Flavobacterium johnsoniae GldK, GldL, GldM, and SprA Are Required for Secretion of the Cell Surface Gliding Motility Adhesins SprB and RemA

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***Flavobacterium johnsoniae* cells move rapidly over surfaces by gliding motility. Gliding results from the movement of adhesins such as SprB and RemA along the cell surface. These adhesins are delivered to the cell surface by a *Bacteroidetes*-specific secretion system referred to as the type IX secretion system (T9SS). GldN, SprE, SprF, and SprT are involved in secretion by this system. Here we demonstrate that GldK, GldL, GldM, and SprA are each also involved in secretion. Nonpolar deletions of *gldK*, *gldL*, or *gldM* resulted in the absence of gliding motility and in T9SS defects. The mutant cells produced SprB and RemA proteins but failed to secrete them to the cell surface. The mutants were resistant to phages that use SprB or RemA as a receptor, and they failed to attach to glass, presumably because of the absence of cell surface adhesins. Deletion of *sprA* resulted in similar but slightly less dramatic phenotypes. *sprA* mutant cells failed to secrete SprB and RemA, but cells remained susceptible to some phages and retained some limited ability to glide. The phenotype of the *sprA* mutant was similar to those previously described for *sprE* and *sprT* mutants. SprA, SprE, and SprT are needed for secretion of SprB and RemA but may not be needed for secretion of other proteins targeted to the T9SS. Genetic and molecular experiments demonstrate that *gldK*, *gldL*, *gldM*, and *gldN* form an operon and suggest that the proteins encoded by these genes may interact to form part of the *F. johnsoniae* T9SS.**

Cells of *Flavobacterium johnsoniae* move rapidly over surfaces by gliding motility. *Flavobacterium* gliding motility does not involve flagella or pili but instead relies on novel machinery that appears to be confined to members of the large and diverse phylum *Bacteroidetes* (1, 2). Genetic experiments identified Gld, Spr, and Rem proteins with roles in motility. A motor comprised of some of the Gld proteins is thought to propel cell surface adhesins such as SprB and RemA. These adhesins interact with the substratum or with polysaccharides that coat the substratum, and the action of the motor on the adhesins propels the cell (3, 4).

Some of the Gld and Spr proteins form a novel protein secretion system originally referred to as the Por protein secretion system (5–7). More recently, this has been called the type IX secretion system (T9SS) (2, 8), because the known components are not similar in sequence to proteins of the previously described type I to type VIII secretion systems (9–11). T9SSs are common in members of the phylum *Bacteroidetes* but are apparently not found outside this phylum (2). Proteins secreted by T9SSs have predicted N-terminal type 1 signal peptides and are thought to rely on the Sec system for export across the cytoplasmic membrane. They also have conserved C-terminal domains (CTDs) that appear to direct them to the T9SS for secretion across the outer membrane (4, 12–15).

T9SSs have been studied in the nonmotile periodontal pathogen *Porphyromonas gingivalis* and in *F. johnsoniae*. The *P. gingivalis* T9SS is involved in secretion of gingipain proteases and other virulence factors, and the *F. johnsoniae* T9SS is needed for secretion of the cell surface motility adhesins SprB and RemA and for secretion of a chitinase. In *F. johnsoniae*, GldN, SprE, SprF, and SprT (which are related to the *P. gingivalis* T9SS proteins PorN, PorW, PorP, and PorT, respectively) have been demonstrated to have roles in secretion (5–7, 16). *F. johnsoniae* GldK, GldL, GldM, and SprA were originally identified as proteins required for gliding motility (17, 18). Studies of *P. gingivalis* revealed that homologs of

these proteins (PorK, PorL, PorM, and Sov, respectively) are required for gingipain secretion (7, 19). *F. johnsoniae* GldK, GldL, GldM, and SprA were assumed to also be involved in protein secretion (2), but the evidence supporting this had not been presented. Here we demonstrate that *F. johnsoniae* GldK, GldL, GldM, and SprA are each required for secretion of SprB and RemA and for utilization of chitin. *gldK*, *gldL*, *gldM*, and *gldN* form an operon, and the products of these genes each localize to the cell envelope, where they may interact to form a complex involved in protein secretion.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *F. johnsoniae* ATCC 17061 strain UW101 was the wild-type strain used in this study. The streptomycin-resistant *rpsL* mutant of UW101 (CJ1827) was used to construct strains with unmarked deletions (20). *F. johnsoniae* strains were grown in Casitone-yeast extract (CYE) medium (21) at 25°C. To observe colony spreading, *F. johnsoniae* cells were grown at 25°C on PY2 medium supplemented with 10 g of agar per liter (22). Motility medium (MM) was used to observe movement of individual cells in wet mounts (23). Strains and plasmids used in this study are listed in Table 1, and primers used in this study are listed in Table S1 in the supplemental material. Sites of transposon insertions in *gldK*, *gldL*, *gldM*, and *gldN* are illustrated in Fig. 1, as are the regions carried on plasmids used for complementation experiments. The plasmids used for complementation were all derived from

TABLE 1 Strains and plasmids used in this study

F. johnsoniae strain or plasmid	Genotype and/or description[a]	Reference(s) or source
Strains		
UW101 (ATCC 17061)	Wild type	38, 39
UW102-57	Spontaneous *gldK* mutant	17, 26
UW102-141	Nitrosoguanidine-induced *gldK* mutant	17, 26
CJ1372	*gldK::HimarEm1* (Em[r])	17
CJ1373	*gldK::HimarEm1* (Em[r])	17
CJ1827	*rpsL2* (Sm[r]); wild-type strain used in construction of deletion mutants	20
CJ1922	*rpsL2 ΔsprB* (Sm[r])	20
CJ1984	*rpsL2 ΔremA* (Sm[r])	4
CJ1985	*rpsL2 ΔsprB ΔremA* (Sm[r])	4
CJ2083	*rpsL2 remA::myc-tag-1* (Sm[r])	4
CJ2090	*rpsL2 Δ(gldN gldO)* (Sm[r])	4
CJ2122	*rpsL2 ΔgldK* (Sm[r])	This study
CJ2140	*rpsL2 ΔgldK* in *remA::myc-tag-1* background (Sm[r])	This study
CJ2157	*rpsL2 ΔgldL* (Sm[r])	This study
CJ2262	*rpsL2 ΔgldM* (Sm[r])	This study
CJ2263	*rpsL2 ΔgldM* in *remA::myc-tag-1* background (Sm[r])	This study
CJ2281	*rpsL2 ΔgldL* in *remA::myc-tag-1* background (Sm[r])	This study
CJ2089	*rpsL2 Δ(gldN gldO) remA::myc-tag-1* (Sm[r])	4
CJ2302	*rpsL2 ΔsprA* (Sm[r])	This study
CJ2317	*rpsL2 ΔsprA* in *remA::myc-tag-1* background (Sm[r])	This study
CJ2327	*sprT* disruption in *remA::myc-tag-1* background (Em[r] Sm[r])	This study
KDF001	*sprT* mutant (Em[r])	7
FJ149	*sprE* mutant (Em[r])	6
Plasmids		
pET30a	Protein expression vector; Km[r]	Novagen
pCP23	*E. coli-F. johnsoniae* shuttle plasmid; Ap[r] (Tc[r])	22
pCP29	*E. coli-F. johnsoniae* shuttle plasmid; Ap[r] (Cf[r] Em[r])	24
pRR51	*rpsL*-containing suicide vector; Ap[r] (Em[r])	20
pJVB4	1.3-kbp fragment of *gldK* amplified with primer pair 704/705 and ligated into EcoRI-, SalI-digested pET30a; Km[r]	This study
pJVB2	0.6-kbp fragment of *gldL* amplified with primer pair 706/707 and ligated into EcoRI-, SalI-digested pET30a; Km[r]	This study
pJVB6	1.3-kbp fragment of *gldM* amplified with primer pair 708/709 and ligated into EcoRI-, SalI-digested pET30a; Km[r]	This study
pAB18	2.4-kbp region downstream of *gldL* amplified with primers 1199 and 1200 and ligated into XbaI-, SalI-digested pRR51; Ap[r] (Em[r])	This study
pAB19	Construct used to delete *gldL*; 2.2-kbp region upstream of *gldL* amplified with primer pair 1197/1198 and ligated into BamHI-, XbaI-digested pAB18; Ap[r] (Em[r])	This study
pAB30	Construct used to delete *sprA*; 2.5 kbp upstream and 2.5 kbp downstream of *sprA* amplified using primer pairs 1333/1334 and 1335/1336, respectively, and ligated into BamHI-, SalI-digested pRR51; Ap[r] (Em[r])	This study
pJJ01	Construct used to delete *gldK*; 1.9-kbp region upstream and 1.9-kbp region downstream of *gldK* amplified using primer pairs 1209/1210 and 1211/1212, respectively, and ligated into BamHI-, SphI-digested pRR51; Ap[r] (Em[r])	This study
pJJ02	Construct used to delete *gldM*; 2.6-kbp region upstream and 2.4-kbp region downstream of *gldM* amplified using primer pairs 1237/1214 and 1215/1238, respectively, and ligated into BamHI-, SphI-digested pRR51; Ap[r] (Em[r])	This study
pTB99	pCP23 carrying *gldK*; Ap[r] (Tc[r])	17
pTB81a	pCP23 carrying *gldL*; Ap[r] (Tc[r])	17
pTB94a	pCP23 carrying *gldM*; Ap[r] (Tc[r])	17
pTB79	pCP23 carrying *gldN*; Ap[r] (Tc[r])	17
pKF002	pCP23 carrying *sprT*; Ap[r] (Tc[r])	7
pSN48	pCP23 carrying *sprA*; Ap[r] (Tc[r])	18

[a] Antibiotic resistance phenotypes are as follows: ampicillin, Ap[r]; cefoxitin, Cf[r]; erythromycin, Em[r]; streptomycin, Sm[r]; tetracycline, Tc[r]. Unless indicated otherwise, the antibiotic resistance phenotypes are those expressed in *E. coli*. The antibiotic resistance phenotypes given in parentheses are those expressed in *F. johnsoniae* but not in *E. coli*.

pCP1 and have copy numbers of approximately 10 in *F. johnsoniae* (21, 22, 24). Antibiotics were used at the following concentrations when needed: ampicillin at 100 μg/ml, cefoxitin at 100 μg/ml, erythromycin at 100 μg/ml, streptomycin at 100 μg/ml, and tetracycline at 20 μg/ml.

Deletion of *gldK, gldL, gldM,* and *sprA*. Unmarked deletions were made as previously described (20). To make the *gldL* deletion, a 2.4-kbp

fragment downstream of *gldL* was amplified by PCR using Phusion DNA polymerase (New England BioLabs, Ipswich, MA) and primers 1199 (introducing an XbaI site) and 1200 (introducing a SalI site). The fragment was digested with XbaI and SalI and ligated into pRR51 that had been digested with the same enzymes, to generate pAB18. A 2.2-kbp fragment spanning the upstream region of *gldL* was amplified by PCR with primers
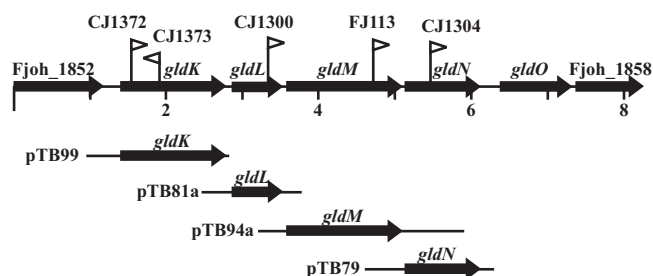
FIG 1 Map of the *gldKLMN* region. Numbers below the map refer to kilobase pairs of sequence. The sites of *HimarEm* insertions are indicated by triangles. Orientations of *HimarEm* insertions are indicated by the direction in which the triangles are pointing, with triangles pointing to the right having inverted repeat 2 (IR2) on the right side. The regions of DNA carried by plasmids used in this study are indicated beneath the map.

1197 (introducing a BamHI site) and 1198 (introducing an XbaI site). The fragment was digested with BamHI and XbaI and fused to the region downstream of *gldL* by ligation with pAB18 that had been digested with the same enzymes, to generate the deletion construct pAB19. Plasmid pAB19 was introduced into streptomycin-resistant wild-type *F. johnsoniae* strain CJ1827 and *remA*::*myc-tag-1* strain CJ2083 (4) by triparental conjugation, and *gldL* deletion mutants were isolated as previously described (20). Deletion of *gldL* was confirmed by PCR amplification using primers 690 and 707, which flank the *gldL* coding sequence. *gldK*, *gldM*, and *sprA* were deleted in wild-type CJ1827 and in *remA*::*myc-tag-1* strain CJ2083 by the same approach, using the primers listed in Table S1 in the supplemental material and the plasmids listed in Table 1.

Transduction of the *sprT* mutation into CJ2083 (*rpsL2 remA*::*myc-tag-1*). The *sprT* mutation in strain KDF001 was transduced into CJ2083 (*rpsL2 remA*::*myc-tag-1*) essentially as previously described (5), using phage φCj54 and selecting for erythromycin resistance.

RT-PCR to characterize the operon comprised of *gldK*, *gldL*, *gldM*, and *gldN*. RNA was extracted from wild-type *F. johnsoniae* UW101 cells as described previously (16). Briefly, wild-type cells were grown in 25 ml of MM overnight at 25°C without shaking. Cells were harvested by centrifugation at 4,000 × *g* for 10 min, and the pellet was suspended in 450 μl of MM. RNAProtect (Qiagen, Valencia, CA) was added, and pellet was collected as described previously (16). RNA was extracted and purified by using the RNeasy minikit (Qiagen) according to the manufacturer's instructions, except that RNasin (Promega Corp., Madison, WI) and RQ1 RNase-free DNase (Promega) were used as described previously (16). RNA concentrations (optical density at 260 nm [OD$_{260}$]) and purity (OD$_{260/280}$) were determined by UV spectroscopy, and RNA integrity was verified by visualizing the intensity of the 16S and 23S rRNA bands on a 1% agarose gel.

Reverse transcriptase PCR (RT-PCR) was used to determine the transcriptional organization of *gldK*, *gldL*, *gldM*, and *gldN*. cDNA was generated by using the SuperScript first-strand synthesis kit for RT-PCR (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instruction. RNA was reverse transcribed by using gene-specific primer 695 essentially as described previously (16). For PCR, antisense primers used to generate the cDNA were used in various combinations with sense primers.

Expression of recombinant GldK, GldL, and GldM for antibody production. A 1,308-bp fragment of *gldK* was amplified by using Phusion DNA polymerase and primers 705 (introducing a SalI site) and 704 (introducing an EcoRI site). The product was digested with EcoRI and SalI and inserted into pET30a (Novagen, Madison, WI) that had been digested with the same enzymes, to generate pJVB4. A 607-bp fragment of *gldL* was amplified by using primers 707 (introducing a SalI site) and 706 (introducing an EcoRI site), and the product was digested and inserted into pET30a as described above, generating pJVB2. A 1,311-bp fragment of

gldM was amplified by using primer 709 (introducing a SalI site) and primer 708. The PCR product was digested with SalI and EcoRI (which cuts within *gldM*) and inserted into pET30a as described above, generating pJVB6. pJVB4, pJVB2, and pJVB6 encode proteins with amino-terminal His-tagged peptides fused to the C-terminal 434, 174, and 426 amino acids of GldK, GldL, and GldM, respectively.

pJVB4, pJVB2, and pJVB6 were introduced into *Escherichia coli* Rosetta2(DE3) (Novagen), which produces seven rare tRNAs required for efficient expression of *F. johnsoniae* proteins in *E. coli* (3, 18). Expression was induced by addition of 1.0 mM IPTG (isopropyl-β-ᴅ-thiogalactopyranoside) and incubation for 3 h at 37°C. Cells were collected by centrifugation and disrupted by using a French pressure cell, and recombinant proteins were purified by Ni affinity chromatography. Polyclonal antibodies against recombinant GldK, GldL, and GldM were produced and affinity purified using the recombinant proteins by Proteintech Group, Inc. (Chicago, IL). Antibodies were also raised against recombinant GldN, as previously described (5).

Detection and localization of GldK, GldL, and GldM. Wild-type and mutant cells of *F. johnsoniae* were grown to mid-log phase in CYE medium at 25°C. Cells were washed twice in sterile distilled water by centrifugation at 4,000 × *g* and were lysed by incubation for 5 min at 100°C in SDS-PAGE loading buffer. Proteins (15 μg per lane) were separated by SDS-PAGE, and Western blotting was performed as described previously (5). To determine protein localization, wild-type cells were grown to mid-log phase in MM at 25°C with shaking. The cells were washed twice with sterile distilled water, and EDTA-free Halt protease inhibitor cocktail was added (Thermo Fisher Scientific, Rockford, IL). The cells were disrupted by using a French pressure cell and fractionated into soluble inner membrane (Sarkosyl-soluble) and outer membrane (Sarkosyl-insoluble) fractions essentially as described previously (25). Proteins were separated by SDS-PAGE, equal amounts of each fraction based on the starting material were loaded per lane, and Western blotting was performed as described previously (5).

Analysis of wild-type and mutant cells for production and secretion of SprB and Myc-tagged RemA. Cells were grown to mid-exponential phase in CYE medium at 25°C. Production of SprB and Myc-tagged RemA was determined by Western blot analyses using antisera against SprB and RemA, respectively, as previously described (4, 5). Secretion of SprB was examined essentially as previously described (6). Briefly, cells were grown overnight in MM at 25°C. Purified anti-SprB (1 μl of a 1:10 dilution of a 300-mg/liter stock), 0.5-μm-diameter protein G-coated polystyrene spheres (1 μl of a 0.1% stock preparation; Spherotech, Inc., Libertyville, IL), and bovine serum albumin (BSA) (1 μl of a 1% solution) were added to 7 μl of cells (approximately 5 × 10⁸ cells per ml) in MM. The cells were introduced into a tunnel slide (4) and examined by phase-contrast microscopy at 25°C. Samples were examined 2 min after spotting, and images were recorded for 30 s to determine the percentage of cells that had anti-SprB-coated spheres attached to them. The major advantage of this method is that proteins are detected on the surface of live cells that have not been chemically or enzymatically altered. Surface-localized Myc-tagged RemA was detected similarly, except that antisera against the Myc tag (EQKLISEEDL; AbCam, Cambridge, MA) was used instead of anti-SprB (4).

Microscopic observations of cell movement. Wild-type and mutant cells were examined for movement over glass by phase-contrast microscopy. Cells were grown overnight in MM at 25°C without shaking, as previously described (23). Simple tunnel slides were constructed by using double-stick tape, glass microscope slides, and glass coverslips, as previously described (4). Cells in MM were introduced into the tunnel slides, incubated for 5 min, and observed for motility by using an Olympus BH2 phase-contrast microscope with a heated stage at 25°C. Images were recorded with a Photometrics CoolSNAP$_{cf}$² camera and analyzed by using MetaMorph software (Molecular Devices, Downingtown, PA).

Measurements of bacteriophage sensitivity. The bacteriophages active against *F. johnsoniae* that were used in this study were φCj1, φCj13,

φCj23, φCj28, φCj29, φCj42, φCj48, and φCj54 (26–28). Sensitivity to bacteriophages was determined essentially as previously described, by spotting 5 μl of phage lysates (10^9 PFU/ml) onto lawns of cells in CYE overlay agar (5). The plates were incubated for 24 h at 25°C to observe lysis.

Measurement of chitin utilization. The ability of *F. johnsoniae* to utilize chitin was assayed as described previously (5). *F. johnsoniae* cells were grown overnight in MM without shaking at 25°C. Two microliters of cells was spotted onto MYA-chitin containing appropriate antibiotics, and plates were incubated for 2.5 days.

Microscopic observations of cell attachment. Wild-type and mutant cells of *F. johnsoniae* were examined for attachment to glass by using a Petroff-Hausser counting chamber as previously described, with slight modifications (5). Cells were grown overnight in MM without shaking at 25°C. The culture was diluted with an equal volume of fresh MM and incubated at 25°C without shaking until a cell density equivalent to an OD_{600} of 0.4 was reached. 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 9 randomly selected 0.03-mm^2 regions of the glass coverslip was determined.

RESULTS

***gldK*, *gldL*, *gldM*, and *gldN* form an operon.** Previous results indicated that *gldL*, *gldM*, and *gldN* are cotranscribed (17). The situation regarding *gldK*, which lies upstream of *gldL*, was less clear. It was suggested that *gldK* was transcribed separately, but the *gldK* transcript was not detected by Northern blot analysis, leaving the operon structure uncertain. Complementation analyses used to determine operon structure were also inconclusive, because moderate overexpression of *gldK* and *gldL* together in wild-type cells resulted in dramatic decreases in growth and motility, complicating analysis of polar effects of mutations (17). To understand the importance of GldK, GldL, and GldM to gliding motility and protein secretion, we revisited the transcriptional organization of these genes.

The ability of pTB99, which carries *gldK*, to complement various *gldK* mutants was examined. Two of the mutants (UW102-57 and UW102-141) had single-base substitutions in *gldK*, whereas the other two (CJ1372 and CJ1373) had *HimarEm* transposon insertions in *gldK*. The sites of the mutations in UW102-57 and UW102-141 were determined by amplification and sequencing. UW102-57 had an "A"-to-"T" substitution at position 1054, numbered from the A of the *gldK* start codon, and UW102-141 had a G-to-A substitution at position 1158, as previously reported (17). The substitutions converted the codons for R_{352} and W_{386}, respectively, to stop codons. pTB99 restored motility to UW102-57 and UW102-141 but failed to restore motility to either of the *gldK HimarEm* mutants (Fig. 2). These results suggested that the base substitution mutations resulted in nonpolar mutations, whereas the *HimarEm* insertions may have exhibited polar effects on downstream genes.

To explore the issue of polarity more directly, we examined the levels of GldK, GldL, GldM, and GldN proteins in *HimarEm*-induced GldK mutants. GldK, GldL, and GldM were overexpressed in *E. coli*, and antisera were raised against each protein. These proteins were each detected in cells of wild-type *F. johnsoniae*, but they were absent from appropriate *gld* deletion mutants (Fig. 3). Western blot analysis revealed that strains CJ1372 and CJ1373, which have transposon insertions in *gldK*, not only lacked GldK protein but also had little if any GldL, GldM, and GldN proteins (Fig. 3 and data not shown). Similarly, strains with transposon insertions in *gldL* produced little GldM or GldN, and strains with
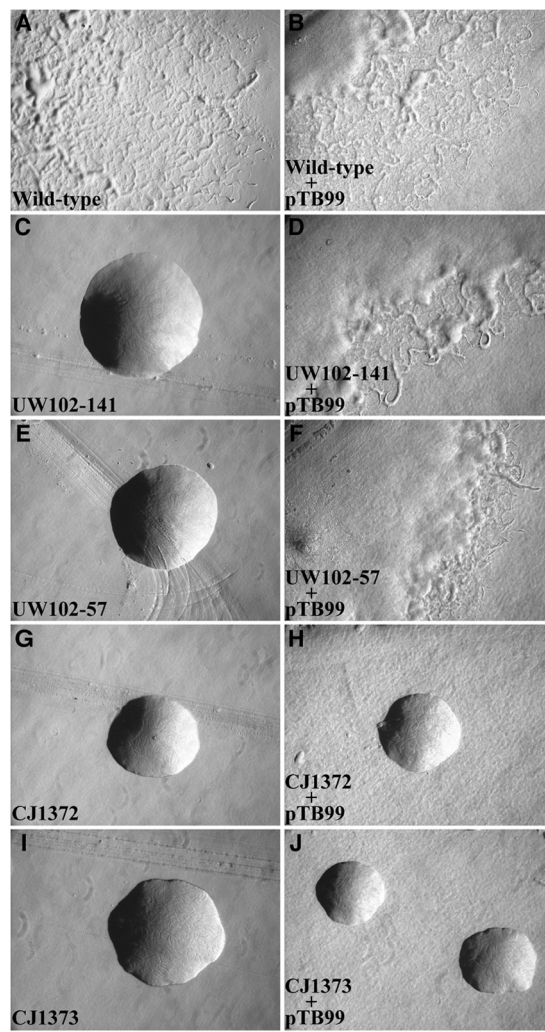

FIG 2 Photomicrographs of *F. johnsoniae* colonies. Colonies were incubated at 25°C on PY2 agar for 36 h. Photomicrographs were taken with a Photometrics Cool-SNAP$_{cf}$^{2} camera mounted on an Olympus IMT-2 phase-contrast microscope. (A) Wild-type *F. johnsoniae* UW101. (B) *F. johnsoniae* UW101 with pTB99, which carries *gldK*. (C) *gldK* point mutant strain UW102-141. (D) UW102-141 complemented with pTB99. (E) *gldK* point mutant strain UW102-57. (F) UW102-57 complemented with pTB99. (G) *gldK Himar* mutant strain CJ1372. (H) CJ1372 with pTB99. (I) *gldK Himar* mutant strain CJ1373. (J) CJ1373 with pTB99. The bar indicates 1 mm and applies to all panels.

transposon insertions in *gldM* were deficient in GldN (data not shown). The results provide additional evidence of polarity of the *gldK* mutations and suggest that *gldK*, *gldL*, *gldM*, and *gldN* may be cotranscribed.

We recently developed a method to generate in-frame deletions of *F. johnsoniae* genes (20). By using this method, strains with deletions of *gldK*, *gldL*, and *gldM* were generated. In contrast to the transposon mutants described above, CJ2122 (Δ*gldK*) produced GldL, GldM, and GldN, indicating that the mutation was nonpolar and that the GldK protein was not required for production of GldL, GldM, and GldN (Fig. 3). The *gldK* deletion mutant produced wild-type levels of GldL and GldM (Fig. 3B and C) but appeared to produce less GldN (Fig. 3D). Complementation of the *gldK* deletion mutant with pTB99, which carries *gldK*, restored
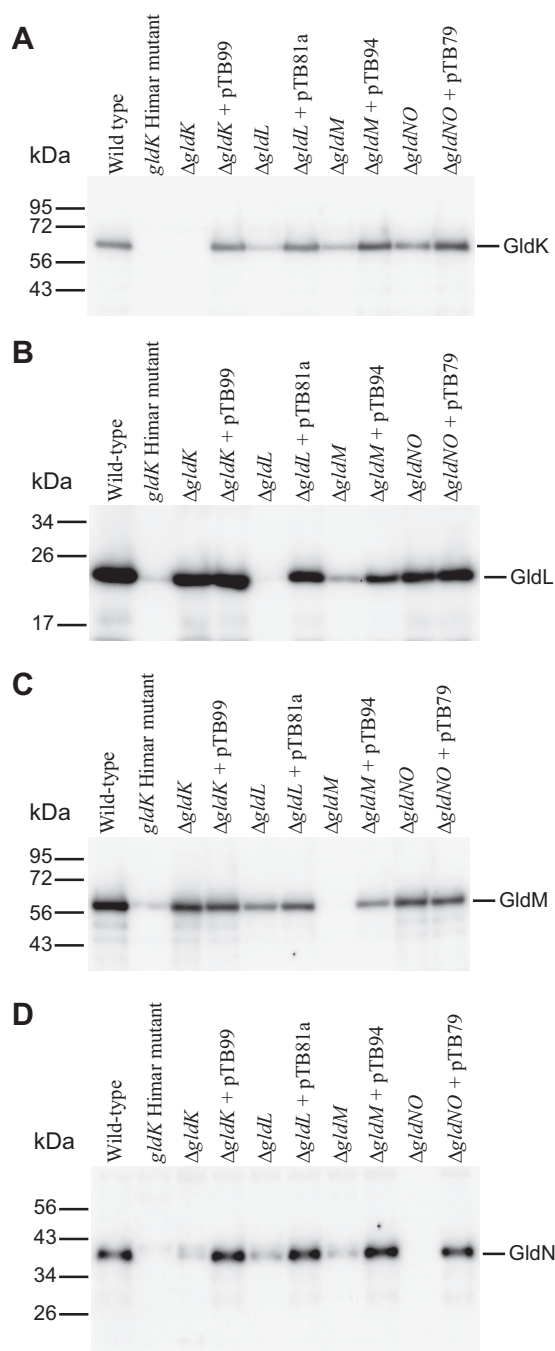
FIG 3 Western blot immunodetection of GldK (A), GldL (B), GldM (C), and GldN (D) in whole-cell extracts of wild-type, mutant, and complemented strains of *F. johnsoniae*. The wild-type strain was CJ1827 (*rpsL2*), the *gldK Himar* mutant was CJ1372, and the strains with deletions in *gldK*, *gldL*, *gldM*, and *gldNO* were CJ2122, CJ2157, CJ2262, and CJ2090, respectively. pTB99, pTB81a, pTB94a, and pTB79 express GldK, GldL, GldM, and GldN, respectively, and thus complement the indicated mutants. Fifteen micrograms of protein was loaded into each lane.
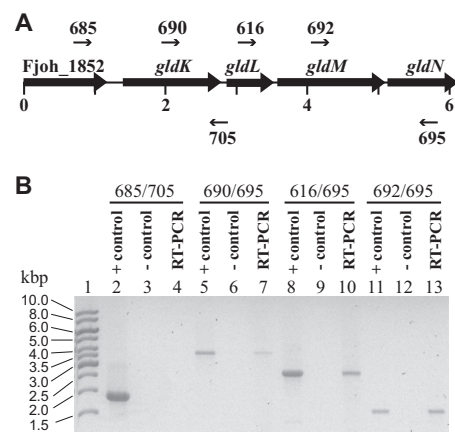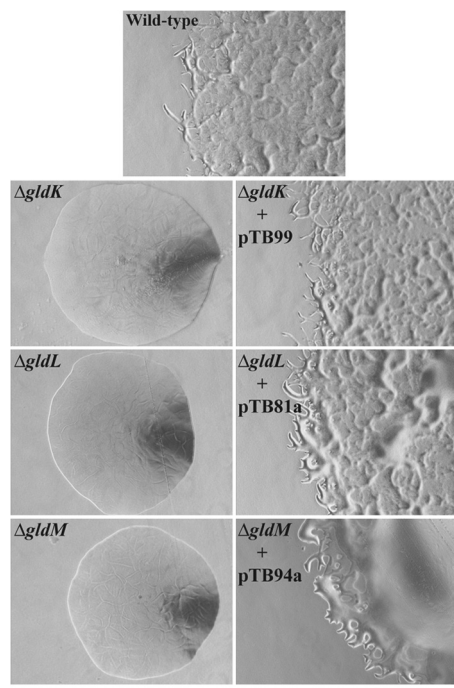

FIG 4 RT-PCR analysis of the *gldKLMN* operon. (A) Map of the *gldKLMN* operon with primers used for RT-PCR (numbered arrows). (B) RT-PCR of wild-type *F. johnsoniae* UW101 RNA. Reverse transcription was carried out with primer 695, which is complementary to a sequence internal to *gldN*. PCR was conducted by using the primer pairs shown. For each primer pair, three reaction mixtures were loaded onto the gel: a positive-control PCR mixture with *F. johnsoniae* chromosomal DNA as the template (lanes 2, 5, 8, and 11), a no-RT control reaction mixture (lanes 3, 6, 9, and 12), and an RT-PCR mixture (lanes 4, 7, 10, and 13). Lane 1, DNA ladder as a size standard; lanes 2 to 4, primer pair 685/705; lanes 5 to 7, primer pair 690/695; lanes 8 to 10, primer pair 616/695; lanes 11 to 13, primer pair 692/695.

produce GldM protein but produced GldN (Fig. 3C and D). The strains with deletions in *gldL* or *gldM* were similar to strains with *gldK* deletions in that they appeared to have low levels of GldN protein. One possible explanation for these results is that GldK, GldL, GldM, and GldN may form a complex, and GldN may be unstable in the absence of any of its partners. This may also explain the low levels of GldL observed in CJ2262 (Δ*gldM*) cells (Fig. 3B) and the reduced levels of GldK protein in strains with mutations in *gldL*, *gldM*, and *gldN* (Fig. 3A).

The results presented above suggest that *gldK*, *gldL*, *gldM*, and *gldN* form an operon. RT-PCR analysis confirmed the presence of a transcript spanning *gldK*, *gldL*, *gldM*, and *gldN* (Fig. 4). A predicted *Flavobacterium* consensus promoter sequence (TANN TTTG [29]) was identified 97 bp upstream of the *gldK* start codon, and 5′ rapid amplification of cDNA ends (RACE) analysis identified the start site of transcription 5 bp downstream of the final G of this promoter sequence (17). An inverted repeat (<u>AAAAAACTC</u> <u>TTACTACC</u>TTTG<u>TAGTAAGAGTTTTTT</u>), which may function as a transcription terminator, was present 22 bp downstream of the *gldN* stop codon. Genetic and molecular evidence previously demonstrated that *gldO*, which lies downstream of the *gldKLMN* operon and which is partially redundant with *gldN*, is transcribed independently. Transposon insertions in *gldN* were not polar on *gldO*, and the *gldN* mutants produced GldO protein (5).
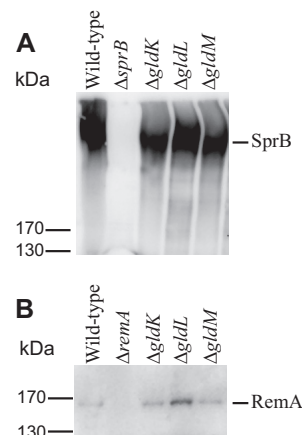
GldK, GldL, and GldM are each required for gliding. As previously reported, transposon insertions in *gldK*, *gldL*, and *gldM* result in a complete loss of motility (17). Given the concerns regarding polarity of these mutations, we analyzed the phenotypes of the in-frame deletions to determine the roles of the individual proteins. Cells with deletion mutations in *gldK*, *gldL*, or *gldM* were completely nonmotile. They formed nonspreading colonies on agar media (Fig. 5) and exhibited no cell movements in wet mounts on glass (see Movies S1 to S3 in the supplemental mate-

GldK and GldN to wild-type levels. Restoration of GldN levels by expression of GldK argues against a polar effect of the *gldK* deletion mutation on expression of *gldN*. Analysis of CJ2157 (Δ*gldL*) and CJ2262 (Δ*gldM*) gave similar results. CJ2157 failed to produce GldL protein but produced GldM and GldN, and CJ2262 failed to

FIG 5 *gldK*, *gldL*, and *gldM* are each required for formation of spreading colonies. Colonies were grown for 36 h at 25°C on PY2 agar medium. Photomicrographs were taken with a Photometrics Cool-SNAP$_{cf}^2$ camera mounted on an Olympus IMT-2 phase-contrast microscope. The wild-type strain was CJ1827. CJ1827, CJ2122 (Δ*gldK*), CJ2157 (Δ*gldL*), and CJ2262 (Δ*gldM*) carried control vector pCP23 so that all strains were resistant to tetracycline and could be grown on identical media. pTB99, pTB81a, and pTB94a express GldK, GldL, and GldM, respectively. The bar indicates 0.5 mm and applies to all panels.

FIG 6 Immunodetection of SprB and RemA in cells of wild-type and mutant *F. johnsoniae* strains. (A) Cell extracts (15 µg of protein) of wild-type (CJ1827), Δ*sprB* (CJ1922), Δ*gldK* (CJ2122), Δ*gldL* (CJ2157), and Δ*gldM* (CJ2262) strains were examined by Western blotting using antiserum against SprB. (B) Cell extracts (15 µg of protein) of wild-type (CJ2083), Δ*remA* (CJ1984), Δ*gldK* (CJ2140), Δ*gldL* (CJ2281), and Δ*gldM* (CJ2263) strains were examined by Western blotting using antiserum against RemA. All strains in panel B except the Δ*remA* strain (CJ1984) were derived from CJ2083 and thus expressed the Myc-tagged version of RemA.

rial). Cells of the deletion mutants were also examined by time-lapse microscopy for possible slow or intermittent movements on glass or agar using methods previously described (2), but no movements were observed. In each case, complementation with the appropriate gene restored motility. Complementation of the *gldM* deletion mutant was incomplete, perhaps because expression of *gldM* from a plasmid did not result in optimal GldM levels or did not allow optimal formation of a complex with the other Gld proteins. The results indicate that GldK, GldL, and GldM are each essential for gliding motility. Previous experiments demonstrated that the presence of GldN, or its paralog GldO, is also required for gliding (5).

GldK, GldL, and GldM are each required for secretion of the cell surface motility adhesins SprB and RemA. Components of the *F. johnsoniae* T9SS, such as GldN, SprE, SprF, and SprT, are required for surface localization of SprB (5–7, 16). GldN is also required for secretion of the mobile cell surface motility adhesin RemA (4, 5). Here we examined the roles of GldK, GldL, and GldM in secretion of SprB and of recombinant RemA that carried a Myc tag to allow easy detection. Cells with deletion mutations in *gldK*, *gldL*, or *gldM* produced SprB and Myc-tagged RemA proteins, as determined by Western blot analyses (Fig. 6), but they failed to secrete these proteins to the cell surface (Tables 2 and 3). Anti-SprB-coated polystyrene spheres attached readily to wild-type cells but failed to attach to cells of an *sprB* deletion mutant or to cells that had deletions in *gldK*, *gldL*, or *gldM* (Table 2). Com-

plementation with a plasmid expressing the appropriate gene in each case restored SprB to the cell surface. Similarly, anti-Myc-coated polystyrene spheres attached readily to CJ2083 cells, which express Myc-tagged RemA, but they failed to attach to cells with mutations in *gldK*, *gldL*, or *gldM* (Table 3).

Cells of *gldK*, *gldL*, and *gldM* mutants were also resistant to infection by bacteriophages that infect wild-type cells (Fig. 7). SprB is thought to be a receptor for phages φCj1, φCj13, φCj23, and φCj29 (3), and RemA appears to be a receptor for phages φCj42, φCj48, and φCj54 (4), so it is not surprising that mutants with defects in secretion of SprB, RemA, and perhaps additional cell surface motility proteins exhibit phage resistance.

Cells of the deletion mutants were also deficient in attachment to glass (Table 4), as has previously been observed for other motility mutants of *F. johnsoniae* (18). This may be the result of the absence of motility adhesins such as SprB and RemA on the cell surface. However, the lack of SprB and RemA on the cell surface cannot explain the entire attachment defect. Cells of an *sprB* mutant or of a *remA* mutant attached to glass almost as well as wild-type cells, and cells lacking both genes were only partially deficient in attachment to glass (Table 4). In contrast, cells with mutations in *gldK*, *gldL*, or *gldM* were completely deficient in attachment to glass. This suggests that cell surface adhesins in addition to SprB and RemA are secreted by the T9SS. Proteins secreted by T9SSs typically have conserved CTDs belonging to the TIGRFAMs TIGR04131 and TIGR04183. Analysis of the *F. johnsoniae* genome revealed 53 proteins with these T9SS CTDs (see Table S2 in the supplemental material). Some of these may be the additional cell surface adhesins that contribute to the ability of wild-type cells to attach to glass.

GldK, GldL, and GldM are each required for chitin utilization. *F. johnsoniae* was originally identified as a chitin-digesting bacterium (30). It produces a variety of proteins involved in chitin utilization, including at least one extracellular chitinase that we refer to as ChiA, encoded by Fjoh_4555. Mutations in the T9SS

TABLE 2 Binding of protein G-coated polystyrene spheres carrying anti-SprB antibodies

Strain	Description	Antibody added	Avg % of cells with spheres attached (SD)[a]
CJ1827/pCP23	Wild type with control plasmid pCP23	No antibody	0.0 (0.0)
CJ1827/pCP23	Wild type with control plasmid pCP23	Anti-SprB	53.7 (3.1)
CJ1922/pCP23	Δ*sprB* with control plasmid pCP23	Anti-SprB	1.0 (1.0)
CJ2122/pCP23	Δ*gldK* with control plasmid pCP23	Anti-SprB	0.3 (0.6)
CJ2122/pTB99	Δ*gldK* with pTB99 carrying *gldK*	Anti-SprB	54.0 (2.0)
CJ2157/pCP23	Δ*gldL* with control plasmid pCP23	Anti-SprB	0.3 (0.6)
CJ2157/pTB81a	Δ*gldL* with pTB81a carrying *gldL*	Anti-SprB	54.3 (3.5)
CJ2262/pCP23	Δ*gldM* with control plasmid pCP23	Anti-SprB	0.0 (0.0)
CJ2262/pTB94a	Δ*gldM* with pTB94a carrying *gldM*	Anti-SprB	56.0 (2.0)
CJ2302/pCP23	Δ*sprA* with control plasmid pCP23	Anti-SprB	0.3 (0.6)
CJ2302/pSN48	Δ*sprA* with pSN48 carrying *sprA*	Anti-SprB	51.3 (4.0)

[a] Purified anti-SprB antiserum and 0.5-μm-diameter protein G-coated polystyrene spheres were added to cells as described in Materials and Methods. Samples were introduced into a tunnel slide, incubated for 2 min at 25°C, and examined by using a phase-contrast microscope. Images were recorded for 30 s, and 100 randomly selected cells were examined for the presence of spheres that remained attached to the cells during this time. Numbers in parentheses are standard deviations calculated from three measurements.

genes *gldN*, *sprE*, and *sprT* result in defects in chitin utilization and defects in secretion of ChiA (5–7). Cells with deletion mutations in *gldK*, *gldL*, and *gldM* were examined for chitin utilization (Fig. 8). Deletion of any of these genes resulted in an inability to digest colloidal chitin, indicating that GldK, GldL, and GldM are each required for chitin digestion and suggesting that they may be required for secretion of ChiA. In each case, complementation with plasmids carrying the appropriate genes restored the ability to digest chitin, although chitin utilization was only partially restored by complementation of the *gldM* mutant.

Localization of GldK, GldL, and GldM. GldK, GldL, and GldM were each present primarily in the insoluble (membrane) fraction of cell extracts (Fig. 9). GldK is a predicted outer membrane lipoprotein, and GldL and GldM have hydrophobic alpha-helical segments that are predicted to anchor them to the cytoplasmic membrane (17). The mild detergent Sarkosyl solubilizes the *F. johnsoniae* cytoplasmic membrane while leaving the outer membrane intact (25). Sarkosyl solubilization was used to examine protein localization. GldL and GldM were enriched in the Sarkosyl-soluble (cytoplasmic membrane) fraction as predicted, whereas GldK was present in both the Sarkosyl-soluble (cytoplas-
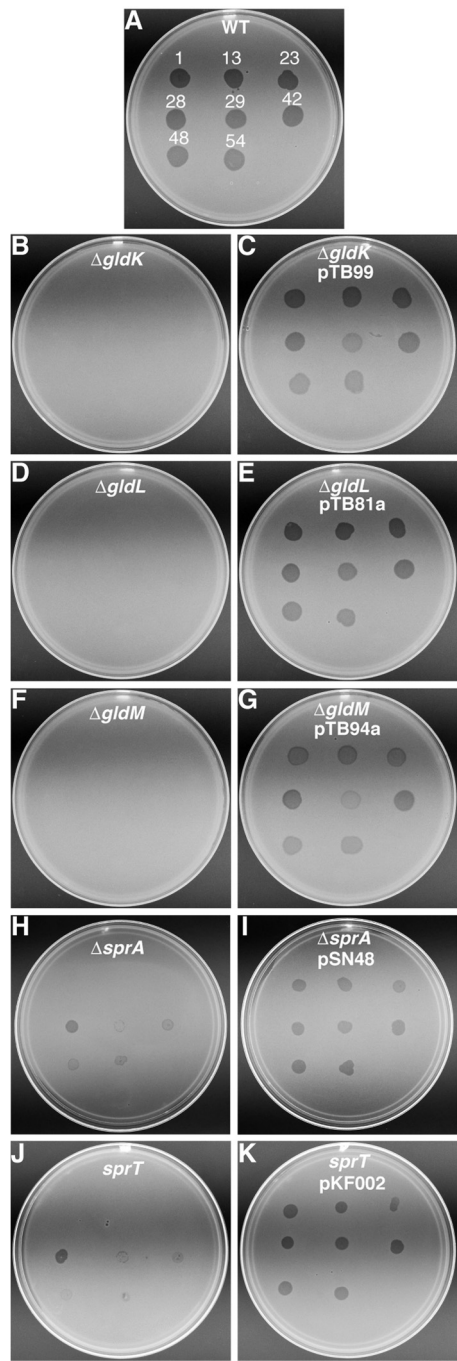
mic membrane) and Sarkosyl-insoluble (outer membrane) fractions. While integral outer membrane proteins of *F. johnsoniae* are generally not solubilized by Sarkosyl, lipoproteins such as GldK might have a more tenuous connection to the membrane and thus might be partially solubilized by this treatment. The absence of GldL and GldM in the outer membrane fraction suggests that the cytoplasmic membrane was completely solubilized by the detergent, and the enrichment of GldK in the outer membrane fraction suggests that GldK may be associated primarily with the outer membrane.

SprA is required for secretion of SprB and RemA and for utilization of chitin. SprA is an outer membrane protein that is involved in gliding motility (18). SprA is similar in sequence to the *P. gingivalis* T9SS protein Sov (18, 19). The similarity between SprA and Sov suggested that SprA might have a role in secretion. *sprA* deletion mutants were constructed and examined for secretion of SprB and RemA. The mutants produced SprB and RemA proteins (see Fig. S1 in the supplemental material) but failed to secrete them to the cell surface (Tables 2 and 3), suggesting that SprA has a role in secretion. Previous results using transposon-induced *sprA* mutants suggested that SprA was not completely essential for

TABLE 3 Binding of protein G-coated polystyrene spheres carrying antibodies against Myc-tagged peptide

Strain	Description	Antibody added	Avg % of cells with spheres attached (SD)[a]
CJ1827	Wild type	Anti-Myc	0.0 (0.0)
CJ1984	Δ*remA*	Anti-Myc	0.0 (0.0)
CJ2083	*remA*::*myc-tag-1*	No antibody	0.0 (0.0)
CJ2083	*remA*::*myc-tag-1*	Anti-Myc	54.0 (4.0)
CJ2140/pCP23	Δ*gldK remA*::*myc-tag-1* with control plasmid pCP23	Anti-Myc	1.0 (0.0)
CJ2140/pTB99	Δ*gldK remA*::*myc-tag-1* with pTB99 carrying *gldK*	Anti-Myc	57.0 (1.0)
CJ2281/pCP23	Δ*gldL remA*::*myc-tag-1* with control plasmid pCP23	Anti-Myc	1.0 (1.0)
CJ2281/pTB81a	Δ*gldL remA*::*myc-tag-1* with pTB81a carrying *gldL*	Anti-Myc	52.7 (2.1)
CJ2263/pCP23	Δ*gldM remA*::*myc-tag-1* with control plasmid pCP23	Anti-Myc	1.0 (1.0)
CJ2263/pTB94a	Δ*gldM remA*::*myc-tag-1* with pTB94a carrying *gldM*	Anti-Myc	53.0 (1.7)
CJ2317	Δ*sprA remA*::*myc-tag-1*	Anti-Myc	0.6 (0.5)
CJ2317/pSN48	Δ*sprA remA*::*myc-tag-1* with pSN48 carrying *sprA*	Anti-Myc	55.3 (2.5)
CJ2327	*sprT remA*::*myc-tag-1*	Anti-Myc	0.0 (0.0)
CJ2327/pKF002	*sprT remA*::*myc-tag-1* with pKF002 carrying *sprT*	Anti-Myc	64.6 (2.0)

[a] Purified anti-Myc tag antiserum and 0.5-μm-diameter protein G-coated polystyrene spheres were added to cells as described in Materials and Methods. Samples were introduced into a tunnel slide, incubated for 2 min at 25°C, and examined by using a phase-contrast microscope. Images were recorded for 30 s, and 100 randomly selected cells were examined for the presence of spheres that remained attached to the cells during this time. Numbers in parentheses are standard deviations calculated from three measurements.

TABLE 4 Effect of deletion of *gldK*, *gldL*, *gldM*, and *sprA* on attachment of cells to glass coverslips

Strain	Description	Avg no. of cells attached to 0.03-mm² region of glass coverslip (SD)[a]
CJ1827/pCP23	Wild type with control vector pCP23	69.1 (4.9)
CJ2122/pCP23	Δ*gldK* with control vector pCP23	0.3 (0.5)
CJ2122/pTB99	Δ*gldK* with pTB99 carrying *gldK*	67.7 (8.6)
CJ2157/pCP23	Δ*gldL* with control vector pCP23	0.3 (0.5)
CJ2157/TB81a	Δ*gldL* with pTB81a carrying *gldL*	60.7 (6.8)
CJ2262/pCP23	Δ*gldM* with control vector pCP23	0.6 (1.0)
CJ2262/pTB94a	Δ*gldM* with pTB94a carrying *gldM*	24.1 (2.5)
CJ2302/pCP23	Δ*sprA* with control vector pCP23	0.1 (0.3)
CJ2302/pSN48	Δ*sprA* with pSN48 carrying *sprA*	64.2 (8.5)
KDF001	*sprT* mutant	1.4 (1.3)
KDF001/pKF002	*sprT* mutant with pKF002 carrying *sprT*	53.0 (5.4)
FJ149	*sprE* mutant	2.6 (1.2)
CJ1922/pCP23	Δ*sprB* with control vector pCP23	66.6 (11.3)
CJ1984/pCP23	Δ*remA* with control vector pCP23	54.8 (7.3)
CJ1985/pCP23	Δ*sprB* Δ*remA* with control vector pCP23	22.6 (4.1)

[a] Approximately 10⁶ cells in 2.5 μl of MM were introduced into a Petroff-Hausser counting chamber and incubated for 2 min at 25°C. Samples were observed by using an Olympus BH-2 phase-contrast microscope, and cells attached to a 0.03-mm² region of the glass coverslip were counted. Numbers in parentheses are standard deviations calculated from 9 measurements.

chitin utilization (18). The *sprA* mutants were severely but incompletely deficient in chitin utilization. Because the mutants analyzed would have produced truncated SprA protein that might have been partially functional, we revisited this phenotype with the deletion mutant. *sprA* deletion mutant strain CJ2302 failed to utilize chitin, suggesting a more severe defect in chitinase secretion than was observed for the transposon-induced mutant (see Fig. S2 in the supplemental material).

SprT and SprE have previously been shown to be required for secretion of SprB and for chitin utilization (6, 7). Like SprA, SprT was also required for secretion of RemA (Table 3, and see Fig. S1 in the supplemental material). The involvement of SprE in RemA secretion is less certain, but the resistance of *sprE* mutants to phages φCj42, φCj48, and φCj54, which are thought to use RemA

FIG 7 Effect of mutations on susceptibility to phages. Bacteriophages (5 μl of lysates containing approximately 10⁹ PFU/ml) were spotted onto lawns of cells in CYE overlay agar. The plates were incubated at 25°C for 24 h to observe lysis. Bacteriophages were spotted in the following order from left to right, as also indicated by the numbers in panel A: top row, φCj1, φCj13, and φCj23; middle row, φCj28, φCj29, and φCj42; bottom row, φCj48 and φCj54. (A) Wild-type *F. johnsoniae* CJ1827. (B) CJ2122 (Δ*gldK*). (C) CJ2122 complemented with pTB99, which carries *gldK*. (D) CJ2157 (Δ*gldL*). (E) CJ2157 complemented with pTB81a, which carries *gldL*. (F) CJ2262 (Δ*gldM*). (G) CJ2262 complemented with pTB94a, which carries *gldM*. (H) CJ2302 (Δ*sprA*). (I) CJ2302 complemented with pSN48, which carries *sprA*. (J) KDF001 (*sprT* mutant). (K) KDF001 complemented with pKF002, which carries *sprT*.
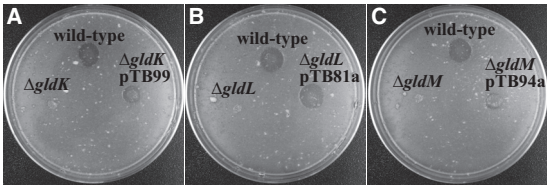
FIG 8 *gldK*, *gldL*, and *gldM* are each required for chitin utilization. Approximately 10⁶ cells of *F. johnsoniae* were spotted onto MYA-chitin medium containing tetracycline and incubated at 25°C for 60 h. (A) *F. johnsoniae* CJ1827 (wild type), CJ2122 (Δ*gldK*), and CJ2122 complemented with pTB99, which carries *gldK* (Δ*gldK* pTB99). (B) *F. johnsoniae* CJ1827 (wild type), CJ2157 (Δ*gldL*), and CJ2157 complemented with pTB81a, which carries *gldL* (Δ*gldL* pTB81a). (C) *F. johnsoniae* CJ1827 (wild type), CJ2262 (Δ*gldM*), and CJ2262 complemented with pTB94a, which carries *gldM* (Δ*gldM* pTB94a). CJ1827, CJ2122, CJ2157, and CJ2262 carried control vector pCP23 so that all strains were resistant to tetracycline.
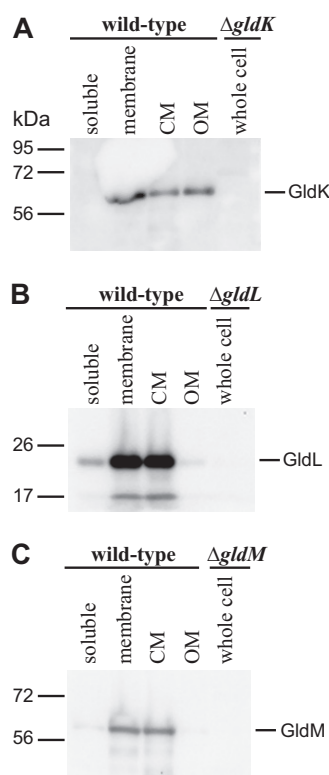
FIG 9 Cell fractionation and immunodetection of GldK, GldL, and GldM. Cell fractions of wild-type *F. johnsoniae* CJ1827 cells (lanes 1 to 4) and extracts of *gld* mutants (lanes 5) were examined for GldK (A), GldL (B), and GldM (C) by Western blot analyses. Lanes 1, soluble fraction of cell extract (cytoplasmic and periplasmic proteins); lanes 2, insoluble fraction of cell extract (primarily membrane proteins); lanes 3, Sarkosyl-soluble fraction of membranes (primarily cytoplasmic membrane proteins [CM]); lanes 4, Sarkosyl-insoluble fraction of membranes (primarily outer membrane proteins [OM]); lanes 5, cell extracts of *gldK*, *gldL*, or *gldM* mutants to demonstrate specificity of the antisera used in each panel.

as a receptor, suggests a role for SprE in RemA secretion also (4, 6). Cells with mutations in *sprA*, *sprE*, and *sprT* were each deficient in attachment to glass (Table 4), presumably as a result of a failure to secrete SprB, RemA, and other adhesins to the cell surface.

SprA, SprE, and SprT may be less central than GldK, GldL, GldM, and GldN to protein secretion and to gliding motility. Cells with mutations in *sprA*, *sprE*, and *sprT* differ from cells of the *gldK*, *gldL*, *gldM*, and *gldNO* deletion mutants in two respects. First, cells of the *gld* mutants are completely nonmotile, whereas cells of *sprA*, *sprE*, and *sprT* mutants are severely deficient in motility but exhibit weak gliding movements on wet glass surfaces (6, 7, 18). This phenotype was verified for the newly constructed *sprA* deletion mutant (see Movie S4 in the supplemental material). Second, cells of the *gld* mutants were completely resistant to infection by all bacteriophages tested, whereas cells of *sprA*, *sprE*, and *sprT* mutants exhibited resistance to some but not all bacteriophages. They exhibited increased resistance to φCj1, φCj13, φCj23, and φCj29, as do *sprB* mutants (3, 16), and they were partially resistant to φCj42, φCj48, and φCj54, as are *remA* mutants (4), but they remained susceptible to φCj28 (Fig. 7). Resistance of *gldK*, *gldL*, *gldM*, and *gldNO* mutants to phages such as φCj28, which infect cells of CJ1985 (Δ*sprB* Δ*remA*) (4), supports the idea suggested above that the T9SS secretes cell surface proteins in addition to

SprB and RemA. The sensitivity of *sprA*, *sprE*, and *sprT* mutants to phages such as φCj28 suggests that although SprA, SprE, and SprT are needed for secretion of some proteins by the T9SS, they may be less central to the function of this secretion system than are GldK, GldL, GldM, and GldN/O. The similar phenotypes of *sprA*, *sprE*, and *sprT* mutants also suggest the possibility that the proteins encoded by these genes may function together to support T9SS function.

DISCUSSION

gldK, *gldL*, *gldM*, and *gldN* were originally discovered as genes required for *F. johnsoniae* gliding motility (17). Later, orthologs of these genes (*porK*, *porL*, *porM*, and *porN*, respectively) were demonstrated to be required for secretion of *P. gingivalis* gingipains (7). The *P. gingivalis* secretion system was referred to as the Por secretion system (7) or, more recently, as the T9SS (2, 8). In *F. johnsoniae*, *gldN* and/or its paralog *gldO* was shown to be essential for secretion of the motility adhesins SprB and RemA (4, 5). The results presented here demonstrate that *F. johnsoniae gldK*, *gldL*, and *gldM* are cotranscribed with *gldN* and that the products of each of these genes are required for secretion of SprB and RemA. The outer membrane motility protein SprA, an ortholog of the *P. gingivalis* T9SS protein Sov, is also shown to be required for secretion of SprB and RemA. The results suggest that GldK, GldL, GldM, GldN, and SprA are each components of the *F. johnsoniae* T9SS. Other components of this system, previously shown to be involved in secretion in *F. johnsoniae*, include SprE, SprF, and SprT (6, 7, 16), which are similar in sequence to the *P. gingivalis* T9SS proteins PorW, PorP, and PorT, respectively (7, 31). The demonstration that the *F. johnsoniae* orthologs of the *P. gingivalis* T9SS proteins have roles in protein secretion provides independent confirmation of the function of these proteins. Similar proteins are found in many members of the large and diverse phylum *Bacteroidetes*, suggesting that T9SSs are common in this phylum (2).

The phenotypes of the *F. johnsoniae* mutants suggest that GldK, GldL, GldM, and GldN are essential for T9SS function and that SprA, SprE, and SprT are essential for secretion of many, but perhaps not all, proteins targeted to this system. Cells lacking GldK, GldL, GldM, or GldN and its paralog GldO failed to secrete SprB and RemA and were completely deficient in gliding motility and attachment to glass. They were also deficient in chitin utilization. The defect in chitin utilization is likely the result of a failure to secrete the chitinase encoded by Fjoh_4555, as previously reported for an *sprT* mutant (7). SprB and RemA appear to function as receptors for some but not all *F. johnsoniae* phages. Cells lacking GldK, GldL, GldM, or GldN and GldO were resistant to all phages tested, suggesting that some other proteins secreted by the T9SS also serve as phage receptors. In contrast, cells with mutations in *sprA*, *sprE*, and *sprT* exhibited partial phage resistance. They were resistant to phages φCj1, φCj13, φCj23, and φCj29 (indicating the absence of SprB on the cell surface) and were partially resistant to φCj42, φCj48, and φCj54 (suggesting the absence of RemA on the cell surface). They remained fully susceptible to φCj28. These results support the suggestion that GldK, GldL, GldM, and GldN (or GldO) are essential components of the T9SS, whereas SprA, SprE, and SprT are required for secretion of SprB and RemA but not for secretion of some other cell surface phage receptors secreted by the T9SS.

GldK, GldL, GldM, and GldN may form a complex. Orthologs of *gldK*, *gldL*, *gldM*, and *gldN* are present in many members of the phylum *Bacteroidetes*, and they appear to be invariably clustered together on the genomes in this order, suggesting that, as in *F. johnsoniae*, they may be transcribed as a unit. Of 27 sequenced genomes analyzed that had each of these genes (2), 26 had *gldK*, *gldL*, *gldM*, and *gldN* organized as in *F. johnsoniae*, and the 27th (*Chitinophaga pinensis*) had the same organization except that a 201-bp open reading frame of unknown function was present between *gldK* and *gldL*. Unlike the highly conserved gene order, the sequences of the individual proteins have diverged substantially (see Fig. S3 to S6 in the supplemental material). This argues against recent horizontal gene transfer as an explanation for the conserved gene order. Phylogenetic trees based on GldK, GldL, GldM, and GldN sequences are similar to those based on 16S rRNAs (see Fig. S7 to S11 in the supplemental material), suggesting that the T9SS genes have primarily been passed vertically. The genetic organization of *gldK*, *gldL*, *gldM*, and *gldN* has probably been conserved because it is important for function. For example, the products of conserved gene clusters often interact (32). GldK, GldL, GldM, and GldN may interact to form a T9SS complex, and coordinated expression may be required for efficient assembly of this complex.

Several experimental observations support the suggestion that GldK, GldL, GldM, and GldN interact. First, as indicated above, cells with nonpolar mutations in *gldL*, *gldM*, and *gldN* had decreased levels of GldK protein. Similarly, cells with mutations in *gldM* had decreased levels of GldL protein, and cells with nonpolar mutations in *gldK*, *gldL*, and *gldM* appeared to have decreased levels of GldN protein (Fig. 3). These results suggest the possibility of instability of the proteins in the absence of their partners. Second, moderate overexpression (less than 10-fold) of *gldK* and *gldL* together, but not individually, in wild-type cells resulted in adverse effects on growth and motility, suggesting interaction between the encoded proteins (17). Third, a *gldK* mutant was identified that could be partially suppressed by moderate overexpression of *gldL* (17). Finally, the *P. gingivalis* T9SS proteins PorK, PorL, PorM, and PorN (orthologs of *F. johnsoniae* GldK, GldL, GldM, and GldN, respectively) migrate as a large complex by blue native PAGE (7).

Comparative analysis of GldK, GldL, GldM, and GldN proteins. GldK, GldL, GldM, and GldN are novel proteins, and their exact functions in secretion and motility are not known. Analysis of genome sequences revealed orthologs of the genes encoding each of these proteins in many members of the phylum *Bacteroidetes* but not in bacteria belonging to other phyla (2). GldK is the most highly conserved of the four proteins. Fifty-six residues are completely conserved among the 26 sequences examined (see Fig. S3 in the supplemental material). Each of the proteins has an N-terminal type 2 signal peptide followed immediately by a cysteine, characteristic of bacterial lipoproteins. GldK is similar to sulfatase-modifying enzymes such as human formylglycine-generating enzyme (FGE). FGE activates sulfatases by catalyzing the conversion of the active-site cysteine to formylglycine. GldK is not thought to have this activity, however, because it lacks the active-site cysteine residues corresponding to C_{336} and C_{341} of FGE (33). The conserved regions of GldK may be important for protein folding and/or for protein-protein interactions, but further studies are needed to determine the exact functions of these regions. GldL and GldM localize to the cytoplasmic membrane, and sequence

analyses suggest that they each have hydrophobic alpha-helical membrane-spanning regions. The N-terminal region of GldL is highly conserved and has two predicted membrane-spanning helices (see Fig. S4 in the supplemental material). The second transmembrane helix has a glutamate residue that is conserved in all 26 sequences. The high level of conservation of this charged residue within a hydrophobic stretch is unusual and suggests the possibility that it may have an important function. For example, it may be involved in harvesting cellular energy to power secretion, although other explanations are possible. The remainder of the GldL protein is predicted to be soluble. Several regions of strong conservation are scattered across this soluble region of the protein, including a pocket of high identity near the C terminus. GldM has a single predicted transmembrane helix. This region lies near the N terminus and is very highly conserved (see Fig. S5 in the supplemental material). The high levels of conservation in the transmembrane regions of GldL and GldM suggest that these have functions in secretion and/or motility beyond simple membrane anchoring. GldN is the least conserved of the four proteins (see Fig. S6 in the supplemental material), with only two residues (N_{77} and N_{265}; numbered based on *F. johnsoniae* GldN) present in each sequence. GldN appears to have a cleavable type 1 signal peptide, but there is little else to assist in prediction of function. The presence of GldN, or the nearly identical protein GldO, is essential for motility and for secretion of SprB, RemA, and chitinase (4, 5). Future studies will be needed to elucidate their roles in these processes.

The results presented indicate that GldK, GldL, GldM, and SprA are each required for secretion of SprB, RemA, and chitinase. These proteins presumably function with other previously known components of the *F. johnsoniae* T9SS, including GldN, SprE, and SprT, that are also required for secretion of the same proteins. The predicted outer membrane protein SprF, which exhibits similarity to the *P. gingivalis* T9SS protein PorP, is also required for secretion of SprB but is not needed for secretion of RemA or for chitin utilization (4, 16). Unlike *P. gingivalis*, *F. johnsoniae* has many *sprF*-like (*porP*-like) genes that may encode semiredundant proteins involved in secretion of different proteins. A speculative model depicting our current understanding of the *F. johnsoniae* T9SS is illustrated in Fig. 10. As shown in this diagram, GldL and GldM are the only known cytoplasmic membrane components of the *F. johnsoniae* T9SS. As such, they have potential roles in harvesting cellular energy to power secretion, as suggested above, but further experiments will be needed to determine their exact functions.

GldK, GldL, and GldM are required not only for secretion but also for gliding motility. The motility defects of cells lacking these proteins may be explained by their inability to deliver motility adhesins, such as SprB and RemA, to the cell surface. However, GldK, GldL, and GldM may also have more direct roles in cell movement. Although the cell surface adhesins involved in motility are known, the motor for cell movement has not yet been identified. Gliding of *F. johnsoniae* and related bacteria requires the proton gradient across the cytoplasmic membrane (34–37), and proteins that span this membrane must be involved in converting this into cell movement. Twelve Gld proteins required for gliding of *F. johnsoniae* have been identified (17), and only four of these (GldF, GldG, GldL, and GldM) are thought to span the cytoplasmic membrane. GldF and GldG do not appear to be central components of the gliding machinery because some relatives of *F.*
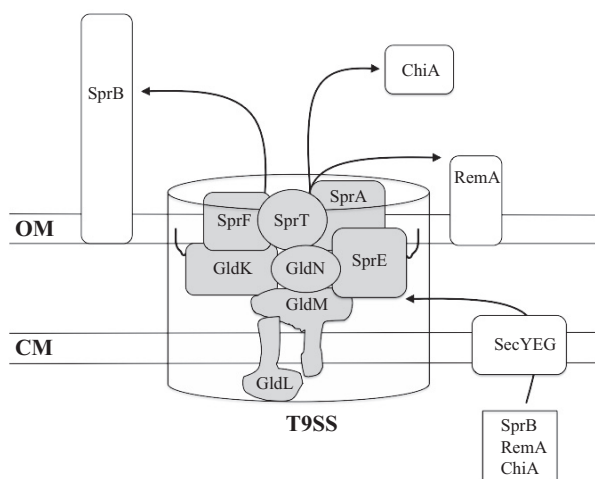
FIG 10 Speculative model of the *F. johnsoniae* T9SS. GldK, GldL, GldM, and GldN form the core components that are required for secretion. GldO (not shown) is nearly identical to GldN and is partially redundant with it. The outer membrane proteins SprA and SprT and the lipoprotein SprE are required for secretion of SprB, RemA, and ChiA, but they may not be required for secretion of all proteins targeted to the T9SS. SprF is needed for secretion of SprB but not for secretion of RemA and ChiA. *F. johnsoniae* has many paralogs of *sprF* that may encode proteins involved in secretion of proteins other than SprB. Proteins secreted by the T9SS all have predicted type 1 signal peptides and are predicted to be exported across the cytoplasmic membrane by the Sec system before being secreted across the outer membrane by the T9SS. Black lines indicate lipid tails on lipoproteins. Proteins are not drawn to scale, and stoichiometry of components is not known.

johnsoniae lack these proteins and yet exhibit rapid gliding motility (2). This leaves GldL and GldM as candidates for the motor proteins that harvest cellular energy and propel SprB and RemA, resulting in cell movement. Alternatively, some other unidentified cytoplasmic membrane proteins may perform these motor functions. Further study is needed to determine whether GldL and GldM have roles in gliding beyond secretion of SprB and RemA and to determine the exact functions of each of the T9SS proteins in protein secretion.

ACKNOWLEDGMENT

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