, and an *rpoS* mutant is extremely acid sensitive (46). Since *S. flexneri* encounters acidic conditions during transit through the stomach and possibly in other locations within the host, we compared the in vitro acid resistances of *dksA* and *rpoS* mutants by examining the survival of stationary-phase cultures in LB at pH 2.5.

Both the *rpoS* and *dksA* mutants were significantly more sensitive to acid than the parental strain. The *dksA* mutant exhibited a sharp reduction in acid survival during the first hour of incubation in LB at pH 2.5 (Fig. 1) and was approximately 100 times more sensitive than the wild type. The *rpoS* mutant was even more sensitive to acid than the *dksA* mutant, exhibiting sensitivity below the detectable level of 0.001% survival. The fact that the *rpoS* mutant was more acid sensitive than the *dksA* mutant suggests that *rpoS* expression was not completely blocked in the *dksA* mutant. This result was confirmed by Western blot analysis, which showed that RpoS was present, yet below wild-type levels, in the *dksA* mutant (data not shown).

If the decrease in acid resistance in a *dksA* mutant was due to decreased expression of *rpoS*, then increased expression of *rpoS* should restore acid resistance in a *dksA* mutant. Western blot analysis indicated that RpoS levels were greater in the *dksA* mutant containing the cloned *rpoS* gene than in the *dksA* mutant without the gene or in the wild-type parent strain (data not shown). To test the effect of overexpression of *rpoS* in the *dksA* mutant, we examined the acid resistance at 1 h of mutants containing either the *dksA* or *rpoS* cloned genes (Fig. 2). Over-

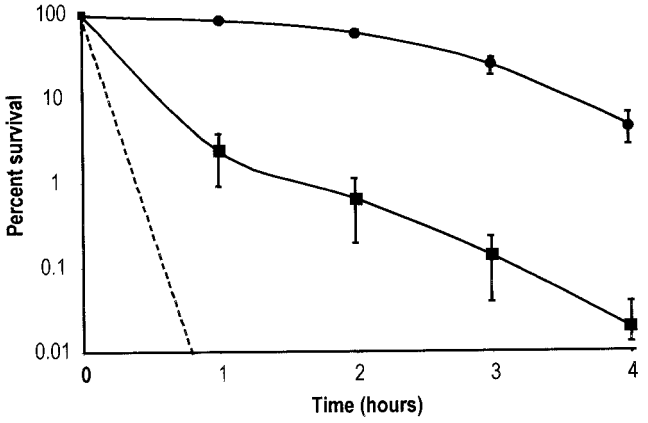

FIG. 1. Effect of *dksA* and *rpoS* on survival of *S. flexneri* in acid. Shown are percentages of survival over time in LB-HCl, pH 2.5, for wild-type SA100 (circles) and *dksA* mutant SA5287 (squares) *S. flexneri*. The *rpoS* mutant SA710 (dashed line) was below detectable levels of 0.001% survival at all time points and is plotted as the theoretical maximal level of survival. The averages of three experiments are shown with the standard deviations.

expressing *rpoS* in the *dksA* mutant was not sufficient to restore acid resistance. The resistance of SA5287/pDEB2 was slightly higher than that of SA5287 (Fig. 2), but the difference was not significant ($P = 0.19$). Additionally, *dksA* carrried on a plasmid was insufficient to restore acid resistance in an *rpoS* mutant (Fig. 2). However, the wild-type *dksA* gene restored acid resistance in the *dksA* mutant, and *rpoS* restored acid resistance in the *rpoS* mutant.

CHP sensitivity of the *dksA* and *rpoS* mutants. In addition to acid resistance, oxidative stress resistance is another RpoS-dependent environmental response that *Shigella* may induce in the host environment. To test the sensitivity of the *dksA* mutant to this stress, we exposed undiluted, stationary-phase cultures to oxidizing agent CHP. All tested strains exhibited sensitivity to oxidative stress, but the *dksA* and *rpoS* mutants were significantly more sensitive than the wild type (Fig. 3). As observed with exposure to acid stress, the *rpoS* mutant exhibited greater sensitivity than the *dksA* mutant.

The ability of *dksA* and *rpoS* carried on plasmids to restore

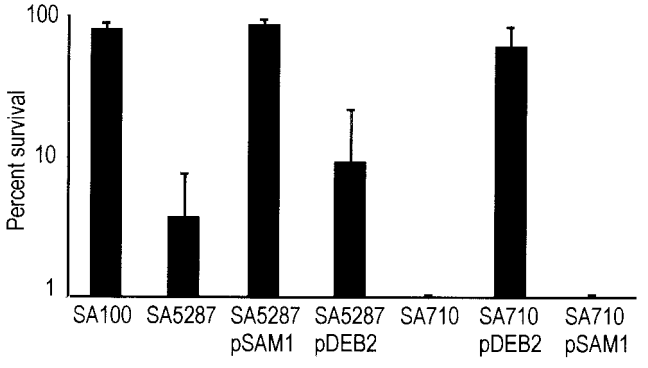

FIG. 2. Effect of *dksA* and *rpoS* on acid resistance. The indicated strains were exposed to LB, pH 2.5, for 1 h, and the percent survival was determined by plating. The averages of at least three experiments are shown with the standard deviations.

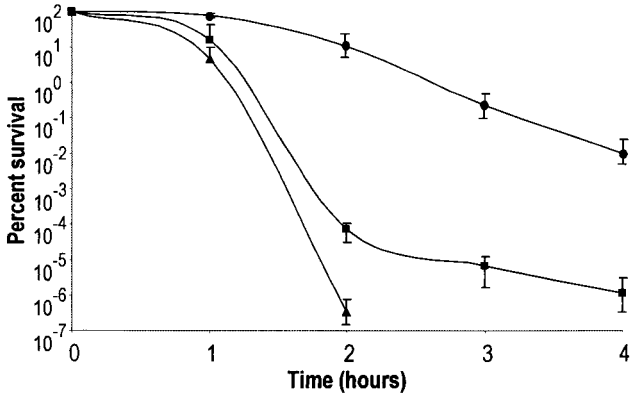

FIG. 3. Effect of *dksA* and *rpoS* on survival of *S. flexneri* in CHP. Shown is percent survival over time in oxidizing agent CHP at an approximately 2 mM concentration. Wild-type SA100 (circles), *dksA* mutant SA5287 (squares), and *rpoS* mutant SA710 (triangles) strains of *S. flexneri* were examined for 4 h. The *rpoS* mutant was below the detectable levels of 10^{-7}% survival at 3 and 4 h, and therefore these data points do not appear on the graph. The averages of three experiments are shown with the standard deviations.

CHP resistance in the mutant strains was examined at 2 h (Fig. 4). As expected, plasmid-expressed *dksA* complemented the CHP-sensitive phenotype in the *dksA* mutant. However, the *dksA*-expressing plasmid failed to restore CHP resistance in the *rpoS* mutant. Interestingly, plasmid-expressed *rpoS* not only complemented sensitivity to oxidative stress in the *rpoS* mutant but also restored resistance to oxidative stress in the *dksA* mutant. These results suggest that DksA and RpoS operate in the same pathway during oxidative stress and that DksA is required upstream of RpoS for oxidative resistance.

Phagocytic vacuole escape of the *dksA* mutant. Increased sensitivity of the *dksA* mutant to acid and oxidative stress appears to be due, at least in part, to the effects of DksA on RpoS expression. However, the fact that *rpoS* mutant SA710 produced plaques on Henle cell monolayers indicates that DksA operates in an RpoS-independent role in intracellular multiplication or spread of *Shigella*. To determine the role of DksA in the intracellular environment, we examined the effect of a *dksA* mutation on the different stages of the intracellular life cycle of *S. flexneri*. The possible stages include escape from the phagocytic vacuole, intracellular survival and multiplication, and spread to an adjacent epithelial cell.

The ability of the *dksA* mutant to escape the phagocytic vacuole was determined by infecting rhodamine-labeled Henle cells with GFP-expressing bacteria (Fig. 5). The 5-(and 6)-carboxytetramethylrhodamine succinimidyl ester probe used in this study forms peptide bonds with cellular proteins located on the outer surfaces of eukaryotic cell plasma membranes (10, 32). The probe does not cross the plasma membrane and thus does not label intracellular proteins. Phagocytic vacuoles that result from the internalization of the plasma membrane are specifically labeled with the rhodamine probe. Labeling the Henle cells with the rhodamine probe did not affect bacterial invasion (data not shown). Following invasion, we visualized GFP-expressing bacteria and rhodamine-labeled Henle cell membranes by confocal scanning laser microscopy. Intracellular bacteria that were unable to lyse the phagocytic vacuole (*E. coli* DH5α/pLR56/pUC-GFP) were surrounded by a rhodamine-labeled membrane (Fig. 5). This strain expresses the *Yersinia enterocolitica invA* gene, which confers on *E. coli* the ability to attach to Henle cells and be internalized but not to lyse the phagocytic vacuole. In contrast, wild-type *S. flexneri* cells, which lyse the vacuole, were located in the Henle cell cytoplasm and were not surrounded by a rhodamine-labeled membrane (Fig. 5). Similarly, no colocalization of GFP and rhodamine in Henle cells infected with the *dksA* mutant was noted (Fig. 5). These results indicate that the *dksA* mutant is capable of lysing the phagocytic vacuole and gaining access to the host cell cytoplasm.

Growth rate and in vivo multiplication of the *dksA* mutant. Although the *dksA* mutant gained access to the Henle cell cytoplasm, the ability of the mutant to grow in the intracellular environment could affect its ability to spread. Initial observations indicated that the *dksA* mutant exhibited a slightly reduced growth rate in LB at 37°C (Fig. 6) and a very long lag in ISM, which contains ions at concentrations comparable to those found in the eukaryotic cell cytoplasm (Fig. 7). Following the extended lag in ISM, however, the growth rate of the *dksA* mutant appeared to be approximately the same as that of wild-type *S. flexneri* (Fig. 7). When the *dksA* mutant population was subcultured from late-exponential or stationary-phase growth in ISM, these cultures also exhibited a 25-h lag (data not shown). Thus, the growth observed in ISM following the delay was not the result of outgrowth of revertants or mutants that had suppressors of the original *dksA* mutation.

Since the *dksA* mutant exhibited a long lag in ISM, we examined whether impaired intracellular growth could account for the failure of the mutant to spread intercellularly. Intracellular growth rate was determined by counting the number of intracellular bacteria per infected Henle cell at various times following invasion. In contrast to the delayed onset of growth in ISM in vitro, the *dksA* mutant multiplied normally within Henle cells, and the average growth rate of the *dksA* mutant was similar to that of the wild type (Fig. 8).

Filamentation of the *dksA* mutant. During examination of intracellular growth, we observed elongation and filamentation

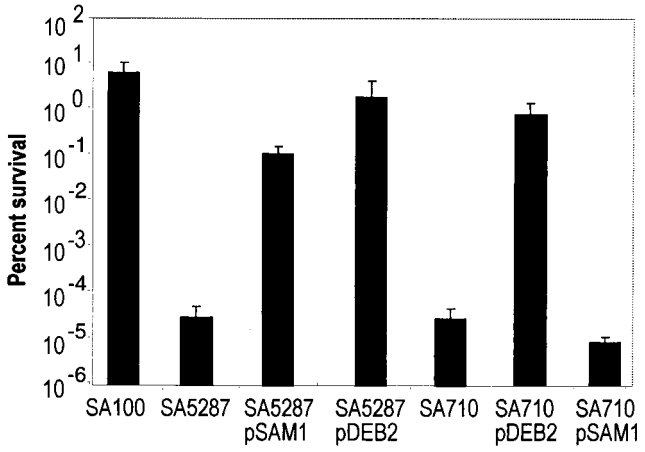

FIG. 4. Effect of *dksA* and *rpoS* on CHP resistance. The indicated strains were exposed to 2 mM CHP for 2 h, and the percent survival was determined by plating. The averages of at least three experiments are shown with the standard deviations.

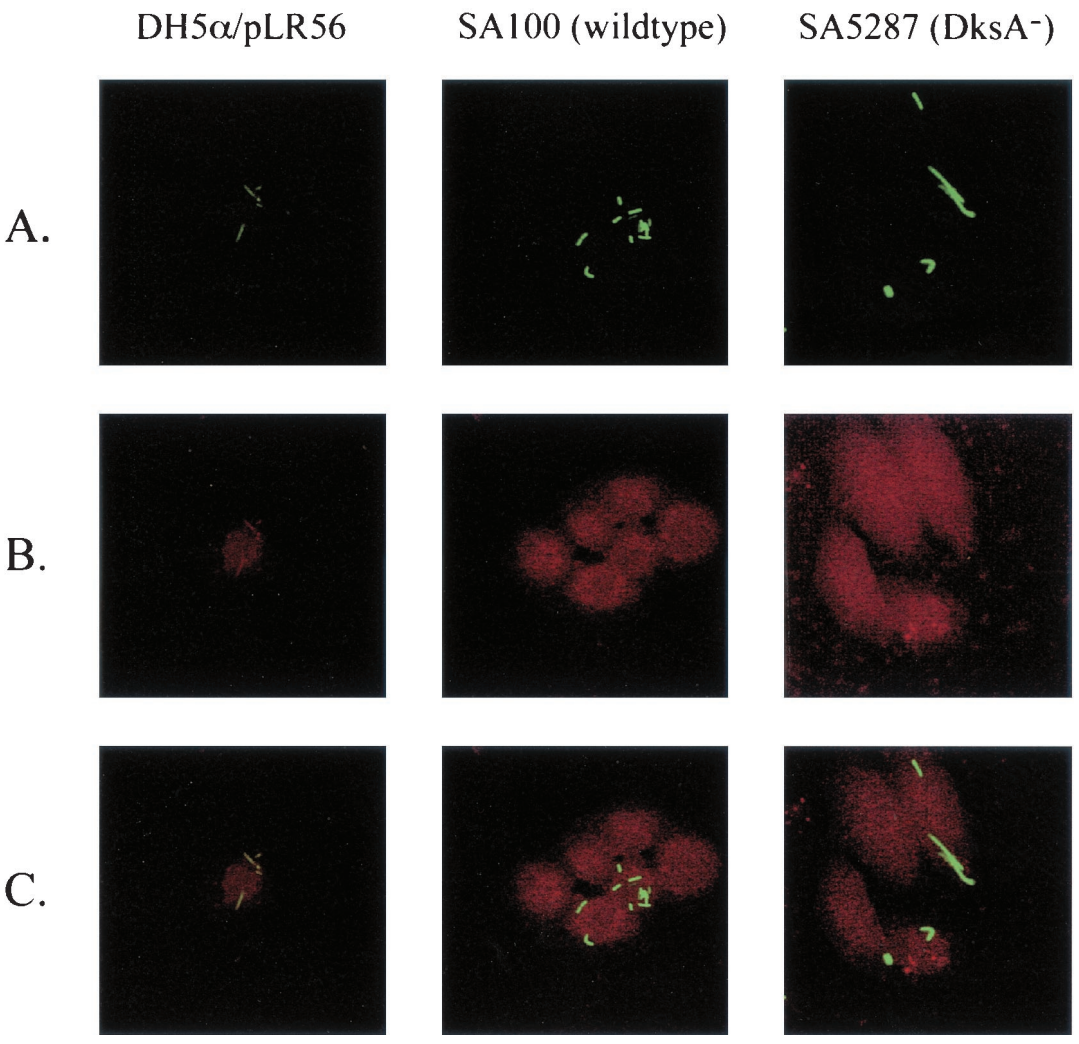

FIG. 5. Escape of wild-type and *dksA* mutant bacteria from the Henle cell vacuole. Henle cells in which the cell membrane had been labeled with 5- and 6-carboxytetramethylrhodamine succinimidyl ester were infected with *E. coli* DH5α/pLR56 (InvA$^+$), wild-type *S. flexneri* SA100, or *dksA* mutant SA5287. Each strain contained plasmid pUC-GFP to provide constitutive production of GFP. (A) GFP-labeled intracellular bacteria. (B) Rhodamine labeling of the Henle cell and vacuole membrane. (C) Overlay of the two images created in Adobe Photoshop.

of the *dksA* mutant. The *dksA* mutant bacteria appeared elongated compared to the wild type in the intracellular environment during the first 2 h postinvasion (Fig. 9). At 3 h postinvasion, the elongation was more pronounced and filamentation of some of the *dksA* mutant cells was observed. Less filamentation was noted at 4 h (Fig. 9), suggesting that this may be a transient phenomenon. Filamentation of the *dksA* mutant may impede bacterial spread to an adjacent cell, but this phenomenon is unlikely to be solely responsible for the inability of the *dksA* mutant to form wild-type plaques, since only a portion of the cells showed filamentation and since fewer filaments were observed at 4 h than at 3 h postinvasion.

IcsA localization in the *dksA* mutant. Because the *dksA* mutant survived and multiplied in Henle cells but did not form wild-type plaques, we examined the localization of IcsA on the bacterial cells. The failure of *S. flexneri* to express or properly localize IcsA, especially on filamentous bacteria, could impede intercellular spread and the ability to form plaques (11). IcsA was observed at a polar location in wild-type *S. flexneri* bacteria grown in vitro (Fig. 10B). The *dksA* mutant grown in broth showed examples of aberrant IcsA surface expression or localization, and fewer mutant cells bound the IcsA antibody than did wild-type bacteria (Fig. 10D and data not shown). IcsA was localized to one pole on some of the mutant bacteria, but others had IcsA located over the entire cell surface or at both poles. The *dksA* cells grown in vitro, like those growing intracellularly, were elongated compared to the wild type, and some filamentation was observed (Fig. 10C). However, there was no clear correlation between elongation or filamentation and aberrant IcsA localization (Fig. 10C and D, and data not shown).

IcsA expression in intracellular bacteria was also examined (Fig. 11). The wild-type bacteria growing within Henle cells showed polar localization of IcsA (Fig. 11A), but the intracellular *dksA* mutant population was a mixture of cells with polar and nonpolar IcsA localization (Fig. 11B). While DksA may have other important functions in the cell, the requirement for consistent polar localization of IcsA may be sufficient to account for the aberrant plaque formation by the *dksA* mutant.

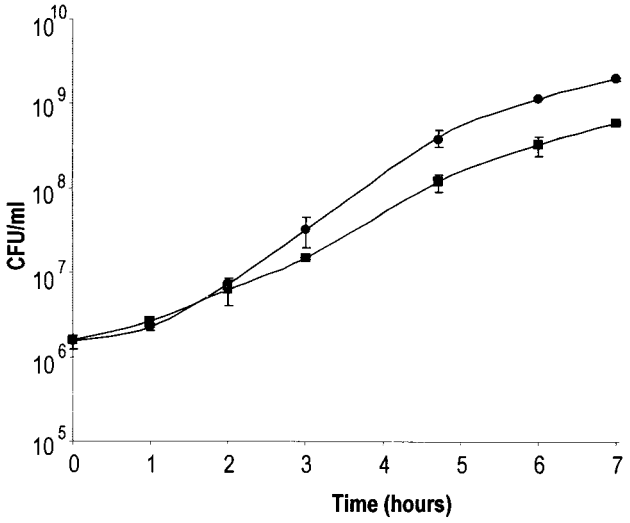

FIG. 6. In vitro growth rates of *S. flexneri* wild-type SA100 (circles) and *dksA* mutant SA5287 (squares) in LB. The averages of three experiments are shown with the standard deviations.

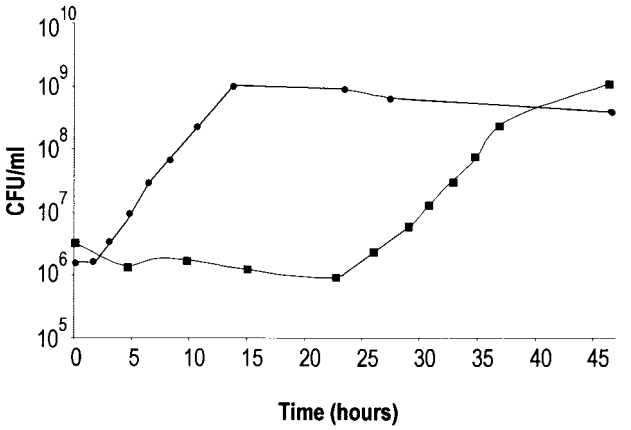

FIG. 7. Growth of *S. flexneri* in ISM. Wild-type (circles) and the *dksA* mutant (squares) bacteria were grown in ISM, and the numbers of bacteria at each time point were determined by plating. The averages of three experiments are shown.

DISCUSSION

Here, we identify DksA as a protein necessary for intercellular spread of *S. flexneri.* DksA appears to influence or function in a variety of pathways in enteric bacteria. The phenotypes include a failure to properly localize IcsA, sensitivity to environmental stress, and cell elongation or filamentation. Two-dimensional gel analysis of the *S. flexneri dksA* mutant showed that expression of several proteins is altered compared to that for the wild type (data not shown), which is in agreement with a previous report for *S. enterica* serovar Typhimurium (47). Because the *dksA* mutant exhibited phenotypic deficiencies in several assays and exhibited altered protein expression relative to that of the wild type, we hypothesize that DksA may function in a central pathway within the cell.

The *dksA* gene was initially isolated in *E. coli* as part of a locus that, when overexpressed, could suppress the temperature-sensitive growth of a *dnaK* deletion strain at 40.5°C (20). The *dksA* gene (*dnaK* suppressor) encodes a 17.5-kDa protein that comprises 151 amino acids and that has a pI of 4.87. Construction of an *E. coli dksA* deletion mutant indicated that *dksA* is not an essential gene (20). The C terminus of DksA is similar, and possibly related, to that of the F plasmid-encoded TraR protein (5). This region of homology contains a zinc finger domain common to many regulatory proteins, which may give insight into the function of the protein (5). Support for a regulatory role of DksA is provided by two-dimensional gel analysis of proteins expressed by an exponentially growing culture of an *S. enterica* serovar Typhimurium *dksA* mutant. There is differential expression of at least 14 proteins compared to expression by the wild type (47).

Genetic analysis indicates that DksA is required for the optimal translation of RpoS (sigma S), and an *S. enterica* serovar Typhimurium *dksA* mutant has a reduced amount of RpoS in stationary phase (47). Our Western blot analysis indicates that a similar effect is observed in the *S. flexneri dksA* mutant (data not shown). RpoS is the sigma factor serving as a master regulator of stationary-phase and stress response gene expression (23), including genes required for survival during low pH (24, 46), oxidative stress (8), UV radiation (42, 43), and hyperosmolarity (17, 18). Thus, the decreased survival in harsh environmental conditions of the *dksA* mutant may partially be due to decreased levels of RpoS.

The role of RpoS in virulence appears to be dependent on the pathogen as well as the stage of the infection process. RpoS is required for the intracellular survival of *Legionella pneumophila* in its host amoeboid species (15) as well as for virulence in *S. enterica* serovar Typhimurium (1, 4, 9, 48). However, the role of RpoS in virulence is rarely due to the regulation of specific virulence genes. For example, fewer *E. coli rpoS* mutant than wild-type cells are isolated following passage through mouse and bovine hosts (35). This observation is proposed to be due to the significant reduction in acid resistance of the bacterium (35). RpoS has been reported to be important during the initial colonization of *S. enterica* serovar Typhimurium

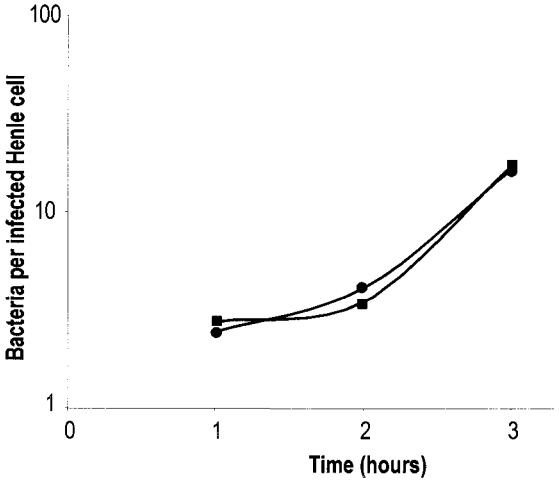

FIG. 8. In vivo growth rate of *S. flexneri*. Wild-type SA100 (circles) and *dksA* mutant SA5287 (squares) strains of *S. flexneri* were isolated from Henle cells at the indicated times, and the numbers of intracellular bacteria were determined by plating. The average numbers of intracellular bacteria in three independent experiments are shown.

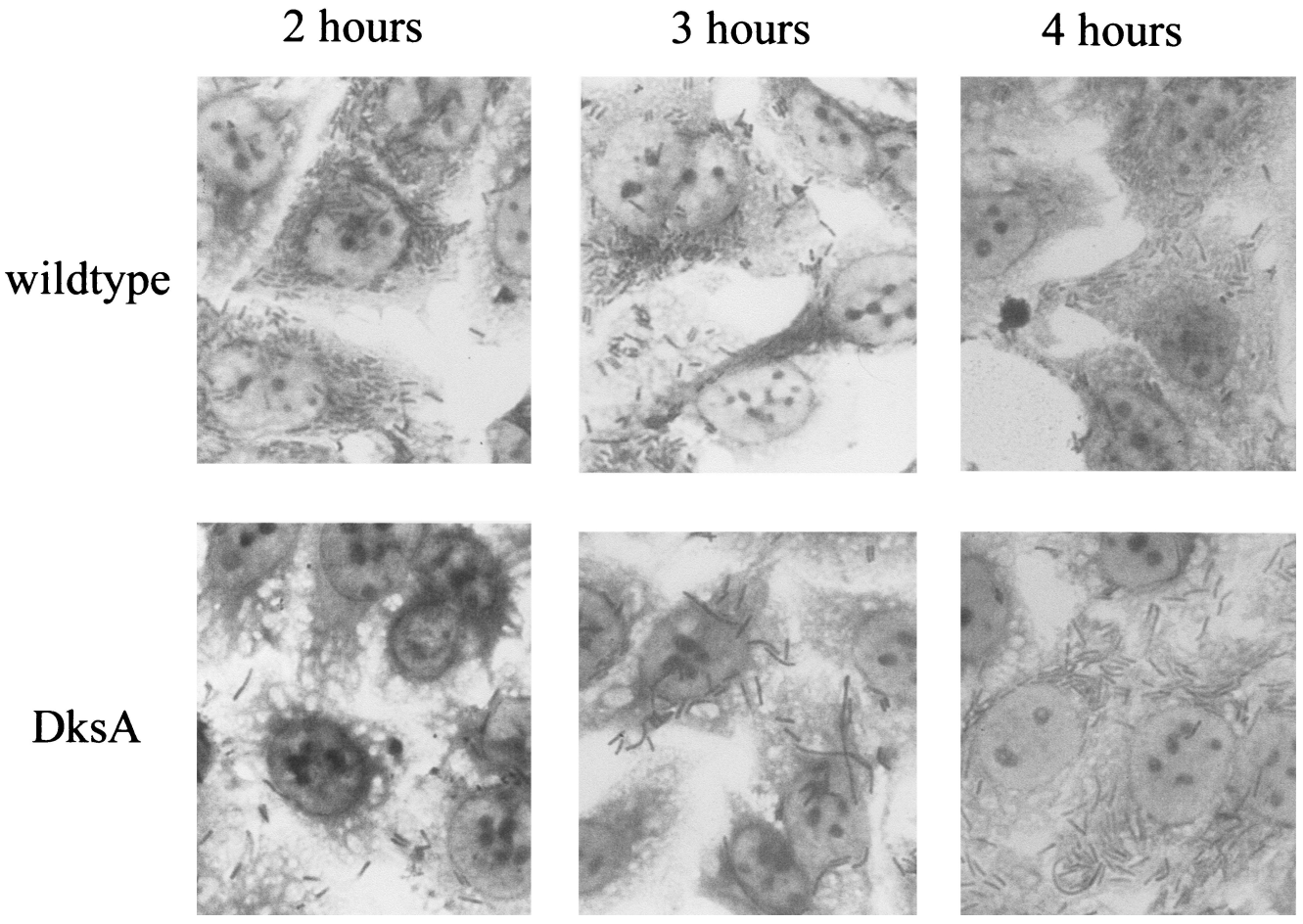

FIG. 9. Growth of the *S. flexneri dksA* mutant in Henle cells. Henle cells infected with wild-type SA100 and *dksA* mutant SA5287 bacteria were stained with Wright-Giemsa stain and observed at 1,000× magnification at 2, 3, and 4 h postinvasion. Representative images are shown.

of the gut-associated lymphoid tissue, but RpoS does not affect the ability of the bacterium to attach to and invade Int-407 cells and J774 macrophage-like cells or the ability to survive in macrophages (30). RpoS has been excluded from any role during the long-term colonization by *E. coli* of the mouse large intestine (21). Interestingly, the presence of this sigma factor appears to inhibit the virulence of *Pseudomonas aeruginosa* since an *rpoS* mutant survives as well as wild-type bacteria in rat lungs yet causes greater damage to lung tissue (40).

DksA is necessary for *S. enterica* serovar Typhimurium colonization of 3-week-old chicks and for virulence in 1-day-old chicks, which lack a complex intestinal flora (41). There also is a significant increase in the 50% lethal dose values for oral infection of mice by the *S. enterica* serovar Typhimurium *dksA* mutant (47). To date, the avirulent phenotype of *dksA* mutants has only been examined in animal models, and the molecular mechanism responsible has not been investigated. However, it has been proposed that the avirulence of an *S. enterica* serovar Typhimurium *dksA* mutant is partially due to DksA regulation of RpoS (47).

Since the *dksA* mutant has reduced resistance to environmental stresses, it is likely that DksA regulation of RpoS may be required for full virulence of *S. flexneri* during infection of the host, particularly during passage through the acidic human stomach. However, the ability of the *S. flexneri rpoS* mutant to form plaques on Henle cell monolayers excluded DksA regulation of RpoS as the molecular mechanism responsible for failure of the *dksA* mutant to spread in Henle cell monolayers. Additionally, since the *rpoS* mutant is extremely sensitive to environmental stresses, the results of the *rpoS* plaque assay suggest that *S. flexneri* does not experience extreme acid or oxidative stress during invasion, growth in the intracellular environment, or intercellular spread in cultured cells. Thus, the sensitivity of the *dksA* mutant to environmental stress, even in an RpoS-independent pathway, is not responsible for the inability of the mutant to spread intercellularly. These results are supported by experiments in which we were able to isolate viable *dksA* mutant bacteria from infected Henle cells (data not shown).

The *dksA* mutant, like wild-type *Shigella*, was able to lyse the phagocytic vacuole and is capable of dividing in the intracellular environment. Unlike the wild type, however, the *dksA* mutant did not show consistent polar location of IcsA. IcsA induces the continuous polymerization of F-actin. This process is responsible for propelling the bacterium into an adjacent epithelial cell. The mechanics of this type of movement necessitate a single focus of IcsA to produce unidirectional forces. Therefore, polymerization of actin around the entire surface or

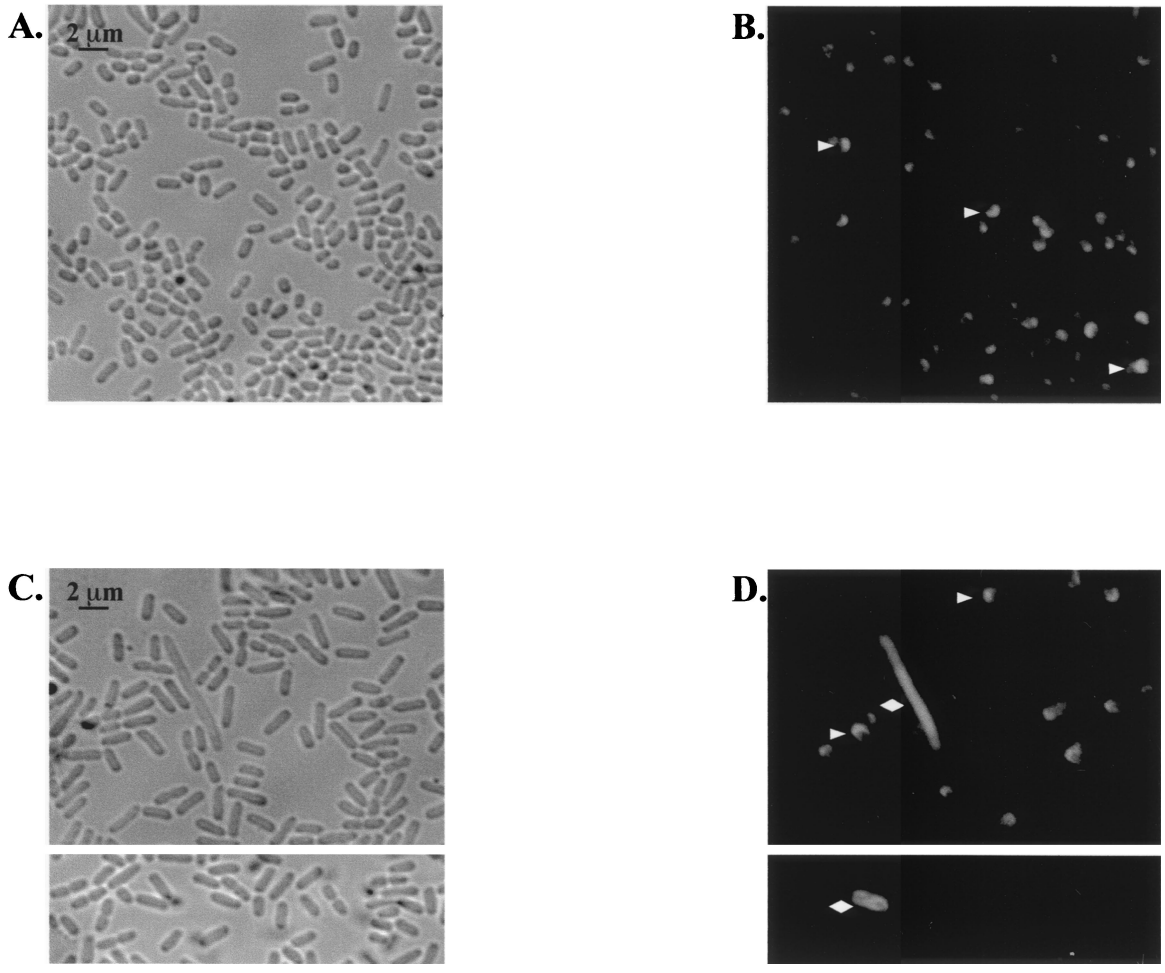

FIG. 10. Localization of IcsA on *S. flexneri* grown in vitro. Phase-contrast and immunofluorescence images of SA100 (A and B) and SA5287 (C and D) followed staining with anti-IcsA are shown. Cells were observed at 1,000× magnification. The SA100 images are from 5-h cultures, and the SA5287 images are from two separate fields at 4 and 5 h. Arrowheads, representative bacteria with polarized IcsA; diamonds, representative bacteria with aberrant localization of IcsA (either partially polarized or nonpolarized).

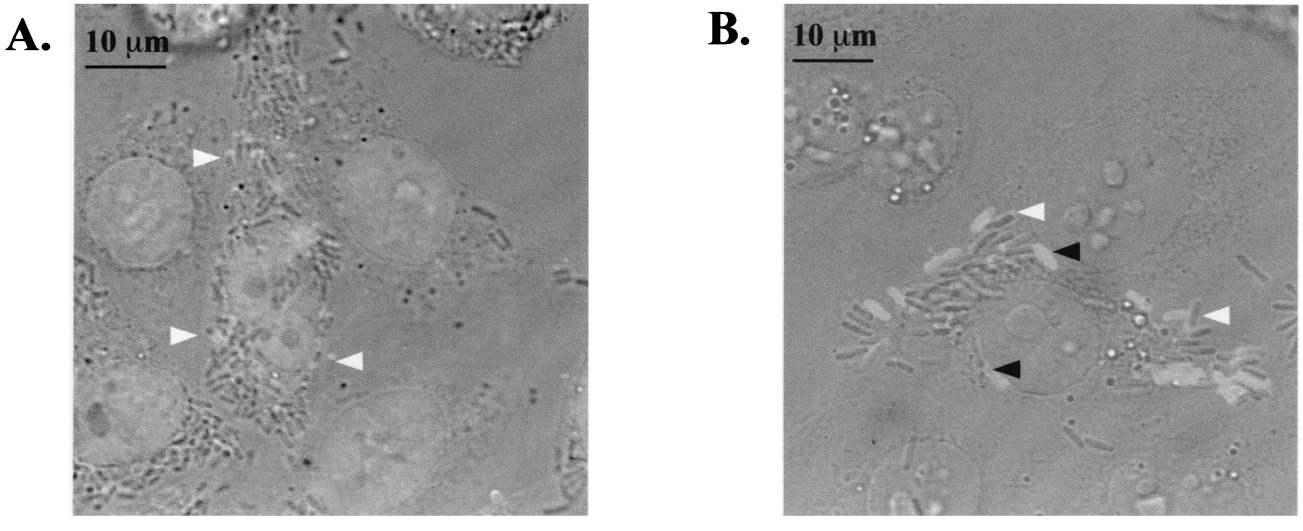

FIG. 11. Localization of IcsA on *Shigella* growing within Henle cells. Phase-contrast and immunofluorescence images of SA100- (A) and SA5287-infected (B) Henle cells were overlaid using Adobe Photoshop. Cells were observed at 1,000× magnification. White arrowheads, representative bacteria with polarized IcsA; black arrowheads, representative bacteria with aberrant localization of IcsA (either partially polarized or nonpolarized).

at both poles of the *dksA* mutant would prevent net movement of the bacterium in one direction through the host cell cytoplasm and thus prevent spread into an adjacent epithelial cell.

The specific mechanism by which DksA affects IcsA localization is unknown, although it may be related to the effect of the *dksA* mutation on cell division and filamentation. SopA (IcsP), which cleaves IcsA on the entire bacterial surface, may play a role in the polar localization of IcsA (7). However, IcsA, when expressed in *E. coli* in the absence of SopA, exhibited polar localization (36), indicating that SopA is not necessary for this process in *E. coli*. IcsA appears to be targeted directly to the old pole of the bacterium in an unknown process in both *S. flexneri* and *E. coli* (39). Here, we have identified DksA as a protein necessary for this process. The inability of a *dksA* mutant to properly localize IcsA likely contributes to the inability of the *dksA* mutant to form wild-type plaques on tissue culture monolayers.

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