A Shigella flexneri Virulence Plasmid Encoded Factor Controls Production of Outer Membrane Vesicles

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ABSTRACT Shigella spp. use a repertoire of virulence plasmid-encoded factors to cause shigellosis. These include components of a Type III Secretion Apparatus (T3SA) that is required for invasion of epithelial cells and many genes of unknown function. We constructed an array of 99 deletion mutants comprising all genes encoded by the virulence plasmid (excluding those known to be required for plasmid maintenance) of Shigella flexneri. We screened these mutants for their ability to bind the dye Congo red: an indicator of T3SA function. This screen focused our attention on an operon encoding genes that modify the cell envelope including virK, a gene of partially characterized function. We discovered that virK is required for controlled release of proteins to the culture supernatant. Mutations in virK result in a temperature-dependent over-production of outer membrane vesicles (OMVs). The periplasmic chaperone/protease DegP, a known regulator of OMV production in Escherichia coli (encoded by a chromosomal gene), was found to similarly control OMV production in S. flexneri. Both virK and degP show genetic interactions with mxiD, a structural component of the T3SA. Our results are consistent with a model in which VirK and DegP relieve the periplasmic stress that accompanies assembly of the T3SA.

KEYWORDS Shigella outer membrane vesicles virulence

Shigella is a genus of pathogenic bacteria that causes the diarrheal disease shigellosis, which places an onerous and ongoing burden on the developing world. A study performed in 2006 that focused on Southeast Asia showed that the incidence of shigellosis in this part of the world has not subsided and that there is a high incidence of Shigellae that is multidrug resistant (von Seidlein et al. 2006). Hallmarks of shigellosis include high fever and a mucopurulent diarrhea with blood. The latter symptom is a result of bacterial invasion and destruction of colonic epithelium.

Shigella flexneri contains a 220-kb virulence plasmid encoding approximately 100 genes (Buchrieser et al. 2000). This plasmid encodes components of a Type III Secretion System and its effectors, which are necessary for invasion into epithelial cells (Sansonetti et al. 1982), survival inside macrophages, and a myriad of effects that modulate the host immune response (Parrot 1999; Phalipon and Sansonetti 2007). Many of the genes encoded by the virulence plasmid are uncharacterized or poorly characterized. The Type 3 Secretion Apparatus (T3SA) of S. flexneri is well-defined due partly to the fact that Shigella spp. bind the dye Congo red when the T3SA is active, providing a phenotype that is correlated with Type III secretion and virulence (Parrot et al. 1995; Sakai et al. 1986). The mechanism by which Congo red induces activity of the T3SA is unknown; it is presumed that proteins secreted from S. flexneri bind Congo red resulting in a red colony. The T3SA spans the bacterial cell envelope to create a structure capable of transporting effectors from inside the bacterium to the host cell cytosol. In Gram-negative bacteria, the cell envelope consists of the inner membrane, the cell wall, which is composed of a layer of heteroplymer glycan chains cross linked by amino acids (peptidoglycan), and the bacterial outer membrane, which is comprised of a phospholipid inner leaflet and a glycolipid outer leaflet (reviewed in Silhavy et al. 2010)).
Gram-negative bacteria possess multiple envelope stress responses (ESRs) that maintain envelope integrity. These ESRs can be triggered by separate environmental insults and often are interconnected. Accumulation of unfolded proteins in the periplasm triggers activation of an alternate sigma factor, σE, that controls gene expression directed toward proper folding of proteins and repair of envelope damage. The production of outer membrane vesicles (OMVs) has been shown to represent a unique ESR (McBroom and Kuehn 2007). OMV production is increased in cells that cannot adequately deal with periplasmic stress, as in the case of deletions of the dual function DegP chaperone/protease (McBroom and Kuehn 2007). Hypervesiculating mutants show reduced fitness when combined with mutations that limit OMV production, indicating OMV production is required for maintaining envelope integrity (Schwechheimer and Kuehn 2013). OMVs can play a role in pathogenesis because they can transport toxins or deliver inflammatory components of the cell envelope to host cells (Kulp and Kuehn 2010).

Collections of deletion mutants, or “deletion collections,” are commonly used to identify novel gene functions by researchers who study organisms such as *Saccharomyces cerevisiae* (Giaever et al. 2002) or *Drosophila melanogaster* (Ryder et al. 2004). Deletion collections and similar tools created for the pathogens *Salmonella enterica* and *Shigella flexneri* have been used to discover new virulence genes and aid in development of antimicrobial agents (Donald et al. 2009; Santiviago et al. 2009). Here we report the creation of the pWR100 collection: a collection of precise mutants encompassing all genes encoded by the virulence plasmid of *Shigella flexneri*. Furthermore, we demonstrate the utility of the pWR100 collection by using it to identify new phenotypes associated with virK, a virulence plasmid-encoded gene. We find that loss of virK is associated with defects in T3SA function and hypervesiculation, suggesting that VirK supports T3SA function.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains and growth conditions**

A streptomycin-resistant strain of *Shigella flexneri* serotype 5a (M90T-Sm) was used as the parent strain for all mutants in the pWR100 collection (Onodera et al. 2012). *S. flexneri* was routinely cultured in or on Trypticase soy broth (TSB) plus 0.01% Congo red, with or without 20 mg/mL agar. Tetracycline was used at a concentration of 2494 | S. Sidik et al.
Integration of the knock-out cassette at the desired location was confirmed by PCR using primers for the P1 or P2 sequence in the knock-out cassette with a primer upstream (in the case of P1) or downstream (in the case of P2) of the region being deleted. Obtaining a product from such a PCR indicated correct integration of the knock-out cassette.

Figure 1  Fidelity of the deletion collection to established phenotypes. (A) Results from a screen for the ability of mutants from the pWR100 collection to bind the dye Congo red. CR units are defined as the density of wild-type S. flexneri (M90T-Sm) minus the density of the indicated mutant. Error bars indicate standard deviation of three replicates. (B) Ninety-six S. flexneri mutants were grown on Trypticase soy broth containing Congo red, then photographed using an excitation wavelength of 430 nm and an emission wavelength of 503 nm. (C) Reverse transcription quantitative polymerase chain reaction was performed to establish the effect of several mutations on transcription of downstream genes. Three replicates were used to establish the mean number of transcripts per 1000 16S rRNA transcripts and a standard deviation. Groups of strains that were analyzed together are separated by horizontal lines. Probability of being the same as M90T-Sm: *0.05 > P > 0.01, **0.01 > P > 0.001 (see the section Materials and Methods for statistical methods).
Tetracycline resistance genes were removed by catalyzing recombination between the flanking FRT sites using the plasmid pRR03. This plasmid was introduced into mutants by electroporation, as described previously, or by mating using the E. coli strain S17-1A pir. In the case of mating, S. flexneri was mixed with S17-1A pir pRR03 in approximately a 1:1 ratio, spotted on TSB and allowed to incubate at 37°C for 5 hr. The mix of bacteria was then plated on M9 minimal media plate supplemented with 10 μg/ml nicotinic acid plate containing kanamycin (to select for S. flexneri containing pRR003) and incubated at 37°C overnight. Colonies were patched onto TSB Congo red plates containing kanamycin, with and without tetracycline. Strains that were susceptible to tetracycline were selected, as this indicated loss of the tetracycline resistance gene. In the case of electroporation, S. flexneri was allowed to recover for 90 min, then plated on TSB Congo red plates containing kanamycin. Single colonies were patched onto TSB Congo red plates containing kanamycin, with and without tetracycline.

Construction of a nonpolar ipaH9.8 mutant

The ipaH9.8ΔNterm::tetRA mutant was created as described above with the exception that only approximately the middle third of ipaH (amino acids 183–337, inclusive) was replaced by the knock-out cassette. The primers used to create such a knock-out cassette are listed in Table S1 as ipaH9.8-NtermΔdelFlr and ipaH9.8-C337delFlr.

Congo red screens

Strains from the pWR100 collection were pinned onto 14-cm diameter, round TSB Congo red plates in triplicate. Strains were pinned in different locations on each of the three plates to avoid edge effects. As controls for the screen of the pWR100 collection, the same 96 strains were pinned onto Luria broth plates without Congo red, and 94 strains from the Keio E. coli deletion collection were pinned onto a Congo red TSB plate. Strains were grown for 16 hr at 37°C. Plates were imaged using a VersaDoc imaging system from BioRad with an excitation wavelength of 430 nm and an emission wavelength of 503 nm. Densitometry was performed using Image Lab software from BioRad. The density of the spot corresponding to each strain was determined using local background subtraction, these values were averaged for each strain, and the average for each mutant was subtracted from the average for M90T-Sm to obtain a score in “CR units.”

Reverse transcriptase quantitative PCR (RT-qPCR)

Bacterial cultures were grown overnight at 37°C with shaking at 200 rpm, then diluted at a ratio of 1:100 in TSB and grown for an additional three hours under the same conditions. In experiments 1, 2, and 3 (Figure 1C) Congo red was added to the cultures to a final concentration of 0.05% after 2 hr, and the cultures were then grown for an additional hour. RNA was extracted using an RNasey mini kit from QIAGEN (cat. no. 74014). cDNA was then synthesized from 1 μg of total cellular RNA using qScript cDNA SuperMix from Quanta BioSciences (cat. no. 95048-25). Two microliters of cDNA (equivalent to 100 ng of total RNA) was used as template for qPCR using PerFeCt SYBR Green FastMix from Quanta BioSciences (cat. no. 995072-250). Samples were run on a RotorGene 3000 from Corbett Research. Primer sequences are listed in Table S1 as the name of the gene they amplify, followed by “RT1,” followed by either “F” for “forward” or “R” for “reverse.”

Gentamicin protection assays

HeLa cells were cooled to room temperature for 15 min before infection, then infected at an MOI of 10 using strains of S. flexneri containing the afimbrial adhesin E gene (afae) from E. coli (Labigne-Roussel et al. 1984). Infected cells were allowed to incubate at room temperature for 15 min, then incubated at 37°C with 5% CO2 for 1 hr. Medium was then removed from the cells and replaced with Dulbecco’s Modified Eagle’s medium containing 10% fetal bovine serum and 50 μg/mL gentamicin, and the cells were incubated at 37°C with 5% CO2 for an additional hour. Cells were washed once with phosphate-buffered saline, then lysed in 500 μL of NP-40 lysis buffer (0.1% NP-40; 50 mM Tris-HCl, pH 7.5; 5 mM ethylenediaminetetraacetic acid; 10% glycerol, 100 mM sodium chloride). Fifty microliters of 1:10 serial dilutions of the cell lysates were plated on TSB agar media containing Congo red and incubated at 37°C overnight. Colony-forming units were counted the next day.

Secretion assays

Bacterial strains were grown to mid-log phase, and approximately 4 × 108 bacteria were pelleted at 3220g for 10 min and resuspended in 1 mL of phosphate-buffered saline. Congo red was added to a final concentration of 425 μg/mL unless otherwise indicated, and the bacteria were incubated at 37°C or 30°C for 30 min. Bacteria were pelleted at 3000 g for 10 min, then the supernatant was collected and filtered through a 0.45-μM filter. Bacterial pellets were resuspended in 250 μL of sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl, pH 6.8; 2% SDS; 100 mM dithiothreitol; 10% glycerol) and boiled for 5 min. Supernatant samples were mixed with SDS sample buffer in a 3:1 ratio of sample to buffer and boiled for 5 min before gel loading. All samples were run on 10% SDS-polyacrylamide gel electrophoresis gels. Gels were stained using either a silver stain kit from BioRad (cat. no. 161-0449), in the case of supernatant samples, or Coomassie in the case of bacterial pellet samples.

β-galactosidase assays

Bacterial strains were grown to mid-log phase, then 500 μL of culture was transferred to a 1.5-ml tube and incubated at 37°C for 30 min. One hundred microliters of bacterial culture was then mixed with 500 μL of Z-buffer (0.06 M Na2HPO4·7H2O, 0.04 M NaH2PO4·H2O, 0.01 M KCl, 1 mM MgSO4·7H2O, 0.05 M β-mercaptoethanol), 10 μL of 0.1% SDS, and 10 μL of chloroform. Cells were aspirated, then incubated at room temperature for 15 min. One hundred microliters of bacterial solution was mixed with 25 μL of ONPG (10 mg/mL), and the cells were incubated at room temperature until the mixture produced a yellow color. The reaction was stopped through the addition of 50 μL of 1 M Na2CO3, and the OD600 was read using an Eon microplate spectrophotometer from BioTek. The OD600 of each bacterial strain was also obtained to allow normalization for the number of cells.

Determination of periplasm protein content

Periplasm protein content was determined using a Bradford assay as described (Schwechheimer and Kuehn 2013).

Electron microscopy

Unfixed whole cells, taken directly from broth cultures with minimum disturbance, were allowed to interact with the surface of Formvar and carbon-coated copper grids for up to 5 min. After excess fluid was drawn from the surface of grids with a triangular piece of filter paper, grids were floated on drops of a saturated and unbuffered solution of ammonium molybdate for 20–30 sec. Grids were then air dried and immediately observed in a JOEL JEM-1230 transmission electron microscope equipped with a Hamamatsu ORCA-ER high-resolution (2K by 2K) digital camera. Images captured were saved as TIFF files. For quantification of OMVs, images from the virK or degP mutants or wild type bacteria grown at 37°C were coded and analyzed by counting the proportion of bacteria that were associated with OMVs and expressed as a percentage.
Statistics
In Figure 1C, three biological replicates were performed for each experiment. One-way analyses of variance (ANOVAs) were performed for each experiment, and, if significance was concluded, these were followed by Dunnett’s tests. P-values resulting from one-way ANOVAs were as follows: Expt. 1: 0.0025, Expt. 2: 0.4423, Expt. 3: 0.0103, Expt. 4: 0.1210, Expt. 5: 0.1969.

In Figure 2, A and B, three biological replicates were performed for each experiment. Invasion of a virK mutant was compared with that of the wild type using a one-way ANOVA followed by a Dunnett’s Multiple Comparison Test. Invasion of a phoP mutant was compared with that of wild-type using a one-way ANOVA followed by a Dunnett’s multiple comparison test, **P ≤ 0.0094. In Figure 2D, The optical densities (ODs) of five biological replicates were measured at 420 nm and 600 nm. The OD₆₀₀ of a “blank” well containing only media was subtracted from that of each experimental well. A strain of wild-type S. flexneri containing a plasmid encoding lacZ with no promoter also was included in the experiment. The average OD₄₂₀ of this strain was subtracted from all other strains. Miller units were calculated using the equation:

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\text{Miller Units} = 1000 \times \frac{\text{OD}_{420}}{\text{OD}_{600} \times \text{volume (mL)} \times \text{development time (min)}}
\]

A one-way ANOVA was performed followed by a Dunnett’s test. The P-value resulting from the one-way ANOVA was < 0.0001. In Figure 4, 10 biological replicates were performed. One-way ANOVA followed by a Bonferroni’s multiple comparison test was used, ***P ≤ 0.001. In Figure 5, C and D, three biological replicates were used in each experiment. One-way ANOVA followed by a Dunnett’s multiple comparison test was used. In Figure 5C, protein concentration was compared with virK. In Figure 5D, protein concentration was compared with degP mxij.

RESULTS
Construction of the pWR100 collection
We used λ red-mediated recombination (Figure S1A) (Datsenko and Wanner 2000) to create an ordered array containing mutants for 99 of the 106 genes encoded by pWR100, as identified according to Genbank accession number AL391753 (Buchrieser et al. 2000). Seven
mutants essential for maintenance of the virulence plasmid are not included in the pWR100 collection, namely ccdA, mvpA mvp7, repB, copA, tapA, and repA (Bahassi et al. 1999; Blomberg et al. 1992; Cevallos et al. 2008; Sayeed et al. 2000). Figure S1B, adapted from Buchrieser et al. (2000), displays the genes targeted for deletion in the pWR100 collection.

Each gene included in the pWR100 collection was replaced with a tetracycline resistance gene tetRA (Karlinsey 2007). A similar strategy was used previously by Baba et al. 2006 in the creation of the Keio E. coli deletion collection and has been established as an efficient method for creating a saturated collection of nonessential mutants. The tetRA cassette can easily be removed from pWR100 by using a plasmid-encoded flippase gene to catalyze homologous recombination between flanking FRT sites (Cherepanov and Wackernagel 1995). After removal of tetRA, a “scar sequence” encoding the amino acid sequence MIPGIRPPAVRSSTLSGIQSOKQLQP7 plus the last six amino acids of the gene targeted for replacement is encoded in place of the original gene.

Analysis of the pWR100 deletion collection for Congo red binding

Shigella spp. bind the dye Congo red, and binding is associated with virulence and secretion of T3SA effectors (Benjelloun-Touimi et al. 1995). The ability to bind Congo red routinely is used to ensure that strains retain T3SA function after genetic manipulation, and has been used as an assay to investigate the structure of the T3SA (Kenjale et al. 2005). We measured the ability of the mutants of the pWR100 collection to bind Congo red (Figure 1A). We made the fortuitous discovery that the dye Congo red can be imaged at a wavelength of 503 nm when illuminated with a wavelength of 430 nm, providing a quantitative assay for this phenotype that is associated with T3SA function. This allowed us to obtain grayscale images of the mutant strains in which the color density of the colonies indicated the ability of the mutants to allowed us to obtain grayscale images of the mutant strains in which the color density of the colonies indicated the ability of the mutants to bind Congo red (Figure 1B). The mutant strains also were grown on media without Congo red to ensure that this effect was dependent on the presence of dye (data not shown). The ratio of the standard deviation to the average of the Congo red scores obtained from this control was less than that obtained from the S. flexneri deletion collection plated on Congo red-containing medium (about 0.12, as opposed to 0.2), indicating that Congo red is necessary to observe variation in the fluorescence of the mutants. S. flexneri mutants for whom Congo red phenotypes have been reported behaved as expected in a Congo red binding assay, indicating that our deletion strategy produces the expected results in terms of effector production and secretion. For example, an ipaD mutant, in which effector secretion is constitutive (Menard et al. 1994), was the most Congo red—positive strain screened. A mutant for ospD1, an activator for the transcription factor MxiE (Parsot et al. 2005), also was more Congo red—positive than M90T-Sm. Mutants for the transcription factor-encoding genes virB and virF were among the most Congo red—negative strains (Adler et al. 1989). Many mutants for genes of unknown function were much more Congo red negative than the wild-type parent (M90T-Sm) (e.g., orf185 and orf47) and some mutants for genes of unknown function were more Congo red—positive than M90T-Sm (e.g., orf131a, orf131b, and orf163). These genes may play roles in regulating effector secretion or may affect Congo red binding through a mechanism independent of T3SA function.

Our mutagenesis strategy was designed to minimize polar effects on downstream genes by maintaining an open reading frame in place of each deleted gene, both before and after removal of tetRA. To examine the efficacy of this system, we performed RT-qPCR on four genes using RNA isolated from mutants for genes upstream of these four genes (Figure 1C). Both mutants containing tetRA and those in which tetRA had been removed were analyzed. Of these four genes, one mutant, ipaH9.8, reduced transcription of the downstream gene, ospG, to a statistically significant degree. This effect was observed both with and without tetRA. Transcription of ipaH4.5 was increased upon deletion of the upstream gene, ipaH7.8, although this effect was only observed after removal of tetRA. It is possible that moving the binding site for MxiE, the transcription factor that activates transcription of the ipaHs closer to the ipaH4.5 transcription start site causes a greater number of ipaH4.5 messages to be successfully completed.

The reason for the polar effect of the ipaH9.8 mutation on ospG is unknown. A smaller mutation that replaces approximately the middle third of ipaH9.8 with the scar sequence left behind after removal of tetRA is not polar (Figure 1C Experiment 2). These results reflect mechanisms of S. flexneri gene regulation that are yet to be elucidated (Bongrand et al. 2012). The nonpolar ipaH9.8 mutant is included in the pWR100 collection, with and without tetRA, under the name “ipaH9.8::Nterm.” IpaH9.8 is an E3 ubiquitin ligase, and the catalytic ability of this Type III Secretion System effector depends on a cysteine residue found at amino acid position 337 (Rohde et al. 2007). This cysteine is included in the region that has been deleted, and so the ability of ipaH9.8 to encode an active E3 ubiquitin ligase is eliminated in the ipaH9.8::Nterm strain.

Altered release of proteins to the culture supernatant in a virK mutant

Several mutants for undercharacterized genes were found to be deficient in their ability to bind Congo red (Figure 1A). One of these, ipgF, is encoded within a region of the virulence plasmid that encodes structural elements of the T3SA needle apparatus (Buchrieser et al. 2000). Removal of the tetRA cassette restored a Congo red positive phenotype to the ipgF mutant, suggesting that the ipgF::tetRA mutation had a polar effect that was alleviated when the tetRA cassette was removed. Removal of the tetRA cassette did not restore a Congo red positive phenotype to other mutants, including two mutants, pgdA and virK, that are in an operon that includes: pgdA-wabB-virK-msbB2 (Buchrieser et al. 2000; Goldman et al. 2008; Kaniuk et al. 2004). We found that the pgdA::tetRA mutation resulted in a polar effect on the operon and that, in contrast to the ipgF mutant, the polar effect was still observed when the tetRA cassette was removed. We created a pgdA::kan mutant using the same oligos used to amplify the tetRA cassette. The pgdA::kan mutant did not show a polar effect on downstream genes (Figure S2). These results show that the construction of a complete and non-polar collection of mutants will require a systematic and comprehensive analysis of each gene.

The virK mutant was chosen for further analysis. We found that the virK mutant that we constructed was impaired for the ability to enter cultured epithelial cells and that virK expressed from a plasmid could restore invasion to near wild-type levels (Figure 2A). Transcription of the pgdA-wabB-virK-msbB2 operon is largely controlled by the PhoP-PhoQ two-component system (Goldman et al. 2008). The PhoPQ system is often co-opted to control virulence programs and the PhoPQ regulon includes many factors that modify components of the cell envelope (Alteri et al. 2011; Prost and Miller 2008). PhoPQ is essential for virulence in S. flexneri, although its role in invasion of epithelial cells is ambiguous (Cai et al. 2011; Moss et al. 2000). We found that a phoP mutant was significantly impaired in its ability to invade epithelial cells in the conditions of our experiment (Figure 2B). We tested whether expression of virK from a constitutive promoter
(lac) could restore the phoP invasion defect. The ability of a phoP mutant bearing a virK-expressing plasmid was not increased to a statistically significant degree. We conclude that the invasion defect observed in a phoP mutant may be due to more than one gene under the control of PhoP.

The profile for proteins released to the culture supernatant in the virK mutant was altered compared to the parent strain (Figure 2C). The proteins secreted into the culture supernatant without addition of Congo red included different species than those secreted by wild-type S. flexneri. Also, in culture supernatants from virK we reliably see an increase in a species that corresponds to the autotransporter toxin SepA (Figure 2C and Figure S4) (Benjelloun-Touimi et al. 1995). We observed that the culture supernatant from the virK mutant induced with Congo red contained much fewer proteins than wild type and more closely resembled the culture supernatant from an mxiD mutant that does not assemble a T3SA. When a plasmid that constitutively expressed virK was introduced into the virK mutant, the secretion profile resembled the wild type. Mutations in a regulator of T3SA activity, ipaD, result in a similar phenotype: increased secretion of proteins to the culture supernatant without Congo red induction and an inability to invade epithelial cells (Menard et al. 1994).

To test the effect of virK on T3SA function, we examined the activity of a reporter gene that is controlled by the activity of the T3SA. We used a lacZ reporter gene under the control of the ipaH9.8 promoter, which is known to be constitutive in an ipaD mutant (Le Gall et al. 2005). We found that transcription of the reporter gene was significantly increased in an ipaD mutant but not in a virK mutant (Figure 2D). These data indicate that mutation of virK does not trigger production of effectors that are under the control of the activity of T3SA and argue against VirK regulating the activity of T3SA.

We considered the possibility that the proteins in the culture supernatant of virK mutant cells may be due to lysis of a proportion of cells in the culture. We observed that the virK mutant had a growth rate that was the same as the parent strain. These data suggest that the release of proteins to the culture supernatant by the virK mutant was not due to deregulated T3SA or due to massive cell lysis.

**Release of proteins to the culture supernatant in a virK mutant is temperature dependent**

VirK is a periplasmic chaperone protein that preferentially interacts with misfolded proteins (Tapia-Pastrana et al. 2012). We hypothesized that the release of proteins to the culture supernatant observed in the virK mutant may be associated with assembly of the T3SA. Assembly of the T3SA is temperature-dependent, and does not occur at 30°C (Adler et al. 1989). We performed secretion assays using virK and wild-type S. flexneri at 30°C and 37°C. We found that a virK mutant did not secrete proteins at 30°C, providing a correlation between the virK mutant's prolific secretion and the T3SA (Figure 3A). We observed a basal level of secretion of proteins to the supernatant in wild-type cultures in accord with previous findings (Blocker et al. 1999). By contrast, these proteins were absent in samples prepared from virK cells at 30°C (Figure 3B).

DegP is a chaperone/protease that functions in the periplasm to aid in protein folding and to destroy ones that are improperly folded. Mutations in S. flexneri degP result in a phenotype similar to those that we had observed with virK (Nakata et al. 1992; Purdy et al. 2002). Both virK and degP were identified as virulence factors for S. flexneri based on their impaired intercellular spread, a process that requires both actin polymerization and T3SA (Bernardini et al. 1989; Page et al. 1999). We created a degP mutant and observed a release of proteins at 37°C to the culture supernatant similar to that of virK (Figure 3B).

Because growth at temperatures where no T3SA is assembled suppressed the virK phenotype, we reasoned that deletion of genes required for T3SA assembly may similarly suppress release of proteins to the culture supernatant in virK cultures. We constructed a mutant bearing deletions in both virK and the secretin mxiD that encodes a component of the outer membrane ring of the T3SA and is required for T3SA assembly (Allaoui et al. 1993). Deletion of mxiD did not suppress the release of proteins to the culture supernatant in virK cultures (Figure 3B). Curiously, deletion of virK suppressed the Congo red—negative phenotype of the mxiD mutation. Expression of virK
from a plasmid in *virK* *mxiD* mutants restored a Congo red negative phenotype (Figure 4A).

We examined possible genetic interactions between *degP* and *mxiD*. We found that *degP* *mxiD* mutants are viable at 30°C but are unable to grow at 37°C (Figure 4B). We tested genetic interactions between *degP* and another gene encoding a structural component of the T3SA, the base component *mxiJ*. We observe that a *degP* *mxiJ* mutant is viable at 30°C and 37°C. We observed that *degP* *mxiJ* double mutants were viable at 37°C and that they were Congo red—positive (Figure 4C). We conclude that both *virK* and *degP* show genetic interactions with *mxiD* which encodes a component of the T3SA.

**VirK and DegP control the production of OMVs in *S. flexneri***

In *E. coli*, mutation of *degP* results in an increased production of OMVs (McBroom and Kuehn 2007). OMV production is one type of ESR; vesicle production would be one way to explain the release of proteins to the culture supernatant in *virK* and *degP* mutants. We examined the idea that *virK* may influence production of OMVs. We used transmission electron microscopy of negatively stained, unfixed *S. flexneri* whole cells to observe production of OMVs. We observed very few vesicles in wild-type cells grown at 37°C (Figure 5A). In contrast, many vesicles were associated with *degP* and *virK* cells grown at 37°C, most cells were associated with multiple vesicles. No vesicles were observed in any strains in cells grown at 30°C. We examined *degP* *mxiD* cells that had been grown at 30°C and shifted to 37°C for 4 hr. These cells produced many OMVs, and some cells were completely surrounded by vesicular structures (Figure 5A). We also counted the amounts of vesicles present in several fields around wild-type bacteria, *virK* mutants, and *degP* mutants. Blind scoring of the images from bacteria grown at 37°C showed that a greater proportion of *virK* or *degP* mutants were associated with vesicles than the wild-type control (Figure 5B). The production of OMVs has been shown to be a novel envelope stress pathway that relieves stress that occurs in the periplasm as unfolded proteins accumulate. We examined the amount of proteins present in the periplasm in the various mutants. Mutation of *virK* resulted in a large increase in the amount of periplasmic proteins when compared with the wild type (Figure 5C). This increase could be suppressed by mutation of *mxiD*. We also observed an increase in periplasmic proteins in the *degP* and *degP* *mxiD* mutant strains (Figure 5D). Taken together, these data indicate that *degP* and *virK* relieve envelope stress that accompanies growth at 37°C.

**DISCUSSION**

The mutants that we constructed are easily cured of their tet*RA* cassette to produce markerless mutants. As all of these mutants were constructed in the same manner, in the same genetic background, this collection should prove useful for the construction of multiple gene deletions for the study of genetic redundancy, and for the rational development of vaccine candidates. Importantly, this collection is streptomycin resistant and is compatible with the *S. flexneri* oral model of infection in mice that has been developed by Bernardini and coworkers (Martino et al. 2005). Our strategy was designed to minimize polar effects; however, for reasons that we do not understand, some of the mutants do show polar effects on the expression of downstream genes. DNA topology in regions downstream of the transcription start site has been shown to exert effects on gene expression (Owen-Hughes et al. 1992). Also, multiple genes on pWR100 are produced by a phenomenon known as transcriptional slippage (Pennno et al. 2006). A careful analysis of each mutant will be needed to achieve a comprehensive collection of nonpolar mutants. The construction of such a collection is an achievable goal and will benefit from open collaboration and communication in the Shigella research community.

We used an ordered array of mutants to screen for an *in vitro* phenotype associated with *S. flexneri* virulence *in vitro* and identified an operon with genes under the control of the PhoP-PhoQ two-component system (Goldman et al. 2008). The Congo red phenotype provides an *in vitro* assay associated with the activity of T3SA. Our

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**Figure 4** Mutations in *virK* or *degP* show synthetic phenotypes with structural components of the Type 3 Secretion Apparatus. (A) Congo red associated fluorescence of *virK* *mxiD* mutant strains bearing either an empty vector or one expressing *virK* were grown on Trypticase soy agar (TSA) containing Congo red at the indicated temperatures. The plates were photographed and analyzed according to Materials and Methods. (B). The indicated strains were streaked on TSA media and grown at the temperatures indicated. (C). The indicated mutants were grown on TSA containing Congo red at the indicated temperatures.
results demonstrate that the Congo red phenotype is also associated with production of OMVs. We do not fully understand the role of OMVs in Shigella pathogenesis, but OMV production has practical applications for the production of vaccines or studies of innate immunity (Berlanda Scorza et al. 2012; Irving et al. 2014).

We provide evidence that virK and degP function to reduce the stress imposed by unfolded proteins in the periplasm. The T3SA is a macromolecular machine that spans both the inner and outer membranes, the controlled assembly of the T3SA requires remodeling of the cell envelope that invokes an ESR. This idea is clearly established in Yersinia, where T3SA assembly and the phage shock response are coupled (reviewed in Flores-Kim and Darwin 2012). In this case, phage shock proteins B and C are essential during conditions in which a T3SA is assembled (Horstman and Darwin 2012). By analogy, deletion of degP or virK result in an increase in T3SA components and invoke an ESR that results in the production of OMVs. Both DegP and VirK have been implicated in S. flexneri virulence based on their requirement for cell-to-cell spread. Intracellular spread requires proper function of IcsA and mutations of either degP or virK result in impaired processing of IcsA (Purdy et al. 2007; Wing et al. 2005). Intracellular spread also requires T3SA activity (Page et al. 1999). The genetic interactions between degP/virK and components of the T3SA (mxiD and mxiJ) imply that DegP and VirK may contribute to cell-to-cell spread by influencing T3SA in addition to IcsA. The release of proteins to the culture supernatant in virK and degP mutants is most likely T3SA independent. The proteins are released independent of Congo red induction and the release of proteins is not accompanied by an increased expression of genes that are controlled by the activity of the T3SA. VirK was recently found to be a chaperone for the plasmid-encoded toxin (Pet) of Enterococcosgenic E. coli that is secreted via an autotransporter mechanism (Tapia-Pastrana et al. 2012). Taken together, our data suggest that VirK serves a general quality control function for multiple weapons, including IcsA processing, toxin export, and T3SA assembly.

ACKNOWLEDGMENTS

We thank Dr. Joyce Karlinsey and Dr. Cheryl Patten for their generous gifts of plasmids. This research was supported by Canadian...
Institute of Health Research (MOP-102594). S.S. was an Alexander Graham Bell Canada Graduate Scholar. The authors declare that they have no conflict of interest.

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