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Mutations in *Flavobacterium johnsoniae sprE* Result in Defects in Gliding Motility and Protein Secretion▽†

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Cells of the gliding bacterium *Flavobacterium johnsoniae* move rapidly over surfaces. Transposon mutagenesis was used to identify *sprE*, which is involved in gliding. Mutations in *sprE* resulted in the formation of nonspreading colonies on agar. *sprE* mutant cells in wet mounts were almost completely deficient in attachment to and movement on glass, but a small percentage of cells exhibited slight movements, indicating that the motility machinery was not completely disrupted. SprE is a predicted lipoprotein with a tetratricopeptide repeat domain. SprE is similar in sequence to *Porphyromonas gingivalis* PorW, which is required for secretion of gingipain protease virulence factors. Disruption of *F. johnsoniae sprE* resulted in decreased extracellular chitinase activity and decreased secretion of the cell surface motility protein SprB. Reduced secretion of cell surface components of the gliding machinery, such as SprB, may account for the defects in gliding. Orthologs of *sprE* are found in many gliding and nongliding members of the phylum *Bacteroidetes*, suggesting that similar protein secretion systems are common among members of this large and diverse group of bacteria.

Cells of *Flavobacterium johnsoniae* cells crawl over surfaces in a process called gliding motility. This type of motility is common among members of the phylum *Bacteroidetes*, of which *F. johnsoniae* is a member. Cells of *F. johnsoniae* move at speeds of approximately 2 μm/s over wet glass surfaces. As a result of these cell movements, colonies of *F. johnsoniae* have thin spreading edges.

Gliding of *F. johnsoniae* appears to be mediated by the rapid movement of adhesins, such as SprB, along the cell surface (15, 32). GldK, GldL, GldM, GldN, SprA, and SprT are required for secretion of SprB to the cell surface (38, 41). They also appear to be involved in secretion of extracellular chitinase (38, 41). GldK, GldL, GldM, GldN, SprA, and SprT are similar in sequence to proteins of *Porphyromonas gingivalis* that are involved in secretion of gingipain proteases, which are virulence factors of this periodontal pathogen (40–42). The *P. gingivalis* proteins appear to form a complex, referred to as the Por secretion system (PorSS). Known proteins secreted by the *P. gingivalis* and *F. johnsoniae* PorSSs have N-terminal signal peptides that are typical of proteins targeted to the Sec system. It is thought that these proteins are transported across the cytoplasmic membrane by the Sec system and utilize the PorSS for transit of the outer membrane (38, 41). The PorSS is not closely related to other bacterial protein secretion systems, such as the type I to VI secretion systems (7, 10). Other proteins required for *F. johnsoniae* gliding that are not thought to be part of the PorSS include GldA, GldB, GldD, GldF, GldG, GldH, GldI, and GldJ (1, 5, 11–13, 26, 27). Some of these proteins may comprise the "motor" that propels SprB along the cell surface. The physical relationship of this motor to the PorSS is not known. They may be intimately associated, as is the case for swimming bacteria, where a type III protein secretion system is an integral part of the bacterial flagellum and is involved in its assembly (16). Gliding motility is not unique to members of the phylum *Bacteroidetes* and is observed in many bacteria outside this phylum (24). Gliding of *Myxococcus xanthus* and of *Mycoplasma* species has been studied at the molecular level (20, 23, 29, 43–45). These bacteria lack orthologs to most of the proteins that are required for *F. johnsoniae* gliding, and the mechanism of cell movement is thought to be different for each of these organisms (4, 15).

Previously, 294 Tn*4351*-, *HimarEm1*-, and *HimarEm2*-induced mutants of *F. johnsoniae* that formed nonspreading colonies were isolated (4, 11–13). The mutants were screened for motility defects in wet mounts and were divided into 3 groups. Nineteen of the mutants exhibited cell division defects resulting in the production of filamentous cells that were completely nonmotile. Several of these had insertions in *ftsX* (18), and the rest had insertions in other genes predicted to be involved in cell division (unpublished results). Another 51 mutants were completely nonmotile and had normal cell morphology. These mutants each had mutations in *gld* genes required for motility (1, 4, 5, 11–13, 26, 27). The remaining 224 mutants formed nonspreading colonies but retained some ability to move in wet mounts. The sites of transposon insertions were determined for 32 of these motile nonspreading mutants that were randomly selected for analysis. Six of the mutants had insertions in *sprA* (33), 2 had insertions in *secDF* (31), 16 had insertions in *sprB* (32), 2 had insertions in *sprC* (36), and 1 had an insertion in *sprD* (36), as previously described. Two of the remaining mutants (CJ101-285 and FJ140) had insertions in *fjoh_2111*, which is predicted to encode the lipoprotein localization protein LolA (M. McBride, unpublished results). GldB, GldD, GldH, GldI, GldJ, and GldK are lipoproteins (4, 5, 26, 27), which may account for the motility defects exhibited by *lolA*

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mutants. In this study, the remaining three mutants were analyzed, resulting in the identification of *sprE*. Cells with mutations in *sprE* formed nonspreading colonies and were severely defective in gliding. SprE is similar in sequence to *P. gingivalis* PorSS protein PorW. *F. johnsoniae sprE* mutants were defective in extracellular chitinase and in localization of SprB to the cell surface, suggesting that SprE is a component of the *F. johnsoniae* PorSS.

MATERIALS AND METHODS

Bacterial and bacteriophage strains, plasmids, and growth conditions. *F. johnsoniae* strains MM101 and FJ1, which are direct descendants of the *F. johnsoniae* type strain ATCC 17061, were the wild-type strains used in this study (26). *F. johnsoniae* strains MM101 and FJ1 are essentially identical, except that strain MM101 has a partial defect in chitin utilization. *F. johnsoniae* FJ114 and FJ156 (32, 36) were the *sprB* mutant strains used. *F. johnsoniae* strains were grown in Casitone-yeast extract (CYE) medium at 30°C, as previously described (25). To observe colony spreading, *F. johnsoniae* was grown on PY2 agar medium (1) at 25°C. MM medium was used to observe movement of individual cells in wet mounts (21). The *F. johnsoniae* bacteriophages used in this study were ϕCj1, ϕCj13, ϕCj23, ϕCj28, ϕCj29, ϕCj42, ϕCj48, and ϕCj54 (6, 35, 46). Sensitivity to *F. johnsoniae* bacteriophages was determined as previously described by spotting 5 μl of phage lysates (10^9 PFU/ml) onto lawns of cells in CYE overlay agar (38). *Escherichia coli* strains were grown in Luria-Bertani medium at 37°C. The following antibiotics were used at the indicated concentrations when needed: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; erythromycin, 100 μg/ml; kanamycin, 35 μg/ml; and tetracycline, 20 μg/ml.

Transposon mutagenesis and identification of sites of insertion. Tn*4351* and *HimarEm2* were introduced into wild-type *F. johnsoniae* by conjugation from *E. coli* as previously described (4, 11, 13). Mutants were selected by plating cells on PY2 agar containing erythromycin, and nonspreading colonies were isolated. Chromosomal DNA was isolated from mutants, and sites of transposon insertion were determined as previously described (4, 11, 18).

Sequence analysis. Sequences were analyzed with MacVector software (Cary, NC), and comparisons to database sequences were made using the BLAST algorithm (2). Predictions regarding cellular localization were made using the PSORTb (8) TMpredict (9), and CELLO (47) tools, and tetratricopeptide repeat (TPR) domains were predicted using the TPRpred tool (17). The phylogenetic distribution of *sprE* orthologs was determined using the U.S. Department of Energy Joint Genome Institute Integrated Microbial Genomes (IMG; version 3.3) gene profile tools (22) and by BLASTP analyses of completely sequenced microbial genomes available via NCBI (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi).

Cloning of *sprE*. A 2.92-kb region of *F. johnsoniae* DNA which spans *sprE* was amplified using Fideli *Taq* DNA polymerase (USB Corp.) and primers 712 (5′ TTTGCCGGATCCGACCCAACAACAGTAAGCCG 3′; the BamHI site is underlined) and 713 (5′ CACTTTGTCGACCATTATATTATGGGTTTTTTGGGG 3′; the SalI site is underlined). This fragment was digested with SalI and BamHI and ligated into pBCSK+ (Stratagene) that had been cut with the same enzymes to generate pNap1. pNap1 was digested with XbaI and KpnI, and the fragment containing *sprE* was inserted into pCP23 (1), which had been digested with the same enzymes to generate pNap2.

Protein expression and antibody production. A 2,568-bp fragment encoding the C-terminal 825 amino acids of SprE was amplified using ExTaq (Takara Bio Inc., Otsu, Japan) and primers 921 (5′ GCTAGGGATCCATGGAGGTCTTGGACTTGAG 3′; the BamHI site is underlined) and 923 (5′ GCTAGGTCGACGTCTCCTACAATAGAGGTTCC 3′; the SalI site is underlined). The PCR product was digested with BamHI and SalI and cloned into pET30-C that had been digested with the same enzymes, generating pEVG11D. pEVG11D was introduced into *E. coli* Rosetta 2(DE3) cells (Novagen, Madison, WI), which expressed seven rare tRNAs required for the efficient expression of SprE. To isolate recombinant SprE, cells were grown to mid-log phase at 37°C in rich medium plus glucose (10 g tryptone, 5 g yeast extract, 5 g NaCl, 2 g glucose/liter), induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and incubated for 4 h at 37°C. Cells were disrupted using a French press, and inclusion bodies containing recombinant SprE were collected by centrifugation at 6,000 × *g* for 15 min at 4°C. The inclusions were boiled in SDS-PAGE loading buffer, and SprE was visualized by $CuCl_2$ staining, after separation on a 10% acrylamide gel by SDS-PAGE (19). The SprE band was cut from the gel and destained in 0.25 M Tris (pH 9.0), 0.25 M EDTA, and the protein was electroeluted at 60 mA for 5 h into 25 mM Tris, 192 mM glycine, 0.1% SDS using a model 422 Electro-Eluter (Bio-Rad). Polyclonal antibodies against recombinant SprE were produced and affinity purified using the recombinant protein by Proteintech Group, Inc. (Chicago, IL).

Cell fractionation and Western blot analysis. *F. johnsoniae* cells were grown to mid-log phase in MM medium at 25°C without shaking. Cells were disrupted using a French press and fractionated into soluble and membrane fractions as previously described by centrifugation at 352,900 × *g* for 30 min (13). When whole cells were analyzed, they were pelleted at 4,000 × *g*, resuspended in SDS-PAGE loading buffer, and boiled for 5 min. Proteins (50 μg) were separated by SDS-PAGE, and Western blot analyses were performed as previously described (38) using crude antisera against SprB and SprE at 1:1,000 dilutions.

Microscopic observations of cell attachment to glass and of gliding motility. Attachment of wild-type and mutant cells to glass was measured as previously described (33). In brief, cells were grown in MM medium without agitation overnight at 25°C to a density of 5×10^8 cells/ml. Cells (2.5 μl) were added to a Petroff-Hausser counting chamber, covered with a glass coverslip, and allowed to incubate for 2 min at 25°C. The number of cells attached to 12 randomly selected 0.03-mm^2 regions of the glass coverslip was determined. Wild-type and mutant cells were examined for movement on glass by phase-contrast microscopy at 25°C essentially as previously described (32), except that standard glass slides and coverslips were used instead of Palmer cells.

Binding and movement of anti-SprB-coated polystyrene spheres. Purified antibody against SprB (1 μl of a 1:10 dilution), 0.5-μm-diameter protein G-coated polystyrene spheres (1 μl of a 0.1% stock; Spherotech Inc., Libertyville, IL), and bovine serum albumin (BSA; 1 μl of a 1% solution) were added to 7 μl of cells (approximately 5×10^8 cells per ml) in MM medium. The cell mixture was spotted on a glass slide and covered with a glass coverslip, and images were recorded and analyzed using MetaMorph software as previously described (32).

Immunofluorescent localization of SprB. Immunofluorescence was used to visualize SprB on the surface of formaldehyde-fixed cells as previously described (38), except that cells were collected on white 0.8-μm-pore-size Isopore membrane filters (Millipore, Billerica, MA). In brief, fixed cells were exposed to purified anti-SprB, followed by the F(ab′) fragment of goat anti-rabbit IgG conjugated to Alexa-488 (Invitrogen). InSpeck relative intensity fluorescent beads (Invitrogen-Molecular Probes, Eugene, OR) were added as controls. The filters were mounted on a glass slide with VectaShield with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA), and samples were observed using a Nikon Eclipse 50i microscope. Images were captured with a Photometrics CoolSNAP$_{ES}$ camera with exposure times of 100 to 200 ms (DAPI) and 300 ms (Alexa-488).

Chitin utilization. Chitin utilization on agar was observed as previously described (38). Chitinase activities in culture medium and in cell extracts were measured as previously described (38) using the synthetic substrate 4-methylumbelliferyl β-D-*N*,*N*′-diacetyl-chitobioside [4-MU-$(GlcNAc)_2$; obtained from Sigma-Aldrich (St. Louis, MO)]. Activities in the cell-free supernatants (secreted chitinase) and in cell extracts are indicated as pmol 4-methylumbelliferone released per μg total protein in the original cell suspension. Protein concentrations were determined by the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA).

Nucleotide sequence accession number. The sequence reported in this paper has been deposited in the GenBank database (accession no. EF111025).

RESULTS

Identification and analysis of *sprE*. Analysis of Tn*4351*- and *HimarEm2*-induced mutants that formed nonspreading colonies composed of cells that retained some ability to move resulted in the identification of three mutants (FJ149, FJ160, and CJ986) that had insertions in *fjoh_1051*, which we named *sprE* (Fig. 1). *sprE* encodes a predicted primary product of 870 amino acids. The amino-terminal sequence contains a hydrophobic region terminated by a cysteine, which is characteristic of bacterial lipoproteins. The region between amino acids 119 and 379 displays six tetratricopeptide repeats (TPRs) as determined by TPRpred (17). TPR domains are degenerate 34-amino-acid repeated sequences found in proteins from many organisms and are often involved in protein-protein interactions (3). SprE is similar in sequence to *P. gingivalis* PorW,

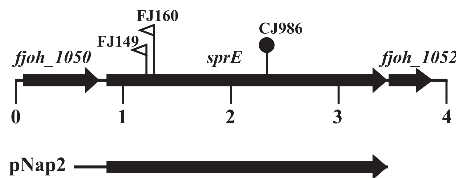

FIG. 1. Map of the *sprE* region. Numbers below the map refer to kilobase pairs of sequence. The sites of *HimarEm2* and Tn*4351* insertions are indicated by open triangles and closed circles, respectively. The orientations of *HimarEm2* insertions are indicated by the direction in which the triangles are pointing. As drawn, FJ149 and FJ160 both have inverted repeat IR1 on the right side of the transposons. The *sprE* mutants FJ149 and FJ160 were derived from wild-type strain FJ1, and the *sprE* mutant CJ986 was derived from wild-type strain MM101. The region of DNA carried by the complementing plasmid pNap2 is indicated beneath the map.

TABLE 1. Effect of mutations in *sprE* on attachment of cells to glass coverslips

Strain	Description	Avg no. of cells attached to 0.03-mm^2 region of glass coverslip[a]
FJ1 with control plasmid pCP23	Wild type	52.9 (11.6)
FJ114 with pCP23	*sprB* mutant	44.4 (14.8)
FJ149 with pCP23	*sprE* mutant	2.0 (1.2)
FJ149 with pNap2	Complemented *sprE* mutant	58.3 (12.7)

[a] Approximately 10^6 cells in 2.5 μl of MM medium were introduced into a Petroff-Hausser counting chamber and incubated for 2 min at 25°C. Samples were observed using an Olympus BH-2 phase-contrast microscope, and cells attached to a 0.03-mm^2 region of the cover glass were counted. Numbers in parentheses are standard deviations calculated from 12 measurements.

which is required for secretion of gingipain proteases (41). *F. johnsoniae* SprE and *P. gingivalis* PorW exhibit 22% amino acid identity and 42% similarity over the entire length of SprE (BLASTP E value of 4×10^{-39}). *P. gingivalis* PorW is 290 amino acids longer than SprE, as a result of a C-terminal extension that is not found on the *F. johnsoniae* protein. *fjoh_1050*, which lies upstream of *sprE*, encodes a predicted ATP-binding-cassette transporter, and *fjoh_1052*, which is downstream of *sprE*, encodes a protein of unknown function. There is no evidence linking *fjoh_1050* and *fjoh_1052* to motility, protein secretion, or SprE function.

Mutations in *sprE* result in defects in attachment and motility. Cells of *sprE* mutants formed completely nonspreading colonies on agar, whereas wild-type cells formed colonies with thin spreading edges (Fig. 2). Introduction of *sprE* on pNap2 restored the ability to form spreading colonies. Wild-type and *sprE* mutant cells of *F. johnsoniae* were examined for attachment to and movement on glass, as previously described (33). Wild-type cells of *F. johnsoniae* readily attached to glass (Table 1) and glided at speeds of 2 μm/s (see Movie S1 in the supplemental material). Under these conditions, greater than 95% of the cells that attached to the glass exhibited gliding movements over a 2-min period. Cells of *sprE* mutants attached poorly to glass (Table 1; see Movie S1 in the supplemental material). The few cells that did attach occasionally exhibited brief gliding movements that were similar to those exhibited by wild-type cells (see Movie S2 in the supplemental material), indicating that unlike completely nonmotile *gld* mutants, cells of *sprE* mutants retain some limited ability to move. Complementation with pNap2 restored the ability to attach to and move efficiently on glass surfaces. The attachment and motility defects exhibited by cells of *sprE* mutants were similar to those observed for cells of *sprA* mutants (33). In contrast, cells of *sprB* mutants attached well to glass (Table 1), and most cells exhibited limited movements (32).

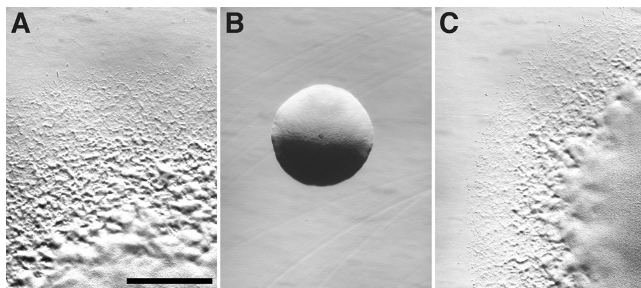

FIG. 2. *sprE* is required for the formation of spreading colonies. Colonies were grown for 48 h at 25°C on PY2 agar medium. Photomicrographs were taken with a Photometrics CoolSNAP$_{cf}{}^2$ camera mounted on an Olympus IMT-2 phase-contrast microscope. (A) Wild-type *F. johnsoniae* FJ1; (B) *sprE* mutant FJ149; (C) FJ149 complemented with pNap2. Cells in panels A and B carried control vector pCP23. Bar in panel A, 1 mm (the bar applies to all panels).

SprB is improperly localized in cells of *sprE* mutants. Components of the *F. johnsoniae* PorSS such as GldN and SprT are required for surface localization of SprB (38, 41). Since SprE is similar in sequence to the *P. gingivalis* PorSS protein PorW, we examined the effect of mutations in *sprE* on surface localization of SprB. Antibodies were used to detect SprB on wild-type cells and on cells of *sprE* mutants. Each of these strains produced SprB protein (see Fig. S1 in the supplemental material). Protein G-coated polystyrene latex spheres and antibodies against SprB were used to detect surface-exposed SprB on live cells and to observe movement of SprB protein along the cell surface (Table 2; see Movie S3 in the supplemental

TABLE 2. Effect of mutations in *sprE* on binding of protein G-coated polystyrene spheres carrying antibodies against SprB

Strain	Description	Antibody added	Avg % of cells with spheres attached[a]
FJ1 + control plasmid pCP23	Wild type	No antibody	0.3 (0.6)
FJ1 + control plasmid pCP23	Wild type	Anti-SprB	72.3 (7.6)
FJ114 + pCP23	*sprB* mutant	Anti-SprB	2.0 (1.0)
FJ149 + pCP23	*sprE* mutant	Anti-SprB	0.7 (0.6)
FJ149 + pNap2	*sprE* mutant complemented with pNap2	Anti-SprB	67.7 (3.2)

[a] Affinity-purified anti-SprB- and 0.5-μm-diameter protein G-coated polystyrene spheres were added to cells as described in Materials and Methods. Samples were spotted on a glass slide, covered with a glass coverslip, incubated for 1 min at 25°C, and examined using a phase-contrast microscope. Images were recorded for 30 s, and 100 randomly selected cells were examined for the presence of attached spheres during this period. Numbers in parentheses are standard deviations calculated from three measurements.

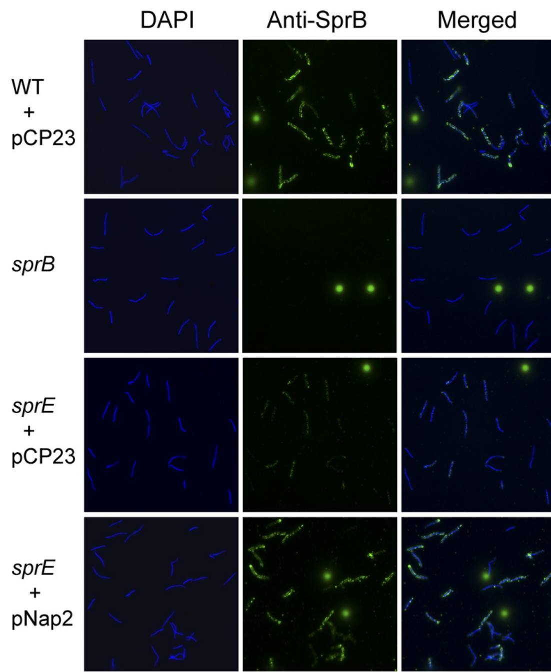

FIG. 3. Detection of surface-localized SprB protein by immunofluorescence microscopy. Cells of wild-type and mutant *F. johnsoniae* were exposed to DAPI and to anti-SprB antibodies, followed by secondary antibodies conjugated to Alexa-488. The fluorescent spheres are InSpeck relative intensity control fluorescence beads. WT, wild-type *F. johnsoniae* FJ1; *sprB*, *sprB* mutant FJ156; *sprE*, *sprE* mutant FJ149. pCP23 is a control vector; pNap2 carries *sprE*. Bar, 20 μm.

material). As previously reported (32), antibody-coated spheres attached specifically to wild-type cells expressing SprB and were rapidly propelled along the surfaces of the cells. Also as previously reported (32), such spheres failed to attach to *sprB* mutant cells, and protein G-coated spheres without antibodies failed to bind to wild-type cells. Antibody-coated spheres failed to bind to cells of the *sprE* mutants (Table 2; see Movie S3 in the supplemental material), indicating that SprB was not exposed on the surface of these cells. Complementation of the mutants with pNap2, which carries *sprE*, restored surface localization of SprB.

Analysis of surface exposure of SprB by immunofluorescence microscopy of fixed cells confirmed that *sprE* mutant cells are defective in surface localization of SprB (Fig. 3). However, small amounts of SprB were detected on cells of the *sprE* mutants by immunofluorescence microscopy. This may be the result of damage to cells during fixation and staining or could indicate that the immunofluorescence assay is more sensitive than the antibody-coated sphere assay and that the *sprE* mutant cells are only partially defective in secretion of SprB. In any case, the decreased levels of SprB and perhaps of other motility proteins on the cell surface may explain some of the motility defects exhibited by cells of *sprE* mutants.

Cells of *sprE* mutants are defective in chitin utilization. *F. johnsoniae* produces a suite of enzymes for chitin utilization, including at least one extracellular chitinase, encoded by *fjoh_4555*, and several cell-bound enzymes (28, 41). Mutations in *F. johnsoniae porSS* genes, such as *gldN* and *sprT*, result in defects in chitin utilization and defects in secretion of extracellular chitinase (38, 41). Cells of the *sprE* mutant FJ149 were deficient in chitin utilization, and complementation with pNap2 restored the ability to digest chitin to wild-type levels (see Fig. S2 in the supplemental material). The defect in chitin utilization appears to be a result of a deficiency in extracellular chitinase activity (Table 3). These results suggest that SprE is involved in secretion not only of SprB but also of extracellular chitinase by the *F. johnsoniae* PorSS.

Bacteriophage resistance of *sprE* mutants. Sensitivity to *F. johnsoniae* bacteriophages was determined as previously described (38) by spotting phage lysates onto lawns of cells. Wild-type cells were lysed by all of the bacteriophages, whereas cells of *sprE* mutants exhibited complete resistance to four of the bacteriophages (ϕCj1, ϕCj13, ϕCj23, and ϕCj29) and partial resistance to three others (ϕCj42, ϕCj48, and ϕCj54) (see Fig. S3 in the supplemental material). Introduction of *sprE* on pNap2 restored phage sensitivity. The cell surface motility protein SprB is thought to function as a receptor for ϕCj1, ϕCj13, ϕCj23, and ϕCj29 (32), and additional SprB-like motility proteins may function as receptors for other phages (38). Mutations in *sprE* result in defects in secretion of SprB and perhaps of other cell surface proteins, which may explain the phage resistance exhibited by *sprE* mutants.

Identification and localization of SprE. Recombinant SprE was produced in *E. coli*, and polyclonal antiserum was raised against it. The antiserum was used to detect SprE in cell extracts. SprE migrated with an apparent molecular mass of approximately 105 kDa, which was slightly larger than the predicted size of 98.7 kDa for the processed protein after removal of the signal peptide (Fig. 4A). SprE is a predicted lipoprotein, and the covalently attached fatty acids may account for the apparent increase in molecular mass. SprE was detected in extracts of wild-type cells but was absent from extracts of *sprE* mutants. Introduction of *sprE* on pNap2 restored production of SprE. SprE was found in the particulate (membrane) fraction of cell extracts (Fig. 4B), as expected for a lipoprotein.

DISCUSSION

Gram-negative bacteria have evolved many different machines to transport proteins across biological membranes. The *sec* and *tat*

TABLE 3. Effect of mutations in *sprE* on chitinase activity

Strain	Chitinase activity[a]	
	Extracellular	In cell extracts
FJ1 (wild type)[b]	223 (54)	241 (32)
FJ149 (*sprE* mutant)[b]	0 (0)	272 (51)
FJ149 complemented with pNap2	234 (86)	279 (50)

[a] Chitinase activities were determined using the synthetic substrate 4-MU-(GlcNAc)$_2$. Equal amounts of each sample, based on the protein content of the original cell suspension, were incubated with 10 nmol of 4-MU-(GlcNAc)$_2$ for 4 h at 37°C, and the amount of 4-MU released was determined by measuring fluorescence emission at 460 nm following excitation at 360 nm. Activities in the cell-free supernatants (extracellular chitinase) and in cell extracts are indicated as pmol 4-methylumbelliferone released per μg total protein in the original cell suspension. Numbers in parentheses are standard errors calculated from three measurements.

[b] Strains without pNap2 carried control vector pCP23 so that all cultures could be grown under identical conditions in the presence of tetracycline.

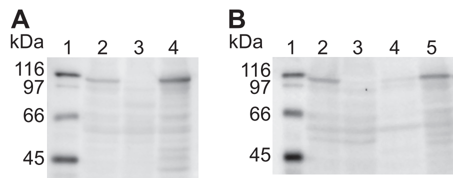

FIG. 4. Immunodetection of SprE. (A) Whole-cell extracts were examined for SprE by Western blot analysis. Lane 1, molecular mass markers; lane 2, wild-type *F. johnsoniae* FJ1 with control vector pCP23; lane 3, *sprE* mutant FJ149 with pCP23; lane 4, FJ149 complemented with pNap2. Fifty micrograms protein was loaded in each lane. (B) Cell fractions were examined for SprE by Western blot analysis. Lane 1, molecular mass markers; lane 2, whole cells of wild-type *F. johnsoniae* FJ1; lane 3, whole cells of *sprE* mutant FJ149; lane 4, wild-type *F. johnsoniae* FJ1, soluble fraction; lane 5, wild-type *F. johnsoniae* FJ1, membrane fraction. Equal amounts of each fraction based on the starting material were loaded in each lane. All strains in panel B carried empty control vector pCP23, which had no effect on expression of SprE.

systems transport proteins into or across the cytoplasmic membrane (30), and a wide variety of protein secretion systems facilitate movement of proteins across the outer membrane. These include the type I to type VI protein secretion systems, as well as systems that have not been codified with a numeric type, such as the chaperone-usher pathway (7, 10). Some of these rely on the *sec* or *tat* systems for delivery of proteins across the cytoplasmic membrane, whereas others secrete proteins across both membranes. Recently, an apparently novel protein secretion system, the PorSS, was identified in two distantly related members of the phylum *Bacteroidetes*, *F. johnsoniae* and *P. gingivalis* (41). GldK, GldL, GldM, GldN, SprA, and SprT are components of the *F. johnsoniae* PorSS. Disruption of the genes encoding any of these proteins results in defects in secretion of the cell surface motility protein SprB and of extracellular chitinase. SprF is also required for secretion of SprB, but not of chitinase (36). SprF may function as an adapter to the PorSS to specifically allow secretion of SprB. The results presented in this paper suggest that *F. johnsoniae* SprE is involved in secretion of SprB and chitinase and thus may be another component of its PorSS. *P. gingivalis* has orthologs to each of the *F. johnsoniae* PorSS proteins mentioned above, and these proteins are required for gingipain secretion (40, 41). Additional *P. gingivalis* proteins that appear to be required for gingipain secretion and that may be part of its PorSS include PorU, PG0027, and PG0534 (14, 39, 41). *F. johnsoniae* has orthologs to the genes encoding each of these proteins, but their roles in protein secretion have not been examined.

Disruption of *F. johnsoniae sprE* results in decreased attachment to glass, severe defects in gliding, an inability to digest chitin, and resistance to some bacteriophages. Each of these defects can be explained by disruption of a protein secretion system that transports the motility protein SprB, related SprB-like adhesins, and chitinase. The decreased attachment to glass suggests that cell surface adhesins in addition to SprB may be mislocalized in *sprE* mutants, since *sprB* mutants do not exhibit an attachment defect to glass (Table 1) (32). Numerous *sprB*-like genes are present in the genome, and it has recently been suggested that these may encode semiredundant cell surface adhesins involved in motility that may be secreted by the PorSS (36, 37). *sprE* mutants also display greater phage resistance than *sprB* mutants. SprB is thought to be a receptor for some phages, such as ϕCj1, ϕCj13, ϕCj23, and ϕCj29 (32). Other SprB-like proteins secreted by the PorSS may serve as receptors for additional phages. The phenotypes of *sprE* mutants are similar to those of cells with mutations in genes encoding other components of the PorSS, such as *gldK*, *gldL*, *gldM*, *gldN*, *sprA*, and *sprT*. *gldK*, *gldL*, *gldM*, and *gldN* are colocalized on the genomes of each of the bacteroidetes that have these genes. This suggests that the proteins encoded by these genes function together. Biochemical evidence for a complex formed by the *P. gingivalis* orthologs of GldK, GldL, GldM, and GldN has been presented (41). *F. johnsoniae* SprA, SprE, and SprT may interact with the predicted GldK, GldL, GldM, and GldN complex, but this has not been examined. Disruption of *gldK*, *gldL*, and *gldM* and deletion of the region spanning *gldN* and its paralog, *gldO*, each results in complete loss of motility and complete resistance to bacteriophages. In contrast, cells with mutations in *sprA*, *sprE*, and *sprT* each exhibit limited motility of individual cells in wet mounts and exhibit sensitivity to some bacteriophages. GldK, GldL, GldM, and GldN may be core components of the PorSS, whereas SprA, SprE, and SprT may be less crucial, with some limited function occurring in their absence. None of the proteins are similar to proteins of known function, so their exact roles in protein secretion are difficult to predict. SprE contains a TPR domain, and other TPR domain proteins are involved in protein-protein interactions (3). SprE may be involved in protein-protein interactions among PorSS proteins and could be necessary for formation of a stable secretion system complex.

Each of the proteins of the PorSS is predicted to reside in the cell envelope, as expected for components of a protein secretion system. GldL is predicted to be a cytoplasmic membrane protein (4), and its *P. gingivalis* paralog, PorL, has been demonstrated to localize to the cytoplasmic membrane (41). SprA and SprT are predicted integral outer membrane proteins, and biochemical evidence confirms that *F. johnsoniae* SprA is partially exposed on the surface of the outer membrane (33) and that the *P. gingivalis* SprT paralog PorT localizes to the outer membrane (34). SprE and GldK are predicted outer membrane lipoproteins. SprE localizes to the membrane fraction, as expected of a lipoprotein, as does the *P. gingivalis* GldK paralog PorK (41). A model of the PorSS complex is depicted in Fig. S4 in the supplemental material. Additional experimentation is required to determine the stoichiometry of the secretion system proteins and to elucidate the dynamic interactions between the proteins that result in secretion of SprB, chitinase, and other proteins.

Complete genome sequences are available for 40 species belonging to 29 genera of the phylum *Bacteroidetes*. *sprE* orthologs are present in 35 of the 40 species (see Table S1 in the supplemental material). Thirty-two of the 35 *sprE* ortholog-containing strains also had orthologs for *gldK*, *gldL*, *gldM*, *gldN*, *sprA*, and *sprT*, which encode components of the PorSS. SprE and its orthologs may function with the products of these genes in protein secretion. The five species that lacked *sprE* orthologs were all closely related members of the genus *Bacteroides*. These *Bacteroides* species also lacked orthologs of *sprA* and *sprT*, and four of the five lacked orthologs of *gldL*, *gldM*, and *gldN*, suggesting that absence of functional PorSSs may be characteristic of members of the genus. *sprE* orthologs were not found in organisms outside the phylum *Bacteroidetes*, although not surprisingly, predicted proteins of unknown function with more limited similarity restricted to the TPR domain

of SprE were identified. Orthologs of the other *porSS* genes were also not found in organisms outside the phylum *Bacteroidetes*. Each of the 35 bacteroidetes SprE orthologs mentioned above had a cysteine at or near the end of its hydrophobic signal peptide, which is a requirement for bacterial lipoproteins. This suggests that SprE and its orthologs are all lipoproteins and that membrane anchoring of the proteins by their lipid tails is important for function.

The PorSS of *P. gingivalis* is involved in secretion of gingipain virulence factors, and the PorSS of *F. johnsoniae* is involved in secretion of chitinase and of the cell surface gliding motility protein SprB. Further study will likely identify additional functions for PorSSs. The ease with which *F. johnsoniae* can be genetically manipulated makes it uniquely well suited to serve as a model system for studies of the mechanism of PorSS-mediated protein secretion. Given the novelty of the proteins involved, the PorSS is likely to exhibit significant differences in structure and function from other well-studied bacterial protein secretion systems.

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