INFECTION AND IMMUNITY, Mar. 2005, p. 1404–1410 Vol. 73, No. 3
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.3.1404–1410.2005

Role of the Pst System in Plaque Formation by the Intracellular Pathogen *Shigella flexneri*

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Received 3 October 2004/Returned for modification 29 October 2004/Accepted 4 November 2004

In response to the host cell environment, the intracellular pathogen *Shigella flexneri* induces the expression of numerous genes, including those in the *pst* operon which is predicted to encode a high-affinity phosphate acquisition system that is expressed under reduced phosphate conditions. An *S. flexneri pst* mutant forms smaller plaques in Henle cell monolayers than does the parental strain. This mutant exhibited normal production and localization of the *S. flexneri* IcsA protein. The *pst* mutant had the same growth rate as the parental strain in both phosphate-reduced and phosphate-replete media in vitro and during the first 3 h of growth in Henle cells in vivo. During growth in phosphate-replete media, the PhoB regulon was constitutively expressed in the *pst* mutant but not the parental strain. This suggested that the inability of the *S. flexneri pst* mutant to form wild-type plaques in Henle cell monolayers may be due to aberrant expression of the PhoB regulon. A mutation in *phoB* was constructed in the *S. flexneri pst* mutant, and the *phoB* mutation suppressed the small plaque phenotype of the *pst* mutant. Additionally, a specific mutation (R220Q) was constructed in the *pstA* gene of the *pst* operon that was predicted to eliminate Pst-mediated phosphate transport but allow normal PhoB-regulated gene expression, based on the phenotype of an *Escherichia coli* strain harboring the same mutation. Addition of this $pstA_{R220Q}$ mutation to a *S. flexneri pst* mutant, as part of the *pst* operon, restored normal plaque formation and regulation of *phoA* expression.

Shigella flexneri is a facultative intracellular bacterium that causes bacterial dysentery in humans. While in the host, *Shigella* can invade colonic epithelial cells and survive and multiply within the eukaryotic cytosol (24). This environment is a unique niche for *Shigella*, consisting of a particular set of nutrients and ions, distinct from the extracellular and external environments. Specific *S. flexneri* genes are induced in response to exposure to the eukaryotic cytosol and encode, among other things, proteins involved in metabolism and nutrient uptake (22). Two of these intracellular induced *S. flexneri* genes are the *phoA* and *pstS* genes, which encode proteins that mediate inorganic phosphate acquisition. *phoA* encodes an alkaline phosphatase that cleaves phosphate from organophosphates in the periplasm (7), and *pstS* encodes a periplasmic phosphate binding protein (29). This suggested that genes that mediate inorganic phosphate acquisition might be important for *Shigella* to survive in the intracellular environment and that phosphate levels may be a signal for intracellular induction of *Shigella* genes.

In *Escherichia coli*, which is very closely related to *S. flexneri* phylogenetically (20), the Pst high-affinity inorganic phosphate uptake system has been well characterized. The Pst system is a member of the periplasmic binding protein-dependent ABC transporter family, which transports molecules from the periplasm to the cytoplasm. The proteins in the Pst system are encoded within the five-gene *pst* operon. The system is composed of the periplasmic phosphate binding protein PstS; the two transmembrane proteins PstA and PstC, which transport the phosphate through the inner membrane; the ATP binding protein PstB, which supplies the energy for transport via ATP hydrolysis; and a protein (PhoU) whose role in phosphate transport is not clear (29).

In addition to mediating high-affinity phosphate uptake, the Pst system also influences phosphate-mediated gene regulation. Inorganic phosphate regulation of gene expression in *E. coli* and *S. flexneri* is mediated by the two-component regulatory system PhoR/PhoB (25, 33). This regulatory system consists of the phosphate sensor PhoR and the transcriptional activator PhoB, which binds to PhoB boxes in the promoters of genes that it regulates. PhoR, whose amino terminus is associated with the bacterial membrane, senses phosphate levels outside the cell (10). Under reduced phosphate conditions, PhoR phosphorylates the transcriptional activator PhoB, which enhances the DNA binding activity of this protein (10). Active PhoB directly binds the promoter region of PhoB-regulated genes to activate gene expression in most cases (11). Among the 31 PhoB-regulated genes is the gene encoding alkaline phosphate (*phoA*). When phosphate levels are high outside the cell, the Pst complex maintains PhoR in a conformation that dephosphorylates (and therefore inactivates) PhoB. In *E. coli*, mutations that disrupt the Pst system generally result in constitutive expression of the PhoB regulon (33).

In *S. flexneri*, a polar mutation in the *pstS* gene eliminates expression of the entire *pst* operon and thus of the Pst system, and an *S. flexneri* strain carrying this *pst* mutation forms smaller plaques on Henle cells than those formed by wild-type *S. flexneri* (22). The underlying reason for the small plaque phenotype of the *S. flexneri pst* mutant is currently unknown; however, since other phosphate transport systems may compensate for lack of Pst-mediated phosphate transport, the defect in the *pst* mutant could

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Reference or source
E. coli strains		
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*) *U169 deoR* [Φ80*dlac*Δ(*lacZ*)*M15*]	23
SM10λ*pir*	*pir*R6K	30
MM294/pRK2013	Kan^r; helper strain for matings	4
S. flexneri strains		
SA100	*S. flexneri* wild-type serotype 2a	19
SM100	SA100 Str^r	S. Seliger
SM169	SM100 *pstS*::*cam*	22
UR005	SM169 *phoB*::*tet*	This study
Plasmids		
pHM5	Allelic exchange vector	21
pMTLtet	Tet^r gene from pBR322 in pMTL24	S. Reeves
pLR29	pGTXN3 with RP4 mobilization region from pGP704	22
pLL1	pWKS30 carrying *phoBR*	This study
pLL1::*tet*	pWKS30 carrying *phoB*::*tet*	This study
pLL2	pHM5 carrying *phoB*::*tet*	This study
pLR83	pLR29 carrying the *phoA* promoter	22
pPst1	pWKS30 carrying *pstSCABphoU*	This study
pAB1	pPst carrying R220Q mutation in *pstA*	This study
pWKS30	Low-copy-number cloning vector	32

be due to constitutive expression of the PhoB regulon. The aim of this study was to elucidate the molecular basis of the plaque formation defect of an *S. flexneri pst* mutant strain.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were routinely grown in Luria-Bertani (LB) broth or on LB agar. *S. flexneri* strains were typically grown in LB broth at 30°C or at 37°C when expression of invasion proteins was required. For growth on solid media, *S. flexneri* strains were grown on tryptic soy broth agar plus 0.01% Congo red dye at 37°C. Antibiotics, when required, were generally used at the following concentrations (per milliliter): 12.5 μg of tetracycline, 15 μg of chloramphenicol, 200 μg of streptomycin, and 125 μg of carbenicillin. X-gal was added at 40 μg/ml for blue-white selection in cloning.

To grow strains in various concentrations of phosphate, T medium (26) containing 0.4% glucose, 2 μg of nicotinic acid/ml, and 40 μM ferrous sulfate was made without phosphate, and various amounts of potassium phosphate (pH 7.0) were then added to the media. Complete T media containing 2 or 0.01 mM phosphate was designated high-phosphate media (HPM) and low-phosphate media (LPM), respectively.

Standard recombinant DNA procedures. Small plasmids were routinely isolated using a QIAprep Spin Miniprep kit (QIAGEN, Santa Clarita, Calif.). Chromosomal DNA was isolated using a DNeasy tissue kit (QIAGEN). DNA was extracted from agarose gels and/or purified using either a QIAquick gel extraction kit or a QIAGEN QuiaxII bead kit (QIAGEN). Chromosomal DNA was extracted using a QIAGEN DNeasy tissue kit. All procedures were performed according to the manufacturer's instructions. Restriction enzyme digests were performed using buffers and enzymes from Promega Corp. (Madison, Wis.). Ligations were performed using T4 DNA ligase (Promega) and the buffer supplied by the manufacturer and were incubated at either 16°C for 4 h to overnight for sticky-end ligations or at room temperature (25°C) overnight for blunt-end ligations.

All PCRs were carried out using either *Taq* (Promega) or *Pfu* Turbo polymerase (Stratagene Cloning Systems, La Jolla, Calif.) by using the buffers and instructions supplied by the manufacturer. *Taq* was used for all PCRs unless the fragments were to be cloned or sequenced, in which case *Pfu* Turbo was used. Primers for individual PCRs are listed in the appropriate sections below. The reactions conditions were as follows: (i) 5 min at 95°C; (ii) 30 s at 95°C; (iii) 30 s at 55°C; (iv) 2 min/kb of desired product at 72°; (v) repeat steps two to four 29 times; (vi) 10 min at 72°C; (vii) 4°C.

IcsA localization. Rabbit polyclonal antibody against IcsA (Rabbit 35) was obtained from Edwin Oaks (Walter Reed Army Institute of Research). Bacterial strains were grown to late logarithmic phase, centrifuged, washed two times with Dulbecco's phosphate-buffered saline (PBS-D), and fixed in PBS-D containing 4% paraformaldehyde for 15 min. Then, the bacteria were washed twice with PBS-D, resuspended in 100 μl of IcsA antiserum diluted 1:100 in PBS-D, and incubated for 1 h. After three washes in PBS-D, the bacteria were resuspended in 100 μl of fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (ICN Biomedicals) diluted 1:100 in PBS-D and incubated for 1 h shielded from light. The bacteria were washed twice with PBS and resuspended in a final volume of 100 μl of PBS. One to 10 microliters of the samples was air dried on a slide. Coverslips were mounted on the slide in mounting media containing PBS-D, pH 8, 0.1% phenylenediamine, and 90% glycerol and visualized by fluorescent microscopy.

***phoA* expression.** *phoA* expression was measured using the plasmid-borne *phoA-gfp* fusion pLR83 (22) or by reverse transcription (RT)-PCR. For *phoA-gfp* expression studies, *Shigella* strains containing pLR83 were subcultured 1:100 into HPM or LPM and grown for 5 to 6 h at 37°C. One milliliter of each culture was centrifuged and the pellet was resuspended in 4% paraformaldehyde for 10 min. The samples were then washed twice by centrifugation followed by resuspension of the cell pellet in low-salt PBS. Green fluorescent protein (GFP) levels were quantified using a FACSCaliber (Becton Dickinson, Franklin Lakes, N.J.) fluorescence-activated cell sorter with an excitation at 488 nm. FACSCaliber settings were as follows: forward scatter, E01; side scatter, 505; and relative fluorescence between 515 and 545 nm, 798.

For *phoA* expression studies using RT-PCR, overnight LB cultures were pelleted and concentrated 10-fold in LPM media. The resuspended cells were inoculated 1:100 into either LPM or HPM and incubated at 37°C for 5 h. Total RNA was isolated from the cultures by using a RNeasy Mini kit (QIAGEN) and quantitated by measuring the absorbance at 260 nm. To amplify and quantitate the amount of *phoA* mRNA in each sample, RT-PCR was carried out using a One-Step RT-PCR kit (QIAGEN) according to the manufacturer's instructions. Aliquots of the reactions were removed at cycles 21, 24, 27, and 30 to verify linearity. RT-PCR was performed with 10 ng of total RNA by using the *phoA* primers UR033 (5′ TATT GCACTGGCACTCTTACC 3′) and UR037 (5′ AGCGCATAGTGAGTGTA TTGC 3′). The RT-PCR products were run on a 1.5% agarose gel, and the amount of DNA in each ~0.3-kb *phoA* band was quantified using densitometry analysis.

Construction of UR005 (*S. flexneri pstS phoB* double mutant) by allelic exchange. The *phoBR* genes from strain SM100 were amplified by using PCR with primers phoBR1 (5 ′CGGGATCCAAACTCAGTGGAATGGG 3′) and phoBR2 (5′ CGGAATTCGCATCGGCTGGCTTATGG 3′). The *phoBR* fragment was digested with EcoRI and BamHI and ligated with pWKS30 digested with EcoRI and BamHI to generate pLL1. A 1.5-kb fragment containing a tetracycline resistance gene (*tet*) was isolated from pMTLtet by digestion with PstI and mung bean nuclease and was inserted into the SmaI site in *phoB* on pLL1. *phoBR* with the *tet* resistance cassette in *phoB* was excised as a XhoI/XbaI fragment and ligated into pHM5 digested with SalI/XbaI to generate pLL2.

pLL2 containing the *phoB*::*tet* gene was mated from *E. coli* SM10λ*pir* to *S.*

flexneri SM169, and cells were plated on tryptic soy broth agar containing tetracycline and chloramphenicol. Isolated colonies were then streaked on LB agar plates containing 5% sucrose, tetracycline, and chloramphenicol to select for double-crossover recombinant colonies. Putative mutants were screened for carbenicillin sensitivity on LB agar. Disruption of the chromosomal *phoB* gene was confirmed by PCR analysis.

Generation of the *pstA* point mutation $pstA_{R220Q}$ by overlap extension. To generate the mutation in *S. flexneri pstA* which changes arginine 220 to glutamine ($pstA_{R220Q}$), PCR overlap extension (5) was employed using the overlapping primers UR015 (5′ GCGATTGCCCAAATTGCC 3′) and UR016 (5′ GGCAA TTTGGGCAATCGC 3′) containing the underlined mutations. The two overlapping fragments were generated separately using PCR with the primer pairs UR016-UR013 (5′ GTATCGTTCTTCCGTTCACCA 3′) and UR015-UR014 (5′ ATACGTACCGCTTCGTCAATG 3′). The PCR products were gel purified, and these fragments were used as the template DNA in a second PCR with primers UR013 and UR014 to generate a full-length DNA fragment containing the $pstA_{R220Q}$ mutation.

To replace wild-type *pstA* in pPst1 with the mutated $pstA_{R220Q}$, the 2.1-kb StyI fragment from the $pstA_{R220Q}$ PCR fragment was used to replace the corresponding StyI fragment in pPst1. Sequence analysis at the Nucleic Acid Research Facility at Virginia Commonwealth University confirmed that the $pstA_{R220Q}$ point mutation was the only mutation present on the resulting plasmid pAB1.

Henle cell assays. Monolayers of Henle cells (intestinal 407 cells; American Type Culture Collection, Manassas, Va.) were used in all experiments and were maintained at 37°C in a 5% CO_2 atmosphere in Henle medium, which consists of minimum essential medium (Invitrogen Corp., Carlsbad, Calif.), 2 mM glutamine, nonessential amino acids, and 10% fetal bovine serum (Invitrogen). The intracellular multiplication assay was performed as described previously (6). Briefly, to infect the Henle cells with *Shigella*, subconfluent monolayers were infected with *Shigella* at a multiplicity of infection of 100 and incubated for 45 min. The infected monolayers were washed and incubated in Henle medium containing gentamicin (20 μg/ml). At the indicated time points, bacteria were recovered from one set of infected Henle cells by lysis of the Henle cells with 1 to 5% deoxycholate and plated on Congo red agar. A second set of infected monolayers was stained with Wright-Giemsa stain to quantitate percent invasion (percentage of Henle cells containing three or more intracellular bacteria). The average number of intracellular bacteria per infected Henle cell was calculated as CFU from the Congo red agar divided by the number of infected Henle cells.

Plaque assays were performed as described by Oaks et al. (16) with the modifications described in Hong et al. (6), and plaques were scored after 2 to 4 days.

RESULTS

Virulence-associated phenotypes of the *S. flexneri pst* mutant SM169. We previously constructed an insertion mutation in the *S. flexneri pstS* gene that was polar on all of the downstream genes in the *pst* operon. This *pst* mutant, SM169, forms smaller plaques in eukaryotic cell (Henle) monolayers than does the parental strain (22). Since wild-type plaque formation requires polar localization of the *Shigella* protein IcsA (13), the small plaque defect in SM169 may be due to aberrant IcsA localization. Thus, we examined IcsA localization in SM169 by indirect immunofluorescence. The *pst* mutant SM169 exhibited production and localization of the *S. flexneri* IcsA protein that was similar to that of the wild-type strain SM100 (Fig. 1). This suggested that the small plaque formation is due to an underlying defect other than improper IcsA localization.

Growth of the *S. flexneri pst* mutant SM169 in vitro and in vivo. Wild-type plaque formation also requires normal growth of *Shigella* within the Henle cell cytosol (16). To determine whether the *pst* mutation affected the growth rate of *Shigella*, we compared the growth of the *pst* mutant SM169 to the growth of the wild-type strain SM100 in vitro and inside Henle cells. In T medium containing various concentrations of phosphate (ranging from 2 to 0.001 mM), there was no difference between the optical density of SM100 and that of SM169 at each phosphate concentration (Fig. 2). This suggests that the *pst* mutation does not significantly affect the growth of *Shigella* in vitro.

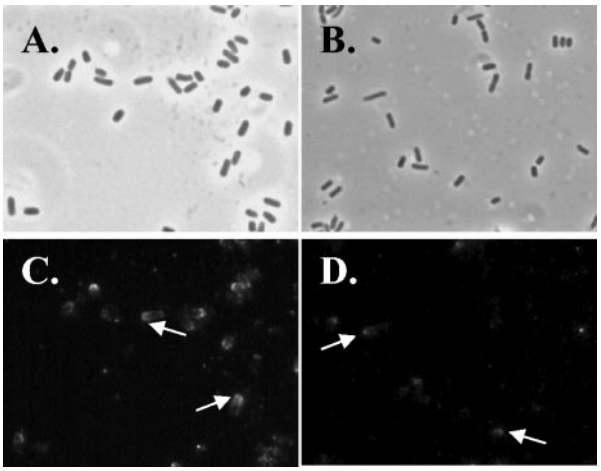

FIG. 1. The *S. flexneri pst* mutant shows polar localization of IcsA. Phase-contrast and immunofluorescence images from the same sample field of SA100 (A and C) and SM169 (B and D) followed staining with anti-IcsA are shown. Cells were observed at ×100 magnification. White arrows indicate representative bacteria with polarized IcsA.

The intracellular multiplication assay was used to measure growth of *Shigella* in Henle cells. Bacteria were recovered from infected Henle cell monolayers at various time intervals postinvasion, and the number of bacteria per infected Henle cell was determined (Fig. 3). The number of SM169 bacteria recovered at each time point was similar to the number of SM100 that were recovered at the same time, suggesting that the underlying cause of the small plaque phenotype in the *pst* mutant is not a reduced growth rate during the first 3 h of Henle cell infection. Intracellular growth of the strains at later time points could not be easily measured using the intracellular multiplication assay because the Henle cells lyse approximately 4 h postinvasion (data not shown). The size of the plaques that *Shigella* forms 2 to 3 days postinfection on Henle cell mono-

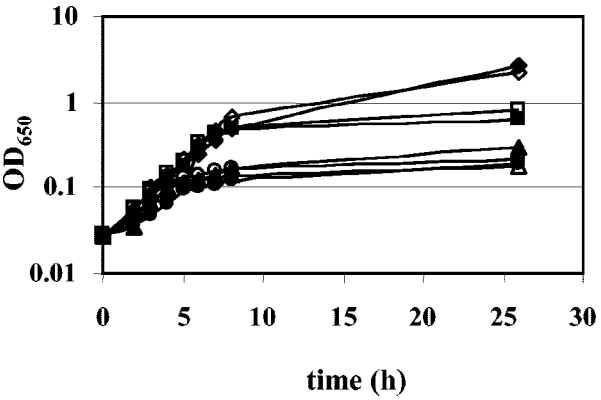

FIG. 2. The *S. flexneri pst* mutant has a normal growth rate in reduced phosphate media. Overnight cultures of the SM100 (closed symbols) or SM169 (open symbols) were subcultured 1:100 into T media containing various concentration of phosphate and grown at 37°C. Phosphate concentrations were 2 (◆, ◇), 0.1 (■, □), 0.01 (▲, △), and 0.001 (●, ○) mM. The optical density of the cultures was measured at various time points. The experiments were performed three times, and a representative experiment is shown.

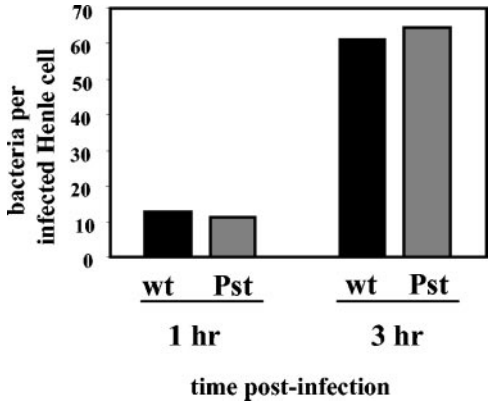

FIG. 3. The *S. flexneri pst* mutant has a normal growth rate in the initial stages of growth within Henle cells. The number of intracellular bacteria (SM100 [wt] or SM169 Pst^- [Pst]) was determined at each time point postinfection by counting the number of Henle cells in each sample by using a hemocytometer and then lysing the infected Henle cells and plating the contents on selective media to determine the number of bacteria present in the sample. This value was normalized to the percentage of infected cells, which was determined by microscopy.

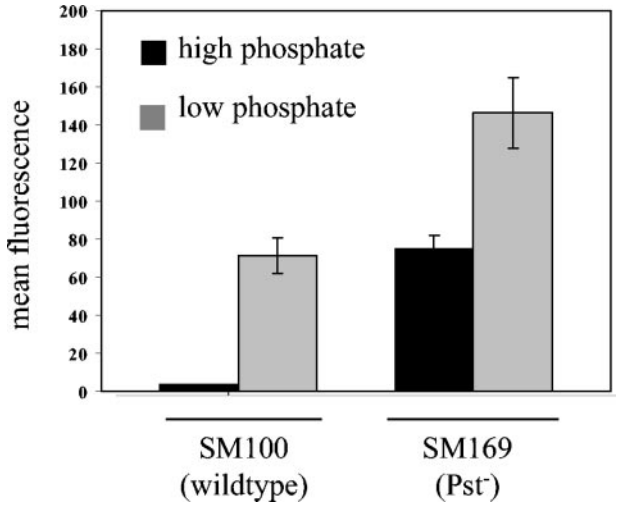

FIG. 4. Aberrant induction of the *S. flexneri phoA* promoter in the *pst* mutant SM169. SA100 or SM169 carrying pLR83 (*phoA-gfp*) was grown in HPM or LPM, and the fluorescence was quantitated by fluorescence-activated cell sorting after 5 to 6 h. Ten thousand bacterial cells were assayed for each experimental condition. The experiments were performed three times, and the standard deviations of the means are shown.

layers is proportional to the intracellular growth rate (6). Extra phosphate (7 mM) was added to the plaque assays in an attempt to suppress the small plaque defect of SM169. However, cells supplemented with additional phosphate had the same size plaques as those that were not supplemented (data not shown).

Expression of the PhoB regulon in the *S. flexneri pst* mutant SM169. In *E. coli*, the Pst system interacts with the PhoBR regulatory system to control phosphate-mediated gene regulation (33). *E. coli pst* mutants constitutively express genes in the PhoB regulon, which are normally repressed in phosphate-replete media. To determine whether this is the case in *S. flexneri*, we used a plasmid-borne fusion of the PhoB-regulated gene *phoA* to the promoterless *gfp* gene (pLR83) to assess expression of the PhoB regulon. Wild-type *S. flexneri* SM100 and the *pst* mutant SM169 carrying pLR83 were grown in HPM or LPM, and GFP reporter activity was measured. In SM100/pLR83, GFP expression driven by the *phoA* promoter was 22-fold lower after growth in HPM, relative to GFP expression after growth in LPM (Fig. 4). In contrast, GFP expression driven by the *phoA* promoter in SM169/pLR83 was aberrantly high when the strain was grown in HPM; specifically, GFP expression was only twofold lower after growth in HPM, relative to GFP expression after growth in LPM, and there was 23-fold more *phoA* promoter activity in SM169, relative to SM100, in HPM (Fig. 4). Thus, the *pst* mutation resulted in increased, and essentially constitutive, expression of *phoA* (Fig. 4) and other genes in the PhoB regulon (data not shown). This suggests that, as in *E. coli*, when phosphate levels are high outside the cell, the *Shigella* Pst complex maintains PhoR in a conformation that dephosphorylates (and therefore inactivates) PhoB.

Suppression of the plaque defect in SM169 by a *phoB* mutation. PhoB mediates induction of *phoA* and other genes in the PhoB regulon under low phosphate conditions (10–12). If constitutive expression of the PhoB regulon in the *pst* mutant SM169 is responsible for the small plaque phenotype, then elimination of PhoB in this strain would be expected to restore normal plaque formation ability to SM169. A *phoB* mutation was generated by allelic exchange in the *S. flexneri pst* mutant strain to test this hypothesis. The resulting *pst phoB* mutant (UR005) formed plaques on Henle cells monolayers that were similar in size to those formed by wild-type SM100, and two times larger in area than the plaques formed by SM169, indicating that eliminating normal PhoB restored the normal plaque formation capability to the *pst* mutant (Fig. 5A to C). This suggests that constitutive expression of a gene (or genes) in the PhoB regulon was responsible for the small plaque phenotype in SM169.

Plaque-forming ability of a *pstA* mutant with uncoupled Pst-mediated phosphate transport and regulation. To provide further evidence that the small plaque defect in the *pst* mutant results from constitutive expression of the PhoB regulon, we introduced a defined mutation ($pstA_{R220Q}$) into the *S. flexneri pstA* gene. $PstA_{R220Q}$ had been shown previously to allow wild-type expression of genes in the PhoB regulon but prevent Pst-mediated phosphate transport in *E. coli* (2). A DNA fragment containing this mutation was used to replace the corresponding wild-type *pstA* sequence on pPst1, which carries the entire *S. flexneri pst* operon.

We confirmed that $pstA_{R220Q}$ conferred wild-type expression of the PhoB regulon in the *S. flexneri pst* mutant by examining expression of the PhoB-regulated gene *phoA* by RT-PCR. *phoA* DNA was amplified by RT-PCR on RNA isolated from various *S. flexneri* strains grown in LPM or HPM. The *pst* mutant strain containing only the vector (SM169/pWKS30) showed consistently high expression of *phoA* regardless of the phosphate levels. The *pst* operon containing the $pstA_{R220Q}$ mutation on pAB1 complemented the constitutive PhoB regulon defect in the *pst* mutant SM169 just as effectively as the

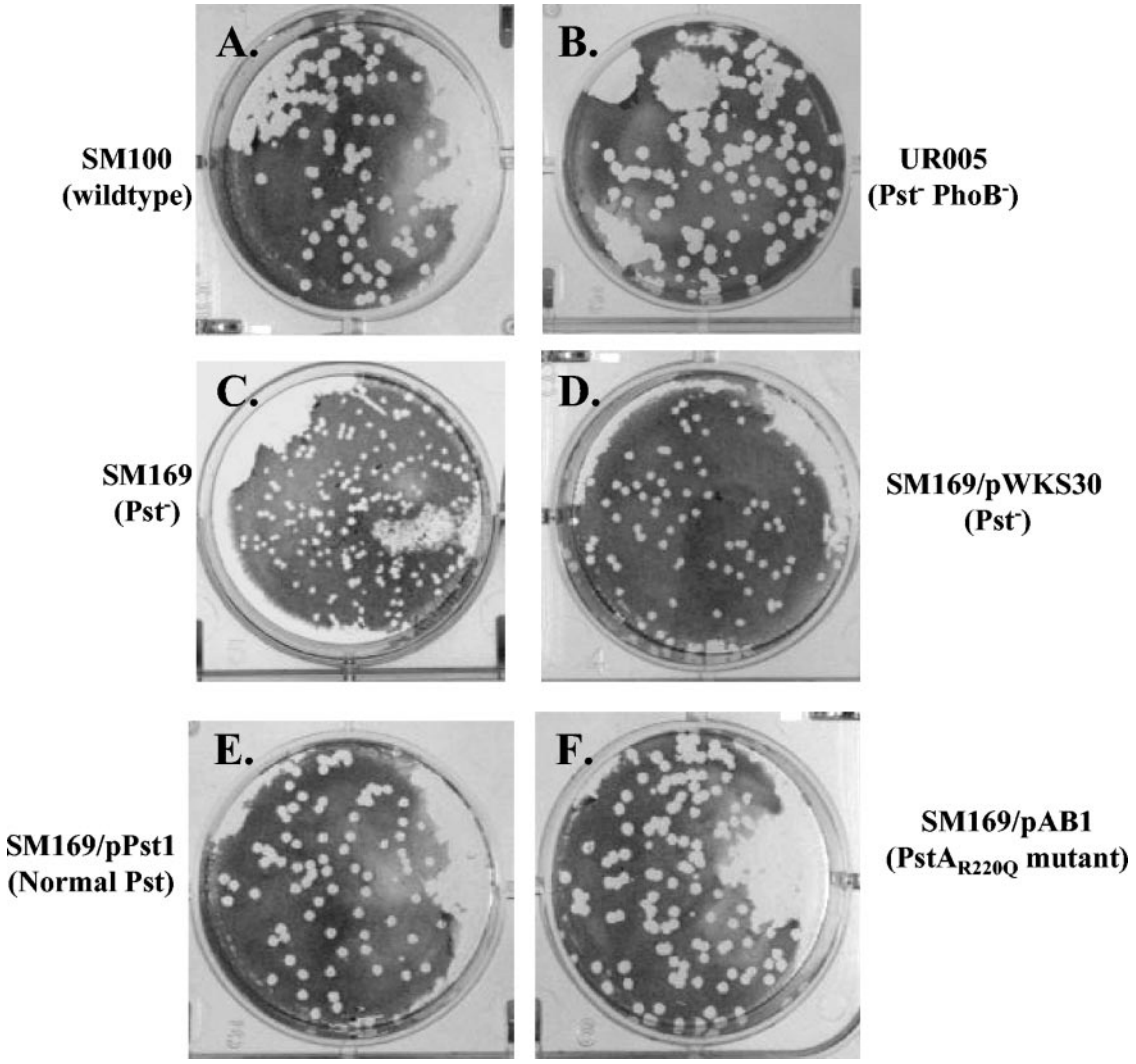

FIG. 5. Plaque assay with *S. flexneri pst* mutants. Confluent Henle cell monolayers were infected with 10^4 bacteria per 35-mm-diameter plate and the plaques were photographed after 3 days. The experiments were performed three times, and a representative experiment is shown.

wild-type *pst* operon on pPst1: SM169 carrying either pAB1 or pPst1 had a wild-type pattern of *phoA* expression (i.e., low levels of *phoA* expression in HPM and high levels of *phoA* expression in LPM) (Fig. 6).

The ability of pAB1 to restore normal plaque formation to SM169 was also assessed. SM169 and SM169/pWKS30 formed plaques that were two times smaller in area than the wild-type plaques, while the $pstA_{R220Q}$ mutant (SM169/pAB1) formed plaques similar in size to those formed by SM100 and SM169/pPst1 (Fig. 5). Since the $pstA_{R220Q}$ mutation confers wild-type expression of genes in the PhoB regulon (Fig. 6) and normal plaque formation (Fig. 5), but defective Pst-mediated phosphate transport (2), this suggests that constitutive expression of a gene or genes in the PhoB regulon was responsible for the small plaque phenotype in SM169.

DISCUSSION

The ability of *Shigella* to cause disease is frequently correlated with the ability to form plaques on eukaryotic cell monolayers. Plaque formation is a complex phenotype that is the result of a sequence of events including invasion and entry into the cytoplasm of eukaryotic cells, multiplication within the host cells, polar localization of the bacterial protein IcsA and subsequent polymerization of host cell actin, and lysis of the vacuoles that surround the double membrane after intercellular spread. Many *Shigella* mutants have been isolated that form small plaques and the underlying defect can be in one or more of the above processes. The *S. flexneri pst* mutant SM169 forms smaller plaques relative to the parental strain SM100 (22). SM169 invades at the same frequency and localizes IcsA in a similar fashion as SM100, suggesting that the small plaque phenotype is not due to defects in these processes. During the first 3 h after infection of Henle cells, SM169 and SM100 multiply at the same rate within the eukaryotic cytosol; however, we cannot eliminate the possibility that growth of the *pst* mutant SM169 slows down at a later time, relative to SM100, possibly due to nonoptimal expression of the PhoB regulon (see below).

E. coli pst mutants are defective in both high-affinity phos-

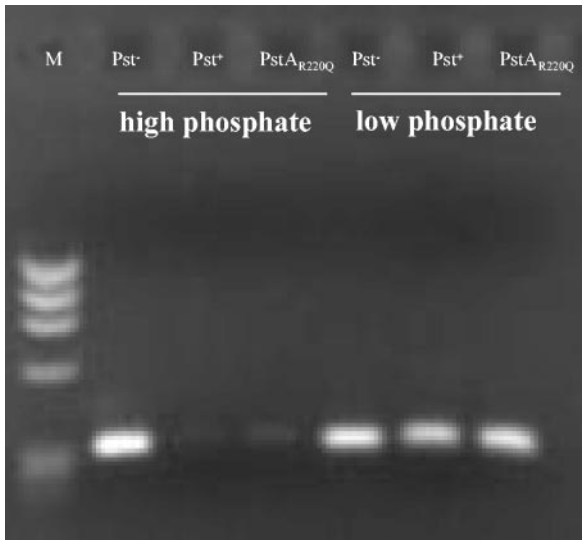

FIG. 6. The *S. flexneri pstA*$_{R220Q}$ mutation confers normal regulation of *phoA* expression. Total RNA was isolated from strains grown for 5 h in HPM or LPM. The *phoA* gene was amplified from the RNA by using RT-PCR, and the amount of *phoA* expressed by each strain was assessed qualitatively using gel electrophoresis and quantitatively using densitometry analysis. Strains were the following: Pst$^-$, SM169/pWKS30; Pst$^+$, SM169/pPst1; and PstA$_{R220Q}$, SM169/pAB1. M is the phiX174 HaeIII DNA size standard.

phate transport and in phosphate-dependent regulation of the PhoB regulon (29, 33). It was not apparent which of these Pst-mediated functions was required for normal plaque formation in *Shigella* since defects in either or both of these tasks could contribute to the small plaque phenotype. Because other phosphate transport systems such as the PitA and PitB systems exist in *S. flexneri* (35), it was possible that these systems compensate for loss of Pst-mediated phosphate transport in *S. flexneri pst* mutants but not necessarily for aberrant regulation of the PhoB regulon. Thus, the failure to form wild-type plaques was hypothesized to be the result of aberrant PhoB-mediated regulation. Two lines of evidence from this study support this model. First, the small plaque defect in the *pstS* mutant was suppressed by mutating the gene encoding the activator of the PhoB regulon (*phoB*) to eliminate PhoB regulon overexpression in the *pst* mutant. This data also suggests that PhoB may not be required for virulence, which is consistent with the observation that some *Shigella dysenteriae* strains have mutations in *phoB* (9). Second, a Pst system with PstA$_{R220Q}$, which confers normal expression of the PhoB regulon but defective Pst-mediated phosphate transport, restored the ability of the *Shigella pst* mutant to form wild-type plaques on Henle cell monolayers. Taken together, these results indicate that the regulatory function of Pst is more important than it's phosphate transport function for *S. flexneri*'s growth and intercellular spread in Henle cells, and the results are consistent with the model of aberrant PhoB regulon expression being responsible for SM169's plaque formation defect.

Optimal growth of a bacterium in its niche requires finely tuned gene expression. *Shigella* has numerous genes that are predicted to be regulators of gene expression (35). If the *Shigella pst* mutant expresses any of the 31 PhoB-regulated genes (33) at aberrantly high levels (due to the defective Pst system) while in the eukaryotic cell, this uncontrolled expression may be detrimental. Two PhoB regulon genes encode known or predicted periplasmic proteins: PsiF and PhoA, a nonspecific alkaline phosphatase (7, 15). Two genes in the PhoB regulon, *psiE* and *phoE*, encode an inner membrane protein and an outer membrane porin, respectively (1, 15, 18). Uncontrolled expression of either of these membrane proteins may disrupt the bacterial membrane or allow too much of a transported ligand to enter *Shigella*. Two PhoB-regulated operons are involved in phosphonate uptake and degradation (33). Phosphonates are a class of organophosphorus compounds that have carbon-phosphorous bonds. The enzymes encoded by these systems may be harmful to intracellular *Shigella* if expressed at abnormally high levels. Further, overexpression of the membrane components of the ABC transporter systems, which transport the substrates for these systems into the bacterial cell, may be toxic to the cell or slow down the growth rate when *Shigella* is intracellular. Finally, the PhoB-regulated protein PhoH contains two motifs that resemble a nucleotide binding pocket and, not surprisingly, PhoH binds ATP (8). PhoH may hydrolyze ATP, since this is a common feature of many ATP binding proteins. Excessive ATP hydrolysis when *Shigella* is intracellular may be detrimental. Interestingly, the genome sequence of *S. flexneri* strain 2457 has two frameshifts in the *phoH* gene, but the *phoH* sequence in *S. flexneri* strain 301 does not have these frameshifts. It is not known whether PhoH is expressed in SM100, the strain used in this study.

Recent studies suggest that the Pst system may modulate the virulence of several other pathogens. Mutations in *pst* cause reduced virulence in the fish pathogen *Edwardsiella tarda* (14, 28) and eliminate virulence in an *E. coli* strain pathogenic to pigs (3). In an enteroinvasive *E. coli* strain, an insertion mutation in the *pst* operon resulted in a hyperinvasive phenotype; however, this is not the case for *S. flexneri* (27). In enteroinvasive *E. coli*, overexpression of the Pst operon may induce an unknown invasion pathway. Expression of the *Streptococcus pneumoniae pstS* gene increases during growth in the murine peritoneal cavity, but it is not known whether the encoded protein contributes to virulence (17). Valdivia and Falkow (31) showed that the *pstS* gene was induced when *Salmonella enterica* serovar Typhimurium was intracellular; however, the *pstS* mutant was not attenuated in virulence. This may reflect a difference in the intracellular location of *Salmonella* compared to *Shigella. Salmonella* resides in an altered macrophage vacuole, while *Shigella* is found in the cytosol of colonic epithelial cells.

Although the *S. flexneri pstS* and *phoA* genes are induced in the eukaryotic cytosol, which suggested that the bacteria may be sensing reduced levels of available phosphate (22), the underlying reason for the small plaque phenotype in the *pst* mutant appears to be aberrant overexpression of the PhoB operon, not phosphate limitation. Previously observed induction of genes in the PhoB regulon when *Shigella* was intracellular was complicated, as only part of the bacterial population that was grown in Henle cells induced the *pstS* and *phoA* genes during the initial stages of infection. This suggested that either individual Henle cells may have different phosphate levels or different parts of the Henle cell may have different phosphate levels (22). In light of our new data that supports aberrant overexpression of the PhoB regulon, not phosphate limitation,

as the underlying reason for the small plaques in the *pst* mutant, it is possible that the bacteria are not severely starved for phosphate in the eukaryotic cytosol. Instead, the intracellular bacteria may be experiencing moderate phosphate limitation which would cause the intermediate induction of the PhoB regulon that appears to be optimal for *Shigella* in the Henle cell cytosol but would not cause complete induction (as in the *pst* mutant) of the PhoB regulon that appears to be detrimental to plaque formation when *Shigella* is intracellular. Finally, intracellular induction of the PhoB regulon may be due not to phosphate limitation but to cross talk with the CreBC two-component regulatory system. In *E. coli* there is a phosphate-independent induction of the PhoB regulon that requires the sensor protein CreC, which may be detecting a central metabolism intermediate, and glucose, acetate, or pyruvate C (34). A similar system may exist in *Shigella*.

ACKNOWLEDGMENTS

We gratefully thank the following individuals for their generous help: Haig Eskandarian for critical reading of the manuscript, Georgiana Purdy for help with IcsA localization assays, Edwin Oaks for anti-IcsA antisera, Stefan Seliger for strain SM100, and Stephanie Reeves for pMTLtet.

This work was supported by Public Health Service Grants AI09918 awarded to L. J. Runyen-Janecky and AI16935 awarded to S. M. Payne and by funding from the University of Richmond School of Arts and Sciences. A. M. Boyle was supported by the Robert F. Smart Award in Biology at the University of Richmond.

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Editor: D. L. Burns