PrgK, a Multidomain Peptidoglycan Hydrolase, Is Essential for Conjugative Transfer of the Pheromone-Responsive Plasmid pCF10

Jenny A. Laverde Gomez, Minny Bhatti, Peter J. Christie

Department of Microbiology and Molecular Genetics, University of Texas Medical School at Houston, Houston, Texas, USA

Peptidoglycan (PG) hydrolases associated with bacterial type IV secretion systems (T4SSs) are thought to generate localized lesions in the PG layer to facilitate assembly of the translocation channel. The pheromone-responsive plasmid pCF10 of *Enterococcus faecalis* encodes a putative cell wall hydrolase, PrgK, and here we report that a *prgK* deletion abolished functionality of the pCF10-encoded T4SS as monitored by pCF10 conjugative transfer. Expression in *trans* of wild-type *prgK* fully complemented this mutation. PrgK has three potential hydrolase motifs resembling staphylococcal LytM, soluble lytic transglycosylase (SLT), and cysteine-, histidine-dependent amidohydrolase/peptidase (CHAP) domains. Complementation analyses with mutant alleles established that PrgK bearing two hydrolase domains in any combination supported near-wild-type plasmid transfer, and PrgK bearing a single hydrolase domain supported at least a low level of transfer in filter matings. When exported to the *Escherichia coli* periplasm, each domain disrupted cell growth, and combinations of domains additionally induced cell rounding and blebbing and conferred enhanced sensitivity to osmotic shock. Each domain bound PG *in vitro*, but only the SLT domain exhibited detectable hydrolase activity, as shown by zymographic analyses and release of fluorescent PG fragments. Genes encoding three T4SS-associated, putative hydrolases, *Lactococcus lactis* CsiA, Tn925 Orf14, and pIP501 TraG, partially complemented the Δ *prgK* mutation. Our findings establish that PrgK is an essential component of the pCF10-encoded Prg/Pcf T4SS and that its hydrolase domains coordinate their activities for full PrgK function. PrgK is indispensable for plasmid transfer in liquid matings, suggestive of a role in formation or stabilization of mating junctions.

The type IV secretion systems (T4SSs) assemble across the bacterial cell envelope to mediate passage of DNA and protein substrates to target cells (1). These systems comprise the conjugation systems present in most bacterial species and the effector translocator and DNA uptake/release systems thus far identified only in Gram-negative (G^-) bacteria (2). Recent structural studies of the *Escherichia coli* pKM101-encoded T4SS identified a channel subassembly that is likely conserved among most T4SSs elaborated by G^- species. Termed the core complex, this subassembly is configured as a large barrel of 185 Å in width and length that spans both membranes and the periplasm and is thought to serve as a structural scaffold for the translocation channel (3, 4). The T4SSs of Gram-positive (G^+) bacteria lack such core complexes, but it is reasonable to predict that these systems also employ a multiprotein channel for conveyance of substrates across the thick cell wall (5).

The low sieving property of peptidoglycan (PG) poses a significant physical barrier to the elaboration of T4SSs as well as other envelope-spanning structures, e.g., flagella, type II and III secretion systems, and type IV pili. Specialized cell wall hydrolases associated with these systems are thought to be important for generating localized lesions in the cell wall early during machine biogenesis (6, 7). In G^- bacteria, T4SS-associated hydrolases are exported, single-domain enzymes with sequence and structural similarity to the *E. coli* soluble lytic transglycosylase Slt70 (7). These enzymes hydrolyze the glycan backbone at the *N*-acetylmuramic acid- β -2,4-*N*-acetylglucosamine (MurNAc-GlcNAc) linkages, resulting in the formation of nonreducing 1,6-anhydromuropeptides on the MurNAc residues (8). T4SS-associated hydrolases characterized to date include VirB1 subunits encoded by the *Agrobacterium tumefaciens* VirB/VirD4 and *Brucella* species VirB systems (9, 10, 11); HP0523, encoded by the *Helicobacter pylori* *cag* pathogenicity island (PAI) (7, 10); LtgX and AtlA, en-

coded by the *Neisseria gonorrhoeae* gonococcal island (GGI) (12, 13); and P19 and TrbN, encoded by the conjugative plasmids R1 and RP4, respectively (7, 14, 15, 16). Genes for such hydrolases are typically located at or near the beginning of the polycistronic operations encoding the translocation complexes, consistent with hydrolase functions acting early during machine assembly. In general, T4SSs deleted of a cognate hydrolase exhibit modest reductions in substrate transfer frequencies of only 10- to 100-fold (9, 15, 16), although Hp0523 was found to be essential for translocation of the CagA effector through the Cag PAI-encoded T4SS (17). Systems functioning in the absence of a cognate hydrolase might recruit a heterologous hydrolase, or machine assembly might proceed during periods of active cell growth and PG remodeling (7).

Previously, we reported that the T4SS-associated hydrolases in G^+ bacteria can be classified into two dominant subfamilies (5). One, exemplified by CwlT, which is encoded by *Bacillus subtilis* integrative conjugative element *BsI* (ICEBsI), ranges in size from 320 to 375 residues and possesses an N-proximal transmembrane (TM) domain and a C-terminal extracytoplasmic region with two potential hydrolase domains (18). Here, we designate protein names with a subscript identifying the encoding mobile element. CwlT_{ICEBsI} possesses a C-terminal CHAP (cysteine-, histidine-dependent amidohydrolase/peptidase) domain with DL-endopepti-

Received 12 August 2013 Accepted 6 November 2013

Published ahead of print 15 November 2013

Address correspondence to Peter J. Christie, Peter.J.Christie@uth.tmc.edu.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JB.00950-13>.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JB.00950-13

dase activity and an N-terminal domain structurally similar to lytic transglycosylases but with *N*-acetylmuramidase activity (18). Other members of this two-domain hydrolase family include *Clostridium perfringens* TcpG_{pCW3} (19), *Streptococcus agalactiae* TraG_{PIP501} (20), *Enterococcus faecium* Orf0032_{pLG1} (21), and Orf14, encoded by Tn916/Tn925 ICEs (22, 23). These proteins carry CHAP domains with a predicted endopeptidase or amidase activity and SLT- or FlgJ-like domains with predicted glycosylase or muramidase activities (see Fig. S1 in the supplemental material).

The second hydrolase subfamily includes the subject of this study, *Enterococcus faecalis* PrgK, encoded by the pheromone-responsive plasmid pCF10 (24). Other members include CsiA, encoded by a 60-kb ICE, termed the sex factor, carried by *Lactococcus lactis* strain MG1363, and VirB1, encoded by the 89K PAI of *Streptococcus suis* strain 05ZYH33 (see Fig. S1 in the supplemental material). These proteins are much larger (>870 residues) than the two-domain hydrolases and carry predicted TM domains within the first third of the protein. CsiA_{ICELIMG1363} was reported to have a C-terminal CHAP domain, but deletion of this domain did not abolish hydrolase activity (25). Deletion of another motif, an 89-residue sequence termed the highly conserved domain (HCD), abolished transfer of the sex factor, and mutation of a conserved arginine in this motif also abolished a cell lysis phenotype accompanying protein overexpression. CsiA_{ICELIMG1363} also was reported to bind D-Ala-D-Ala analogues and inhibit the action of penicillin binding proteins (PBPs). These findings led to a proposal that CsiA_{ICELIMG1363} functions as a cell wall synthesis inhibitor to enable localized assembly of the T4SS translocation complex (25).

Here, we extend the characterization of the novel PrgK/CsiA/VirB1 subfamily of hydrolases associated with G⁺ bacterial T4SSs. We report that PrgK and its homologs in fact possess three putative hydrolase domains whose sequences or structures are most closely related to staphylococcus LytM, the N-terminal domain of bacteriophage ϕ 29 tail protein gp13, “goose-type” soluble lytic transglycosylase (here termed the SLT domain), and CHAP motifs (Fig. 1; also see Fig. S2 in the supplemental material). The SLT domain encompasses the previously identified HCD of CsiA (see Fig. S1). We demonstrate that PrgK is required for conjugative transfer of pCF10, and we define contributions of individual and combined hydrolase domains to PrgK function in *E. faecalis* and *E. coli* cells. *In vitro* studies showed that each domain binds PG, while the SLT domain also hydrolyzes PG. Intriguingly, genes encoding CsiA_{ICELIMG1363}, TraG_{PIP501}, and Orf14_{Tn925} partially complemented a Δ prgK mutation in specific mating conditions. Together, our findings establish that PrgK is an essential component of the Prg/Pcf T4SS, but also that a striking array of PrgK derivatives and heterologous hydrolases support assembly of partially or fully functional translocation channels.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains, plasmids, and oligonucleotides used in this studies are listed in Table 1. *E. coli* DH5 α and EC1000, a strain that produces the pWV01 RepA protein (26), were used as hosts for plasmid constructions. *E. coli* strains were cultured in Luria broth (LB) or brain heart infusion broth (BHI; Difco Laboratories) at 37°C with shaking. *E. faecalis* strains were cultured in BHI at 37°C without shaking. *E. faecalis* strains were grown in the presence of the following antibiotics as needed: erythromycin (100 μ g/ml final concentration for plasmid markers, 10 μ g/ml for chromosomal markers), fusidic acid (25

μ g/ml), rifampin (200 μ g/ml), spectinomycin (1,000 μ g/ml for plasmid markers, 250 μ g/ml for chromosomal markers), streptomycin (1,000 μ g/ml), and tetracycline (10 μ g/ml). *E. coli* strains were cultured with the following antibiotics as needed: carbenicillin (50 μ g/ml), chloramphenicol (20 μ g/ml), erythromycin (100 μ g/ml), kanamycin (50 μ g/ml), and spectinomycin (50 μ g/ml). All antibiotics were obtained from Sigma Chemical Co.

Construction of pCF10 Δ prgK. prgK was deleted from pCF10 as follows. A 568-bp region immediately 5' of prgK (upstream fragment) was amplified with the primer pair prgKNO1/prgKNO2. A 584-bp region immediately 3' of prgK (downstream fragment) was amplified with the primer pair prgKNO3/prgKNO4. The two fragments were cloned sequentially into pCJ47 using the primer-added XbaI/BamHI (upstream fragment) and BamHI/NcoI (downstream fragment) restriction sites, resulting in pJGP2. pJGP2 was used to delete prgK from pCF10 by marker exchange (27) to obtain plasmid pCF10 Δ prgK.

Construction of prgK alleles for expression in E. faecalis. Wild-type prgK and prgK mutant alleles were cloned into pMSP3545 or pMSP3545S for expression from the nisin-inducible promoter, P_{nis}, in *E. faecalis* (28). Plasmid pJL1 expressing wild-type prgK was constructed by PCR amplification of prgK with the primer pair F-NcoI-prgK and R-prgK-XhoI and pCF10 as a template. The PCR product was introduced into NcoI/XhoI-digested pPC914KSNcoI, and then a kanamycin resistance (Kan^r) cassette was excised from pUI188 with SalI digestion and cloned into XhoI-digested pPC914-prgK. A prgK-Kan^r fragment was isolated by digestion with KpnI and blunt ending with Klenow followed by digestion with NcoI; this fragment was introduced into NcoI and blunt-ended SphI sites of pMSP3545. prgK alleles encoding PrgK with amino acid substitution E573A, C766A/H828A, or E573A/C766A/H822A were expressed from plasmids pJL2, pJL4, or pJL6, respectively. These prgK alleles were amplified by overlapping PCR as follows. The 5' fragment was amplified using F-prgK-NcoI and a reverse internal primer containing a codon change (R-E573A, R-C766A, or R-H828A); the 3' fragment was amplified with a forward internal primer containing a codon change (F-E573A, F-C766A, or F-H828A) and R-prgK-XbaI-XhoI. The 5' and 3' fragments were used as templates for overlapping PCR using primers F-prgK-NcoI and R-prgK-XbaI-XhoI. For construction of the double or triple substitution mutants, templates were used that already contained one or two codon substitutions. The amplified prgK alleles were cloned into pMSP3545S digested with NcoI-XbaI. prgK deleted of coding sequences for the LytM, SLT, CHAP, or both CHAP and SLT domains were expressed from plasmid pJL28, pJL3, pJL5, or pJL7, respectively, which were constructed as described below. To generate prgK Δ LytM, fragments 5' or 3' of the LytM coding region were PCR amplified using primers F-prgK-NcoI and R-prgK-LytM-del or F-prgK-LytM-del and R-prgK-XbaI-XhoI, respectively. The 5' and 3' fragments were used as templates for overlapping PCR using primers F-Nco-prgK and R-prgK-XbaI-XhoI. To generate prgK Δ SLT, fragments 5' or 3' of the SLT domain were PCR amplified using primers F-prgK-NcoI and R-prgK-SLT-del or F-prgK-SLT-del and R-prgK-XbaI-XhoI, respectively. The 5' and 3' fragments were used as templates for overlapping PCR using primers F-Nco-prgK and R-prgK-XbaI-XhoI. prgK Δ CHAP was generated by PCR with primers F-prgK-NcoI and R-prgK-CHAPdel. prgK Δ SLT/ Δ CHAP was generated by PCR with primers F-prgK-NcoI and R-prgK-C'-del. The prgK Δ LytM, prgK Δ SLT, prgK Δ CHAP, and prgK Δ SLT/ Δ CHAP fragments were cloned as NcoI/XbaI-digested fragments into pMSP3545S. Plasmid pJL35, carrying prgK Δ 2-170 lacking the N-terminal 170 amino acids, was generated by PCR amplification using F-prgK-Nterm-del-NcoI and R-prgK-XhoI-XbaI and cloning the amplified region into NcoI/XbaI-digested pMSP3545S. Plasmid pJL29, expressing 3 $\times$ FLAG-prgK, was constructed by PCR amplification of prgK with primers F-Nde-prgK and R-prgK-XbaI-XhoI and introduction of the NdeI- and XhoI-digested fragment downstream of a 3 $\times$ FLAG sequence carried in a pBluescript plasmid. The 3 $\times$ FLAG-prgK allele was then introduced as an NcoI/KpnI fragment into similarly digested pMSP3545 to generate pJL29. Plasmids pJL30 (3 $\times$ FLAG-

FIG 1 PrgK is essential for pCF10 transfer. (A) Schematic showing locations of the predicted transmembrane domain (TM) and hydrolase domains of PrgK, with residue numbers listed relative to the beginning of the protein. PrgK domains displayed the greatest structural similarity to *Staphylococcus aureus* LytM, the N-terminal domain of bacteriophage ϕ 29 tail protein gp13 (SLT), and a CHAP/NlpC-P60 hydrolase domain of *Streptococcus saprophyticus*. Alanine substitution mutations of the SLT and CHAP catalytic site residues are indicated with black dots. (B and C) Histograms depicting results of *E. faecalis* mating experiments monitoring transfer of pCF10 or pCF10ΔprgK alone or when complemented with wild-type or mutated prgK alleles. Panel B shows results of complementation with alleles encoding PrgK mutants bearing the hydrolase domains or substitution mutations listed; ΔN2-170 is PrgK deleted of its N-terminal 170 residues. Hash marks are used to group the types of mutations. Panel C depicts results of complementation with alleles encoding N-terminally 3× FLAG-tagged mutant proteins. Transfer frequencies are presented as the number of transconjugants per donor cell (Tc's/Donor). Experiments were repeated 3 times in triplicate, and the histogram depicts average results with standard deviations.

prgK_{E573A}), pJL32 (3×FLAG-prgK-LytM), and pJL34 (3×FLAG-prgKΔ2-170) were generated by PCR (or overlapping PCR) using pJL29 as a template and primers listed in Table 1. Plasmids pJL31 (prgK-CHAP) and pJL33 (prgK-SLT) were generated by overlapping PCR with pJL1 as the template and primers listed in Table 1. Plasmid pJL36, expressing 3×FLAG-prgK_{E573A/C766A/H828A}, was generated by PCR with pJL6 as the template and primers listed in Table 1. The hydrolase genes *csiA*_{ICELMG1363}, *orf14*_{Tn925}, and *traG*_{PIP501} were PCR amplified using primers F-csiA/R-csiA, F-orf14/R-orf14, and F-traG/R-traG, respectively, and cloned as NcoI/XbaI fragments into similarly digested pMSP3545S to generate plasmids pJL25, pJL26, and pJL27.

Construction of plasmids for delivery of hydrolase domains to the *E. coli* periplasm. prgK sequences encoding one or more of the putative hydrolase domains were cloned into pASK-IBA4 in-frame downstream of the coding sequence for the OmpA signal sequence for delivery of the domains to the periplasm of *E. coli*. The entire extracytoplasmic (EC) region of PrgK (residues 269 to 871) as well as the LytM (residues 322 to 528), SLT (residues 532 to 720), and CHAP (residues 720 to 871) domains

were cloned into pASK-IBA4 to generate pJL13, pJL8, pJL9, and pJL11, respectively. Likewise, regions encoding the domain pairs LytM/SLT (residues 322 to 720) and CHAP/SLT (residues 532 to 871) were cloned into pASK-IBA4 to generate pJL14 and pJL15, respectively. The catalytic-site mutants SLT_{E573A}, CHAP_{C766A/H828A}, EC_{E573A}, EC_{C766A/H828A}, and EC_{E573A/C766A/H828A} were cloned into pASK-IBA4 to generate pJL10, pJL12, pJL16, pJL17, and pJL18, respectively. PCR products were generated using pJL1 as a template and the primers F-LytM/R-LytM, F-SLT/R-SLT, F-CHAP/R-CHAP, F-EC/R-CHAP, F-LytM/R-SLT, and F-SLT/R-CHAP (Table 1) to amplify the regions of prgK encoding LytM, SLT, CHAP, EC, LytM/SLT, and SLT/CHAP, respectively. The same primers were used to amplify regions encoding prgK domains bearing catalytic-site mutations using pJL2, pJL4, or pJL6 as the template. The amplified regions were digested with NheI and XhoI, and the resulting fragments were introduced into similarly digested pASK-IBA4 to generate the vectors pJL10 through pJL19 (Table 1).

Construction of plasmids for enrichments of prgK domains. Fragments encoding the PrgK domains and EC region were cloned into

TABLE 1 Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant feature(s) or sequence	Source/reference or use
Strains		
<i>E. coli</i>		
DH5 α	F $^{-}$ ϕ 80 Δ lacZ Δ M15 Δ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r $_{K}^{-}$ m $_{K}^{+}$) phoA supE44 λ^{-} thi-1 gyrA96 relA1	Gibco-BRL
BL21(DE3)	F $^{-}$ ompT (r $_{b}^{-}$ m $_{b}^{-}$) DE3	Novagen
EC1000	Cloning host, provides RepA in trans	26
<i>E. faecalis</i>		
CK104	Rif r Fus r , OG1RF Δ upp2	60
OG1ES	Ery r Str r	61
OG1SSp	Str r Spc r	62
Plasmids		
pJGP2	pCJ47 carrying 5' and 3' sequences for constructing Δ prgK	This study
pCF10	Tet r , pheromone-inducible conjugative plasmid	63
pCF10 Δ prgK	Tet r , pCF10 deleted of prgK	This study
pMSP3545	Erm r , shuttle vector, P $_{nis}$ promoter	28
pMSP3545S	Erm r , Spc r , shuttle vector, P $_{nis}$ promoter	64
pASK-IBA4	Amp r , OmpA signal sequence, P $_{tet}$ (AHT)-inducible promoter	Neuromics
pET28b(+)	Kan r , expression vector for His tagging	Novagen
pJL1	Erm r , pMSP3545 expressing prgK	This study
pJL2	Erm r , Spc r , pMSP3545S expressing prgK $_{E573A}$	This study
pJL3	Erm r , Spc r , pMSP3545S expressing prgK-LytM/CHAP; Δ 532-720	This study
pJL4	Erm r , Spc r , pMSP3545S expressing prgK $_{C766A/H828A}$	This study
pJL5	Erm r , Spc r , pMSP3545S expressing prgK-LytM/SLT; Δ 720-871	This study
pJL6	pMSP3545S expressing prgK $_{E573E/C766A/H828A}$	This study
pJL7	Erm r , Spc r , pMSP3545S expressing prgK-LytM; Δ 532-871	This study
pJL8	Crb r , pASK-IBA4 expressing ompAss-LytM; PrgK residues 322-528	This study
pJL9	Crb r , pASK-IBA4 expressing ompAss-SLT; PrgK residues 532-720	This study
pJL10	Crb r , pASK-IBA4 expressing ompAss-SLT $_{E573A}$	This study
pJL11	Crb r , pASK-IBA4 expressing ompAss-CHAP; PrgK residues 720-871	This study
pJL12	Crb r , pASK-IBA4 expressing ompAss-CHAP $_{C766A/H828A}$	This study
pJL13	Crb r , pASK-IBA4 expressing ompAss-EC; PrgK residues 269-871	This study
pJL14	Crb r , pASK-IBA4 expressing ompAss-LytM/SLT; PrgK residues 322-720	This study
pJL15	Crb r , pASK-IBA4 expressing ompAss-SLT/CHAP; PrgK residues 532-871	This study
pJL16	Crb r , pASK-IBA4 expressing ompAss-EC $_{E573A}$	This study
pJL17	Crb r , pASK-IBA4 expressing ompAss-EC $_{C766A/H828A}$	This study
pJL18	Crb r , pASK-IBA4 expressing ompAss-EC $_{E573A/C766A/H828A}$	This study
pJL19	Kan r , pET28b(+) expressing his-LytM	This study
pJL20	Kan r , pET28b(+) expressing his-SLT	This study
pJL21	Kan r , pET28b(+) expressing his-SLT $_{E573A}$	This study
pJL22	Kan r , pET28b(+) expressing his-CHAP	This study
pJL23	Kan r , pET28b(+) expressing his-CHAP $_{C766A/H828A}$	This study
pJL24	Kan r , pET28b(+) expressing his-EC	This study
pJL25	Erm r , Spc r , pMSP3545S expressing <i>csiA</i>	This study
pJL26	Erm r , Spc r , pMSP3545S expressing <i>orf14</i> $_{Tn925}$	This study
pJL27	Erm r , Spc r , pMSP3545S expressing <i>traG</i> $_{pIP501}$	This study
pJL28	Erm r , Spc r , pMSP3545S expressing prgK-SLT/CHAP	This study
pJL29	Erm r , Kan r , pMSP3545 expressing 3 $\times$ FLAG-prgK	This study
pJL30	Erm r , Spc r , pMSP3545S expressing 3 $\times$ FLAG-prgK $_{E573A}$	This study
pJL31	Erm r , Spc r , pMSP3545 expressing prgK-CHAP; Δ 322-720	This study
pJL32	Erm r , Spc r , pMSP3545 expressing 3 $\times$ FLAG-prgK-LytM; Δ 322-871	This study
pJL33	Erm r , Spc r , pMSP3545 expressing prgK-SLT; Δ 322-528, Δ 720-871	This study
pJL34	Erm r , Spc r , pMSP3545 expressing 3 $\times$ FLAG-prgK Δ 2-170	This study
pJL35	Erm r , Spc r , pMSP3545 expressing prgK Δ 2-170	This study
pJL36	Erm r , Spc r , pMSP3545 expressing 3 $\times$ FLAG-prgK $_{E573A/C766A/H828A}$	This study
Oligonucleotides		
prgKNO1	GCTCTAGAGTGTGGGTTTACAAGATA	pJGP2
prgKNO2	CGGATCCGGCGAATACTTTATGCGC	pJGP2
prgKNO3	CGGATCCTTCTACCATATCCTCACATCC	pJGP2
prgKNO4	CATGCCATGGGGTAAGTGTACTGGTGC	pJGP2

(Continued on following page)

TABLE 1 (Continued)

Strain, plasmid, or oligonucleotide	Relevant feature(s) or sequence	Source/reference or use
F-prgK-NcoI	CGCTTAGGATGTGAGGCCATGGTAGAA	pJL1-pJL7, pJL31, pJL33
R-prgK-XhoI	TCCCTCGAGATTATTTCTTTGGTTCTGTGTG	pJL1
R-prgK-XbaI/XhoI	CCCTCGAGTCTAGAATTATTTCTGTTGGTTCTGTGTG	pJL2, pJL3, pJL4, pJL6, pJL29-31, pJL34-36
F-C766A	AGGCAATGCGTATGCGCCAGGGAATGCCACGTGGTATGTGTTTAATCGTT	pJL4
R-C766A	AACGATTAAACACATACCACGTGGCATTCCCTGGCGCATACGCATTGCCCT	pJL4
F-H828A	GGTTCTAGTACGCAATATGGCGCCGTGGCTTATGTGGAGC	pJL4
R-H828A	CATGCTCCACATAAGCCACGGCGCCATATTGCGTACTAGAACC	pJL4
F-E573A	TTAGGCAATTTTTCTGGTAGCAAGTGGCATTAAATCCAAAA	pJL6, pJL30, pJL36
R-E573A	TTTTGGATTAATGCCACTTGCTACCGAAAAATTGCCTAA	pJL6, pJL30, pJL36
R-prgK-CHAPdel	GCTCTAGATTAAGAAGAAATGTAAACCAATTTTG	pJL5
R-prgK-C' del	GCTCTAGATTAAGAAAAATCTGCACTTCCTAAAAAG	pJL7
R-prgK-SLT-del	CACCGATTGGTGTACCCGATCGAGAAGAAAAATCTGCACTTCCTAAAAAG	pJL3
F-prgK-SLT-del	CTTTTAGGAAGTGCAGATTTTTCTTCTCGATCGGGTACACCAATCGGTG	pJL3
R-prgK-LytM-del	AGAAAAATCTGCACCTCCTAAAGAGTATTCATGTAGAAAAAGAACTTCATC	pJL28
R-prgK-LytM-del	GATGAAGTCTTTTCTACATGAATACTCTTTTAGGAAGTGCAGATTTTTCT	pJL28
F-LytM	CTAGCTAGCTATCGGTACGATGATTTTAA	pJL8, pJL14, pJL19
R-LytM	CCGCTCGAGTTACGTTTGGCTGTAGGTTACACG	pJL8, pJL19
F-SLT	CTAGCTAGCGAAGTGCAGATTTTTCTCCT	pJL9-10, pJL15, pJL20, pJL21
R-SLT	CCGCTCGAGTTAAGAAAAAGAAATGTAAACCAATT	pJL9, pJL10, pJL14, pJL20, 21
F-CHAP	CTAGCTAGCTCTCGATCGGGTACACCAATC	pJL11, pJL12, pJL22, pJL23
R-CHAP	CCGCTCGAGTTAGTTAGGCGCATAAAGTATTC	pJL11-13, pJL15-18, pJL22-24
F-EC	CTAGCTAGCTTGAGTGGCGTTGCAAGTAGT	pJL13, pJL16, pJL17, pJL18, pJL24
F-FLAG-prgK	CTCACCATGGATTACAAGGACG	pJL30, pJL32, pJL34, pJL36
R-prgK-Lyt-SLT-del	CACCGATTGGTGTACCCGATCGAGAATTCATGTAGAAAAGAACTTCATC	pJL31
F-prgK-Lyt-SLT-del	CACCGATTGGTGTACCCGATCGAGAATTCATGTAGAAAAGAACTTCATC	pJL31
R-prgK-SLT-CHAP-del	GCTCTAGATTACGTTTGGCTGTAGGTTACACG	pJL32
F-prgK-Lyt-CHAP-del	GTAACCTACACGCAACGACT TCTCGATCGGGTACACCAATC	pJL33
R-prgK-Lyt-CHAP-del	GATTGGTGTACCCGATCGAGAAGTCGTTTGGCTGTAGGTTAC	pJL33
R-prgK-FLAG-Nterm-del	CGTATCTTTTGCAGTTTTCAC CATATGCTTATCATCATCATCC	pJL34
F-prgK-FLAG-Nterm-del	GGATGATGATGATAAGCATATGTAAAACTGCAAAAGATACG	pJL34
F-prgK-Nterm-del-NcoI	catgccATGGTGAAAAGTCAAAAGATACG	pJL35
F-csiA	GATAACCATGGATAAAAAGCAAGCAAAAGAACTTTAAAGCTTCGC	pJL25
R-csiA	CTATATCTAGATTATTTAAAGTGAACAAATGTCATTCCTGCTCCTGG	pJL26
F-Orf14	GATATCCATGGAGTTGAAAACCTTTAGTGATTGGTGGTTCTGG	pJL26
R-Orf14	CTATATCTAGATCATTTGTTTGATTCGTCCTGCTCCC	pJL27
F-TraG	GGTGATTTTCATACCATGGGAGC	pJL27
R-TraG	GCTCTAGATTATTCACATAAATTGC	pJL28

pET28b(+) for expression and subsequent enrichments. Cloning of sequences encoding the EC region and the LytM, SLT, and CHAP domains resulted in plasmids pJL24, pJL19, pJL20, and pJL22. Cloning of sequences encoding the catalytic site variants SLT_{E573A} and CHAP_{C766A/H828A} resulted in plasmids pJL21 and pJL23. The domains were PCR amplified using primers and templates described above for pASK-IBA4 cloning, and the PCR products were introduced as NheI-XhoI fragments into similarly digested pET28b(+). The resulting plasmids were introduced into *E. coli* BL21(DE3) for production and subsequent enrichments by metal affinity chromatography (IMAC) of the N-terminally His-tagged domains.

Conjugation assays. *E. faecalis* donor and recipient cultures grown overnight were diluted 1:10 in fresh BHI and incubated at 37°C for 1 h. Because our initial studies with the *P_{nis}* promoter supplied evidence for gene expression at similar levels in the absence and presence of nisin, except where noted, strains with alleles expressed from *P_{nis}* were not induced with nisin. Donor and recipient cells were mixed at a ratio of 1:10 and allowed to mate for 2 h at 37°C on filters or in liquid. Serial dilutions of the mating mixtures were plated on selective BHI agar plates to obtain CFU of transconjugants and donors. Experiments were repeated at least three times in triplicate, and plasmid transfer frequencies of a representa-

tive experiment were expressed as transconjugants per donor with standard deviations indicated (29).

In vivo lysis during growth. *E. coli* strains induced for synthesis of exported forms of the PrgK hydrolase domains were assayed for growth suppression and cell lysis as follows. Overnight cultures were inoculated into fresh LB media to a final optical density at 600 nm (OD₆₀₀) of 0.2. Cultures (3 ml) were incubated for 3 h at 37°C with shaking, followed by addition of anhydrotetracycline (AHT; 200 ng/ml final concentration) to induce expression of the *ompAss-prgK'* alleles from the *P_{tet}* promoter carried on pABI-ASK4. Fifty microliters of the cultures was added to wells of a 96-well plate containing 50 µl of fresh LB medium with or without AHT. Cell growth was monitored for 6 to 12 h with OD₆₀₀ measurements taken at 20-min intervals with a Biotek synergy mix microplate reader and Gen5 1.09 software.

Effects of exported PrgK domains on *E. coli* cell shape. Morphologies of *E. coli* cells producing exported forms of the PrgK domains were examined by differential interference contrast (DIC) microscopy. Cells were cultured as described above and induced for 30 min with AHT (200 ng/ml), and then 10 µl of cell cultures was added to the same volume of 2% low-melting-temperature agarose (Fisher) in LB on a glass slide and covered with a coverslip. Cells were visualized with an Olympus BX60 micro-

scope equipped with a UPlanSApo 100 $\times$ oil immersion objective, and images were captured and digitalized with HCLImage live (x64) software.

Osmotic shock-induced cell lysis. Osmotic shock-induced lysis of cells producing exported forms of the PrgK domains was assessed as follows. Cells were cultured as described above and induced for 1 h with AHT (200 ng/ml). Aliquots (1 ml) of the cell cultures were pelleted by centrifugation, and cell pellets were resuspended in 200 μ l of phosphate-buffered saline (PBS). Twenty μ l of the resuspended cells then was added to 80 μ l of PBS or water, and cell lysis was assessed after 1 h by comparing OD₆₀₀ values with PBS to those with H₂O using the equation $1 - (\text{OD}_{600} \text{ H}_2\text{O} / \text{OD}_{600} \text{ PBS}) \times 100$ to yield percent lysis.

Enrichment of PrgK domains. The native or mutant LytM, SLT, or CHAP domain as well as the EC region were enriched from *E. coli* BL21(DE3) cells by IMAC using Talon cobalt resin (Clontech) and a batch/gravity flow column protocol (30). In brief, 1-liter cultures were grown to an OD₆₀₀ of 0.4 and induced with isopropyl- β -D-thiogalactopyranoside (IPTG; 0.5 mM final concentration) for 2 h at 37°C. Cell pellets were collected and resuspended in 5 ml equilibration buffer (50 mM NaPO₄, pH 7.0, 300 mM NaCl, 10 mM imidazole) with protease inhibitors (Complete EDTA free; Roche), and the cells were disrupted with a French press. Lysates were cleared by centrifugation at $12,000 \times g$ for 20 min at 4°C. The clarified suspension (5 ml) was incubated with 1 ml of Talon resin suspension (500- μ l bed volume) with gentle shaking at 4°C for 30 min. The suspension was then transferred to a disposable column (poly-prep chromatography columns; Bio-Rad), and the resin was washed extensively with equilibration buffer. Bound protein was eluted with elution buffer (50 mM NaPO₄, pH 7.0, 300 mM NaCl, 150 mM imidazole) in 500- μ l fractions. The protein samples were analyzed by Coomassie blue-stained SDS-polyacrylamide gels, and the presence of the proteins of interest was confirmed by Western transfer and immunostaining of blots with anti-His antibodies as previously described (29). Protein concentrations were measured using a Bio-Rad protein assay based on the Bradford colorimetric determination (31).

Peptidoglycan binding assay. The His-tagged PrgK domains or His-tagged PcfCAN103 (negative control) (29) was dialyzed in binding buffer (0.1 M NaPO₄, 0.1 M NaCl, pH 7.5), and *Micrococcus luteus* PG (Sigma) was resuspended in the same buffer. Fractions with the domains (20 μ g total protein) next were incubated with purified PG (20 μ g) in 50- μ l reaction volumes for 30 min at 37°C. The mixtures were then centrifuged for 5 min at $16,000 \times g$ to pellet the PG and PG-bound proteins. Supernatant fractions containing unbound proteins were removed, and the PG pellets were washed twice with 500 μ l PBS to remove loosely adherent proteins and then resuspended in 50 μ l of PBS (32). The supernatant and PG fractions were boiled in 50 μ l of 2 $\times$ Laemmli's buffer and then subjected to SDS-PAGE, western transfer, and immunostaining with anti-His antibodies for detection of the His-tagged PrgK domains or His-PcfCAN103 (29).

In vitro hydrolase assays. Fractions containing the His-tagged PrgK domains (20 μ g total protein) were electrophoresed through 12.5% SDS-PAGE gels containing 1% (wt/vol) lyophilized/heat-boiled *M. luteus* cells (Sigma) (33, 34). The gels were washed for 1 h in distilled H₂O at room temperature with gentle shaking and then transferred to renaturation buffer (25 mM Tris-HCl, pH 7.5, 0.5% Triton X-100). The gels were incubated for 16 h at 37°C with gentle shaking. The gels were then stained with stain solution (1% methylene blue, 0.01% KOH) at room temperature for 1 h and destained first with H₂O and then briefly with destain solution (10% [vol/vol] acetic acid, 45% [vol/vol] ethanol, 45% [vol/vol] H₂O) until zones of clearing were visible. The hydrolytic activities appeared as clear bands on a blue background (35).

A lysozyme assay kit, EnzChek (Molecular Probes), was used to measure levels of lysozyme activities of PrgK domains (36). Lysozyme activity is proportional to fluorescence release from fluorescently labeled *Micrococcus lysodeikticus* cell walls. Briefly, 2.5-, 5-, 10-, and 20- μ g aliquots of each PrgK domain were mixed with 50 μ l of lysozyme substrate (50 μ g/ml) in a final volume of 100 μ l in reaction buffer (50 ml of 0.1 M NaPO₄,

0.1 M NaCl, pH 7.5, 2 mM NaN₃). A standard curve was prepared with lysozyme over a range of 0 to 500 U/ml. The reaction mixtures were placed in black 96-well microtiter plates (Greiner Bio-One) and incubated, protected from light, for 2 h at 37°C. Fluorescence intensity was measured at excitation/emission wavelengths of 485/530 nm using a Biotek synergy mix microplate reader and Gen5 1.09 software. One unit is defined as the amount of enzyme required to produce a change in the absorbance at 450 nm of 0.01 U per min at pH 6.25 and 25°C, using a suspension of *M. lysodeikticus* as a substrate.

Protein structure analysis. Protein sequence similarity was analyzed using NCBI BLAST. Identification of the putative PrgK hydrolase domains was achieved with the Phyre 2 structural modeling algorithm (37). Comparison of similar domains and conserved residues was done by visual inspection of sequences and alignments. Transmembrane domains were identified with the SMART algorithm (38, 39), and Signal peptides were determined by the SignalP program (40).

RESULTS

Domain architecture of PrgK. PrgK is predicted to span the cytoplasmic membrane such that the bulk of the protein (residues 269 to 871) is extracytoplasmic (EC). The N-terminal cytoplasmic domain (residues 1 to 246) is almost entirely α -helical in secondary structure, and structural modeling did not reveal insights into its function (see Fig. S1A and S2 in the supplemental material). In contrast, the modeling studies established that the EC region has three potential hydrolase domains (Fig. 1A; also see Fig. S1A and S2). The N-proximal domain (residues 322 to 528) has a structure closely similar (16% identity, 100.0% confidence) to that of *Staphylococcus aureus* LytM (Phyre2 fold library code d1qwya), a metalloendopeptidase of the M23 family (41, 42, 43). The central domain (residues 532 to 720) has a structure similar (29% identity, 100.0% confidence) to that of the N terminus of bacteriophage Φ 29 tail protein gp13 (code c3csqC) and C-type goose lysozyme (20% similarity, 92.1% confidence; code d1iiza) (44). The C-terminal domain (residues 720 to 871) has a structure similar (34% identity, 100% confidence) to that of the CHAP/NlpC-P60 hydrolase domain of *Streptococcus saprophyticus* SSP0609 (code c2k3aA) (45, 46) (Fig. 1A). All three domains, designated LytM, SLT, and CHAP, also have predicted secondary structures similar to their structural templates (see Fig. S2), and the SLT and CHAP domains have conserved catalytic site residues, E573 and Cys766/His828, respectively (Fig. 1A). The LytM domain appears to be degenerate, as it lacks the His and Asp residues in two conserved motifs, HX₃D and HxH, that coordinate metal (Zn²⁺) binding in the catalytic sites of active M23 metalloproteases (41).

PrgK is essential for pCF10 conjugative transfer. Donor cells failed to transfer the pCF10 Δ prgK mutant plasmid at detectable levels in 2-h filter or broth matings or in overnight matings (Fig. 1B and data not shown). The Δ prgK mutation was fully complemented by *trans*-expression of wild-type *prgK*, as shown by restoration of plasmid transfer to near-wild-type levels (Fig. 1B), establishing that synthesis of the putative hydrolase is essential for assembly of a functional pCF10-encoded T4SS.

We evaluated the importance of the predicted hydrolase domains for protein function by constructing plasmids expressing *prgK* alleles (i) with coding capacity for only one or two (in any combination) of the hydrolase domains, (ii) bearing alanine codon substitutions of catalytic site residues in the SLT and/or CHAP domains, and (iii) lacking coding sequence for the first 170 residues of the predicted cytoplasmic domain. Strikingly, results of mating assays with OG1RF(pCF10 Δ prgK) donors expressing

the *prgK* variants established that nearly all of the PrgK mutant proteins retained at least some activity. As shown in Fig. 1B, donor cells producing mutant proteins deleted of one hydrolase domain transferred pCF10Δ*prgK* at frequencies of only 1 and 2 orders of magnitude lower than PrgK-producing cells in filter and broth matings, respectively. Similar results were obtained with donor cells producing PrgK_{C766A}, PrgK_{H828A}, or PrgK_{C766A/H828A}, which sustain alanine substitution mutations in the catalytic site of the CHAP domain. Cells producing the SLT catalytic site mutant, PrgK_{E573A}, transferred pCF10Δ*prgK* at lower frequencies in filter matings and were completely transfer-negative (Tra[−]) in broth matings. Cells producing PrgK_{E573A/C766A/H828A} with catalytic site mutations in both the SLT and CHAP domains were completely Tra[−] in filter and broth matings.

We next asked if PrgK mutants bearing only one of the putative hydrolase domains possessed detectable activity. Cells producing PrgK-LytM (PrgK deleted of the SLT and CHAP domains) were Tra[−] in both filter and broth matings. Interestingly, however, cells producing the PrgK-SLT or PrgK-CHAP mutant proteins efficiently transferred pCF10Δ*prgK* (10^{-2} to 10^{-3} transconjugants/donor [Tc's/D]) in filter matings. In broth matings, cells producing PrgK-SLT were Tra[−], and cells producing PrgK-CHAP transferred the plasmid at lower but detectable frequencies (10^{-5} Tc's/D) (Fig. 1B). Finally, we sought to determine whether the N-terminal cytoplasmic domain of PrgK contributes to channel assembly or activity. Interestingly, the PrgKΔ2-170 mutant supported plasmid transfer in filter matings, albeit at low frequencies (10^{-5} to 10^{-6} Tc's/D) (Fig. 1B).

To evaluate whether PrgK derivatives with attenuated function were stably produced, we fused a 3× FLAG tag to the N termini of native PrgK and several of the mutant proteins. Addition of the 3× FLAG tag did not alter the functionality of the native protein, as shown by near-wild-type plasmid transfer by cells producing the tagged protein (Fig. 1C). Addition of the 3× FLAG tag also did not affect functionality of the PrgK_{E573A} or PrgK_{E573A/C766A/H828A} variant (Fig. 1C). Strikingly, however, addition of the tag to PrgKΔ2-170 and PrgK-LytM conferred enhanced plasmid transfer compared to that of the untagged proteins (Fig. 1B and C). Cells producing 3× FLAG-PrgKΔ2-170 transferred the plasmid at levels similar to those of cells producing the native protein in both filter and broth matings. Cells producing 3× FLAG-PrgK-LytM transferred the plasmid at low but detectable levels ($\sim 10^{-6}$ Tc's/D) in filter matings. Unfortunately, despite repeated efforts, we were unable to detect 3× FLAG-PrgK or any of the tagged mutant proteins in whole-cell extracts or enriched membrane or cell wall fractions obtained from uninduced or nisin- and/or pheromone-induced cells by Western blot analyses (data not shown). While the reason for the lack of immunological detection remains unclear, results of the mating assays nevertheless suggest that the 3× FLAG tag enhances the stabilities or activities of the Δ2-170 and PrgK-LytM mutant proteins.

Export of PrgK domains to the *E. coli* periplasm suppresses cell growth. To gain further evidence for *in vivo* biological activity of the PrgK domains, we engineered *E. coli* strains to export native and catalytic-site mutant domains to the periplasm. In the absence of induction from the *P*_{tet} promoter, *E. coli* strains displayed no discernible change in growth (see Fig. S3 in the supplemental material), but upon AHT induction, all strains were strongly suppressed for growth (Fig. 2A). Export of the SLT domain even resulted in a slight but reproducible reduction in OD₆₀₀ values

within 20 to 40 min postinduction, suggestive of cell lysis. Export of the SLT_{E573A} and CHAP_{C766A/H828A} mutant domains also caused a rapid cessation of cell growth/division (Fig. 2A), which could be due to PG-binding activities of these variants (see below). As expected, we also detected a rapid suppression of cell growth in cells induced for export of the entire EC region (Fig. 2A, lower right). Due to the leakiness of the *P*_{tet} promoter, uninduced cells exported levels of the EC region sufficient to perturb growth (see Fig. S3), but AHT induction rapidly blocked growth and caused apparent cell lysis, as evidenced by an initial decrease in OD₆₀₀ values. Cells induced for export of the EC_{E573A/C766A/H828A} variant also displayed a rapid arrest in cell growth/division but without accompanying cell lysis (Fig. 2A).

As anticipated from the growth curves, cells producing the putative hydrolase domains displayed aberrant morphologies compared to the vector-only cells (Fig. 2B). Cells producing the individual domains maintained the rod shape but appeared slightly enlarged and had rough surfaces. In striking contrast, cells producing the EC region were exclusively rounded and enlarged with membrane blebs, suggestive of extensive PG degradation. The EC_{E573A/C766A/H828A} variant with catalytic-site mutations in the SLT and CHAP domains showed no visible alteration in cell shape compared to the vector-only cells (Fig. 2B).

Cells exporting the EC region to the periplasm also were more sensitive to treatment with 1% SDS (data not shown) and lysis upon exposure to H₂O than cells producing the EC_{E573A/C766A/H828A} variant or the vector-only cells (Fig. 2C). Cells exporting the native or catalytic-site mutant forms of the CHAP domain exhibited enhanced lysis in H₂O, albeit not to the extent observed with cells exporting the EC region. In contrast, the LytM domain or native or mutant SLT domain did not induce cell lysis upon hypoosmotic shock (Fig. 2C).

Export of multiple PrgK domains results in extensive PG degradation. We next explored the phenotypic consequences accompanying export of different combinations of PrgK domains to the *E. coli* periplasm. Without induction, cells cosynthesizing LytM/SLT or SLT/CHAP domain pairs displayed near-wild-type growth (see Fig. S3 in the supplemental material) and were rod shaped (data not shown). Induction of both cell types resulted in a rapid suppression of growth (Fig. 3A), cell rounding (Fig. 3B), and enhanced sensitivity to lysis in H₂O (Fig. 3C). Interestingly, cells exporting both domain pairs were pleiomorphic, with shapes including enlarged rods and round cells as well as unusual square- or rectangular-shaped cells that, to our knowledge, have not been reported previously (Fig. 3B).

Cells exporting the EC_{C766A/H828A} variant with CHAP catalytic-site mutations exhibited phenotypes resembling those of cells exporting the unmutated EC region, including growth suppression (Fig. 3A), rounding and blebbing (Fig. 3B), and hypoosmotic sensitivity (Fig. 3C). In contrast, cells exporting the EC_{E573A} variant with the SLT catalytic site mutation showed less severe growth suppression, grew as enlarged rods, and were less sensitive to lysis in H₂O (Fig. 3A, B, and C).

Taken together, our studies with *E. coli* established, first, that each putative hydrolase domain disrupts PG modeling with consequences of complete suppression of cell growth and division. Second, cosynthesis of domain pairs (LytM/SLT or SLT/CHAP) or all three domains (EC region) resulted in extensive PG degradation, as evidenced by disruption of cell shape, membrane blebbing, and enhanced lysis upon hypoosmotic shock. Third, the SLT

FIG 2 Effects accompanying secretion of PrgK domains on *E. coli* cell growth and morphology. (A) Growth curves of *E. coli* strains with pASK-IBA4 empty vector (Vector) or with plasmids encoding the exported forms of the PrgK domains. Cells were induced with AHT (200 ng/ml final concentration) at $t = 0$ for synthesis of the LytM, SLT, or CHAP domain, the EC (extracytoplasmic region with all 3 domains), or domains bearing the catalytic site mutations indicated. (B) Morphologies of cells producing exported forms of the prgK domains as shown by differential interference contrast (DIC) optics. (C) Cell lysis upon osmotic shock. Log-phase cultures of *E. coli* strains producing the PrgK domains were pelleted, and equal numbers of cells were suspended in PBS or H₂O, followed by incubation for 1 h at room temperature. The histogram presents the percentage of cell lysis (see Materials and Methods) of a representative experiment with standard deviations indicated.

domain is important for the catalytic activity of PrgK *in vivo*, as deduced from the observed effects on cell shape and hypoosmotic sensitivity accruing from export of the EC region versus the EC_{E573A} variant.

The SLT domain hydrolyzes peptidoglycan *in vitro*. We assayed for hydrolase activity *in vitro* by engineering *E. coli* cells to overproduce cytoplasmically localized, His-tagged forms of the PrgK domains for subsequent enrichment by IMAC. These strains did not display growth defects, and by IMAC we obtained highly enriched forms of the native and mutant forms of the SLT and CHAP domains but not the LytM domain or EC region (Fig. 4A, lower). The latter domains were detectable in the eluates from the

Talon resin by immunostaining with anti-His antibodies (see below), yet levels of both fragments were low compared to those for SLT and CHAP domains, and the eluates also contained numerous other protein species (Fig. 4A, lower). We were unsuccessful in our efforts to further enrich the LytM domain and EC region by repeated rounds of IMAC followed by gel filtration, possibly because of extensive degradation of both fragments (data not shown).

We subjected the IMAC-enriched PrgK domains to zymographic analyses using SDS-polyacrylamide gels containing PG from *M. luteus* cells. In these gels, PG-hydrolytic activity is detectable as a clear zone upon staining with methylene blue dye. A

FIG 3 Effects accompanying secretion of native or mutant forms of multiple PrgK domains on *E. coli* cell growth and morphology. (A) Growth curves of *E. coli* strains with pASK-IBA4 (Vector) or with plasmids encoding combinations of native or mutant PrgK domains. Cells were induced with AHT (200 ng/ml final concentration) at $t = 0$ for synthesis of the domains indicated, and OD₆₀₀ values were determined at 20-min intervals. (B) Morphologies of cells producing the prgK domains imaged by differential interference contrast (DIC) optics. (C) Cell lysis upon osmotic shock. Log-phase cultures of *E. coli* strains producing the PrgK domains were pelleted, and equal numbers of cells were suspended in PBS or H₂O, followed by incubation for 1 h at room temperature. The histogram presents the percentage of cell lysis (see Materials and Methods) of a representative experiment with standard deviations indicated.

strong zone of clearing was seen at a position corresponding to the SLT domain, and only a weak zone of clearing was evident at a position corresponding to the SLT_{E573A} mutant (Fig. 4A, upper), despite the fact that gels were loaded with comparable amounts of the native and mutant domains (Fig. 4A, lower). We also detected a weak zone of clearing at a position predicted for the LytM domain (Fig. 4A, upper, white arrow) on the basis of expected molecular size (Fig. 5). In several repetitions of these assays, we detected weak zones of clearing in lanes loaded with eluate containing the EC region, either at a position similar to that of the LytM domain (Fig. 4A, upper, white arrow) or of ~45-kDa in size (data not shown), both likely corresponding to degraded forms of the EC fragment. We were unable to detect clearing in the gels at positions of the native or mutant CHAP domains despite the fact that both domains were highly enriched by IMAC (Fig. 4A and B).

To further test for PG hydrolase activity *in vitro*, we assayed for release of fluorescently labeled PG originally obtained from *M. lysodeikticus* cells. As shown in Fig. 4B, the native SLT domain hydrolyzed PG; in contrast, the SLT_{E573A} variant, LytM or CHAP domain, or the EC region did not exhibit detectable levels of activity in this assay. Thus, the SLT domain is a catalytically active cell wall hydrolase, but our data are inconclusive for the LytM and CHAP domains.

The three hydrolase domains bind PG *in vitro*. Despite the absence of detectable hydrolase activities with the LytM and CHAP domains, results of our *in vivo* studies clearly established that both of these domains disrupt *E. coli* growth and contribute to cell rounding and hypoosmotic sensitivity when cosynthesized

with the SLT domain (Fig. 2 and 3). Thus, we tested for PG binding activity of these domains *in vitro* using PG purified from *M. luteus*. As a negative control, we also assayed for PG binding activity of a His-tagged soluble domain of the PcfC coupling protein (His-PcfCΔN103) (29). The LytM, SLT, and CHAP domains, but not His-PcfCΔN103, fractionated with PG in cosedimentation assays (Fig. 5). Interestingly, the LytM domain was detected exclusively in the PG fraction, suggestive of high-affinity binding. Appreciable amounts of the SLT and CHAP domains also cosedimented with PG. We also detected higher-molecular-mass forms of the SLT and CHAP domains migrating at positions, consistent with possible dimer forms of these domains. The catalytic-site variants of these domains migrated exclusively as predicted monomers, but both mutant domains retained PG-binding activity. Finally, we detected an immunoreactive PG-binding species in the reaction mixture containing the EC region, which, on the basis of its apparent molecular size (~45 kDa), might consist of the His-LytM/SLT fragment (Fig. 5).

Heterologous hydrolase genes complement the ΔprgK mutation. As mentioned earlier, for some Gram-negative bacterial T4SSs, heterologous hydrolases have been shown to functionally substitute for the endogenous hydrolase. We assayed for heterologous complementation of the ΔprgK mutation by *trans*-expression of three hydrolase genes associated with Gram-positive conjugation systems, *csiA*_{1CELIMG1363}, *traG*_{IP501}, and *orf14*_{Tn925}, which is identical to *orf14*_{Tn916} (Fig. 6; also see Fig. S1 in the supplemental material). Donor cells producing the heterologous hydrolases failed to transfer pCF10ΔprgK at detectable levels in liquid mat-

FIG 4 SLT causes peptidoglycan lysis *in vitro*. (A) Detection of PG hydrolase activity by zymography as described in Materials and Methods. Equivalent amounts of each Talon column eluate enriched for the PrgK domains listed were loaded onto duplicate gels and analyzed by zymography (upper) and Coomassie blue staining (lower). White arrows highlight detectable hydrolase activity in the LytM- and EC-containing lanes. MW, protein molecular weight markers with sizes (in thousands) listed on the left. (B) Lysozyme activity of each PrgK domain and catalytic site mutants as measured with the EnzChek lysozyme assay. Lysozyme activity is expressed as U/ml detected in a 100- μ l reaction mixture containing 20 μ g of protein.

ings. Interestingly, however, these donor cells transferred the plasmid in filter matings, albeit at levels ($\sim 10^{-5}$ to 10^{-6} Tc's/D) considerably lower than those observed with cells *trans*-expressing wild-type *prgK* ($\sim 10^{-1}$ Tc's/D). These findings establish that PrgK is essential for T4SS-mediated DNA transfer in liquid, whereas heterologous hydrolases can functionally substitute for PrgK upon packing of donor and recipient cells on solid surfaces.

DISCUSSION

We have shown that PrgK is a murein hydrolase required for conjugative transfer of the pheromone-responsive plasmid pCF10 in *E. faecalis*. In *Firmicutes*, hydrolases often are composed of two or more PG-binding or catalytic domains, a feature thought to be important for ensuring regulatory control of the complex events surrounding metabolism of the thick cell wall (47, 48). The T4SS-associated hydrolases in G^+ bacteria also possess multidomain architectures, in this case probably for coordinating ear-stage reactions associated with morphogenesis of the translocation channel (5). These hydrolases can be grouped into two subfamilies, the two-domain hydrolases represented by TraG_{PIP501} (20), CwlT_{ICEBs1} (18), and Orf14_{Tn916} (5) and the three-domain hydro-

FIG 5 PrgK domains bind peptidoglycan. Talon column eluates enriched for the His-tagged PrgK domains listed at the top or His-PcC Δ N103 (the soluble nucleotide-binding domain of pCF10-encoded PcfC coupling protein [29]) as a negative control were incubated with purified *M. luteus* PG. Relative amounts of the His-tagged domains in the supernatant (A) and pellet (B) fractions following centrifugation were detected by immunostaining of Western blots with anti-His antibodies. MW, protein molecular weight markers with sizes (in thousands) listed at the left.

lases represented by PrgK, CsiA_{ICELIMG1363} (25), and VirB1_{PAIS89K} (49) (see Fig. S1 in the supplemental material). Why two subfamilies of T4SS-associated hydrolases exist in G^+ species is not immediately evident, especially because here we have shown that only two of the three domains in any combination suffice for near-wild-type PrgK function in *E. faecalis* (Fig. 1). Even more strikingly, we discovered that PrgK variants with only one putative hydrolase domain supported pCF10 transfer at detectable levels (Fig. 1), as did heterologous two- or three-domain hydrolases as-

FIG 6 $\Delta prgK$ mutation can be partially complemented with hydrolase genes from other T4SSs. Donor cells carrying pCF10 or pCF10 $\Delta prgK$ and plasmids expressing the heterologous hydrolase genes indicated were assayed for plasmid transfer in 2-h filter or broth matings. Transfer frequencies are presented as the number of transconjugants per donor cell (Tc's/D). Experiments were repeated 3 times in triplicate, and the histogram depicts results of a representative experiment with standard deviations.

sociated with other G⁺ bacterial T4SSs (Fig. 6). Despite the essentiality of PrgK, evidently there is considerable flexibility with respect to the PG-binding or degradation activities required for assembly of a functional Prg/Pcf T4SS.

Our studies supplied important insights into the contributions of the LytM, SLT, and CHAP domains to machine biogenesis. With respect to the LytM domain, we were unable to detect PG hydrolase activity (Fig. 4), but LytM strongly bound PG (Fig. 5) and also conferred strong phenotypes in *E. coli* consistent with effects on cell wall modeling, e.g., cell growth suppression and formation of enlarged rod-shaped cells with rough edges (Fig. 2). Perhaps most intriguingly, we found that *E. faecalis* donor cells producing the PrgK-LytM truncation mutant failed to transfer pCF10 at detectable levels, whereas the isogenic donors producing 3× FLAG-PrgK-LytM elaborated a T4SS, albeit with attenuated function, as evidenced by a low level of plasmid transfer in filter matings (Fig. 1B and C). In view of the wild-type activity of 3× FLAG-PrgK and detectable activities of 3× FLAG-PrgK-LytM and 3× FLAG-PrgKΔ2-170 (Fig. 1C), we suspect that our inability to detect the tagged variants immunologically is due not to instability of the tagged proteins but rather to proteolytic cleavage at or near the tag junction. Regardless, the finding that 3× FLAG-LytM supports assembly of a functional T4SS argues strongly that the LytM domain can, in a specific context, supply a requisite activity for machine assembly. The 3× FLAG tag might stabilize the PrgK-LytM mutant or mediate a charge-based protein-protein contact necessary for detectable protein function, and our current studies are examining these possibilities.

Our finding that PrgK-LytM supports a detectable level of machine function is important in view of the fact that the LytM domain is a member of the subfamily of degenerate LytM domains. Active LytM proteins cleave the peptide cross-bridges of the PG cell wall via a reaction requiring Zn²⁺ binding by His and Asp residues in two conserved motifs, HX₃D and HxH (where X is any amino acid) (42, 43). The degenerate LytM proteins lack one or more of the Zn²⁺ ligands in the active site; therefore, they are catalytically inert. Studies in *E. coli* and *Bacillus subtilis* have now shown that degenerate LytM proteins play critical functions as regulators of cell growth and shape or sporulation, respectively. In *E. coli*, the degenerate LytM proteins EnvC and NlpD bind to and activate partner amidases, thereby stimulating PG hydrolysis to promote cell separation during cell division (50). During *B. subtilis* sporulation, the LytM protein SpoIIQ interacts with its partner SpoIIIA to form the core of a channel across the engulfed forespore membranes to allow passage of nutrients from the mother cell into the forespore (51, 52). In addition to PG-binding activity, therefore, degenerate LytM proteins can form protein-protein contacts important for regulation of cell wall modeling or membrane channel formation. In this context, PrgK's LytM domain might both bind PG and form intersubunit interactions of importance for Prg/Pcf channel assembly.

The SLT domain bound and hydrolyzed PG *in vitro*, and as expected, introduction of the E573A catalytic site mutation abolished the hydrolase activity (Fig. 4 and 5). The finding that both the native and mutant SLT domains strongly disrupted *E. coli* cell growth and shape determination (Fig. 2 and 3) suggests minimally that this domain exerts its *in vivo* effects through PG binding. In *E. faecalis*, however, catalytic activity of the SLT domain also appears to contribute to T4SS assembly, as evidenced by the strongly at-

tenuated plasmid transfer phenotype of the PrgK_{E573A}-producing strain (Fig. 1).

Results of our studies of the PrgK SLT domain have broader importance in view of our discovery that the PrgK homolog, CsiA_{ICELIMG1363}, also possesses an SLT domain (see Fig. S1A and B in the supplemental material). Previously, CsiA was shown to carry a C-terminal CHAP domain, as well as an 89-residue highly conserved domain (HCD), that, through mutational analysis, was shown to be essential for protein function (25). Evidence was presented that the HCD mediates localized lysis of *L. lactis* cells upon CsiA overproduction and also binds the peptidyl-D-Ala-D-Ala extremity of a PG precursor substrate. The latter binding reaction inhibited a PBP transpeptidase reaction *in vitro*, prompting a general model that CsiA promotes assembly of the ICE-encoded T4SS by inhibiting PG synthesis at the site of machine assembly (25, 53). Here, however, we determined that the HCD domain in fact is part of a larger SLT structural fold. Furthermore, we note that two CsiA mutations, K576A and R577A, phenocopied PrgK_{E573A} in blocking T4SS function in liquid matings, and that both of the mutated residues are located near the predicted active-site residue Glu570, which aligns with Glu573 in PrgK (see Fig. S1 and B). These observations and results of our PrgK characterization suggest that the CsiA SLT domain in fact is catalytic and most probably binds the peptidyl-D-Ala-D-Ala moiety *in vitro* through relaxed substrate specificity. Thus, we propose that CsiA functions similarly to PrgK in mediating T4SS assembly through PG-binding and PG degradation activities of its LytM, SLT, and CHAP domains.

Regarding PrgK's CHAP domain, our inability to detect hydrolase activity *in vitro* (Fig. 4 and 5) might be due to release of PG fragments too small for detection by zymography or the fluorescent PG release assay. Consistent with this possibility, elsewhere it has been shown that CHAP-domain proteins hydrolyze diverse PG substrates that contain a gamma-glutamyl moiety but vary with respect to the specific amide bonds cleaved (46, 54). In *E. faecalis*, PrgK_{C766A/H828A} and PrgK_{E573A}, but not PrgK_{C766A/H828A/H828A}, supported pCF10 transfer, providing genetic evidence that catalytically active forms of both the SLT and CHAP domains are important for PrgK function *in vivo*. The CHAP domains of two recently characterized hydrolases associated with G⁺ bacterial T4SSs, CwlT, encoded by *B. subtilis* ICEBs1 (18), and TraG, encoded by *S. agalactiae* pIP501 (20), were reported to exhibit hydrolase activity. The CwlT CHAP domain exhibited DL-endopeptidase activity, as detected by mass spectrometry (18), and TraG's CHAP domain was reported to have a low level of hydrolase activity on the basis of solubilization of L-[³H]lysine-labeled PG (20). These findings, coupled with our demonstration that *traG*_{pIP501} partially complemented the Δ*prgK* mutation (Fig. 6), point to a general role for catalytically active forms of CHAP domains in assembly of G⁺ bacterial T4SSs.

Finally, we think that it is biologically relevant that donor cells producing 3× FLAG-PrgK-LytM, PrgK_{E573A}, or PrgK-SLT, as well as cells producing CsiA_{ICELIMG1363}, Orf14_{Tn925}, or TraG_{pIP501}, transferred pCF10 only in filter matings (Fig. 1 and 6). Under these mating conditions, donor and recipient cells are packed on a solid surface, which favors establishment of direct cell-cell contacts. In contrast, efficient mating in liquid requires initial formation of a distant or loose donor-recipient contact that is subsequently converted to a tight mating junction that is refractory to mechanical disruption. In G⁻ bacteria, conjugative pili such as the *E. coli* F plasmid-encoded pilus initiate distant cell-cell contacts in liquid media, and then through pilus retraction cells are brought together

into tight apposition (55, 56, 57). The G^+ bacteria lack conjugative pili and likely have evolved alternative mechanisms for initiating distant donor-recipient cell contacts in aqueous environments (5, 58). It is interesting that most specialized hydrolases associated with T4SSs in G^+ species have a CHAP domain at their C terminus (5), and here we showed that the PrgK-CHAP mutant was the only single-domain variant that supported plasmid transfer in liquid matings. In future studies, it will be interesting to determine whether the multidomain hydrolases, and specifically the CHAP domains, participate not only in channel biogenesis but also in initiation or stabilization of donor-recipient cell contacts.

In sum, we have shown that PrgK is essential for biogenesis of the Prg/Pcf T4SS. Similar findings were reported for CsiA_{ICELMG1363} (25) and TraG_{PIP501} (20), whereas the *S. suis* PAI and *C. perfringens* pCW3 encode functional T4SSs even in the absence of VirB1_{PAIS89K} (49, 59) and TcpG_{pCW3} (19), respectively. The underlying factors governing the importance of specialized hydrolases for biogenesis of cognate T4SSs in both G^- and G^+ bacteria remain unknown, but in the case of *E. faecalis* pCF10 it is interesting that the *prg* and *pcf* genes are highly induced in response to donor cell perception of recipient-produced pheromone. Within 15 min of pheromone induction, the Prg and Pcf subunits build the T4SS, which then mediates very high plasmid transfer frequencies in both solid-surface and liquid matings (24). The essentiality of PrgK might be attributed to a requirement for abundant PG hydrolase activity within this brief kinetic burst of channel assembly.

ACKNOWLEDGMENTS

We thank Mayukh Sarkar for discovery of the three putative hydrolase domains of PrgK through his Phyre 2 structural modeling analysis. We thank Jessica Galloway-Pena for help with strain and plasmid constructions. We thank members of the Christie laboratory for helpful discussions. We thank Bill Margolin and his laboratory members for help with microscopy.

Studies in the Christie laboratory were supported by NIH grants 2R01GM048476 and 1R21AI105454.

REFERENCES

- Alvarez-Martinez CE, Christie PJ. 2009. Biological diversity of prokaryotic type IV secretion systems. *Microbiol. Mol. Biol. Rev.* 73:775–808. <http://dx.doi.org/10.1128/MMBR.00023-09>.
- Cascales E, Christie PJ. 2003. The versatile bacterial type IV secretion systems. *Nat. Rev. Microbiol.* 1:137–150. <http://dx.doi.org/10.1038/nrmicro753>.
- Fronzes R, Schafer E, Wang L, Saibil HR, Orlova EV, Waksman G. 2009. Structure of a type IV secretion system core complex. *Science* 323:266–268. <http://dx.doi.org/10.1126/science.1166101>.
- Chandran V, Fronzes R, Duquerroy S, Cronin N, Navaza J, Waksman G. 2009. Structure of the outer membrane complex of a type IV secretion system. *Nature* 462:1011–1015. <http://dx.doi.org/10.1038/nature08588>.
- Bhatty M, Laverde Gomez JA, Christie PJ. 2013. The expanding bacterial type IV secretion lexicon. *Res. Microbiol.* 164:620–639. <http://dx.doi.org/10.1016/j.resmic.2013.03.012>.
- Koraimann G. 2003. Lytic transglycosylases in macromolecular transport systems of Gram-negative bacteria. *Cell Mol. Life Sci.* 60:2371–2388. <http://dx.doi.org/10.1007/s00018-003-3056-1>.
- Zahl D, Wagner M, Bischof K, Bayer M, Zavec B, Beranek A, Ruckstuhl C, Zarfel GE, Koraimann G. 2005. Peptidoglycan degradation by specialized lytic transglycosylases associated with type III and type IV secretion systems. *Microbiology* 151:3455–3467. <http://dx.doi.org/10.1099/mic.0.28141-0>.
- Thunnissen AM, Isaacs NW, Dijkstra BW. 1995. The catalytic domain of a bacterial lytic transglycosylase defines a novel class of lysozymes. *Proteins* 22:245–258. <http://dx.doi.org/10.1002/prot.340220305>.
- Berger BR, Christie PJ. 1994. Genetic complementation analysis of the *Agrobacterium tumefaciens* *virB* operon: *virB2* through *virB11* are essential virulence genes. *J. Bacteriol.* 176:3646–3660.
- Hoppner C, Liu Z, Domke N, Binns AN, Baron C. 2004. VirB1 orthologs from *Brucella suis* and pKM101 complement defects of the lytic transglycosylase required for efficient type IV secretion from *Agrobacterium tumefaciens*. *J. Bacteriol.* 186:1415–1422. <http://dx.doi.org/10.1128/JB.186.5.1415-1422.2004>.
- Mushegian AR, Fullner KJ, Koonin EV, Nester EW. 1996. A family of lysozyme-like virulence factors in bacterial pathogens of plants and animals. *Proc. Natl. Acad. Sci. U. S. A.* 93:7321–7326. <http://dx.doi.org/10.1073/pnas.93.14.7321>.
- Kohler PL, Hamilton HL, Cloud-Hansen K, Dillard JP. 2007. AtlA functions as a peptidoglycan lytic transglycosylase in the *Neisseria gonorrhoeae* type IV secretion system. *J. Bacteriol.* 189:5421–5428. <http://dx.doi.org/10.1128/JB.00531-07>.
- Cloud-Hansen KA, Hackett KT, Garcia DL, Dillard JP. 2008. *Neisseria gonorrhoeae* uses two lytic transglycosylases to produce cytotoxic peptidoglycan monomers. *J. Bacteriol.* 190:5989–5994. <http://dx.doi.org/10.1128/JB.00506-08>.
- Bayer M, Bischof K, Noiges R, Koraimann G. 2000. Subcellular localization and processing of the lytic transglycosylase of the conjugative plasmid R1. *FEBS Lett.* 466:389–393. [http://dx.doi.org/10.1016/S0014-5793\(00\)01088-7](http://dx.doi.org/10.1016/S0014-5793(00)01088-7).
- Bayer M, Eferl R, Zellnig G, Teferle K, Dijkstra A, Koraimann G, Hogenauer G. 1995. Gene 19 of plasmid R1 is required for both efficient conjugative DNA transfer and bacteriophage R17 infection. *J. Bacteriol.* 177:4279–4288.
- Bayer M, Iberer R, Bischof K, Rassi E, Stabentheiner E, Zellnig G, Koraimann G. 2001. Functional and mutational analysis of p19, a DNA transfer protein with muramidase activity. *J. Bacteriol.* 183:3176–3183. <http://dx.doi.org/10.1128/JB.183.10.3176-3183.2001>.
- Fischer W, Puls J, Buhrdorf R, Gebert B, Odenbreit S, Haas R. 2001. Systematic mutagenesis of the *Helicobacter pylori* *cag* pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8. *Mol. Microbiol.* 42:1337–1348. <http://dx.doi.org/10.1046/j.1365-2958.2001.02714.x>.
- Fukushima T, Kitajima T, Yamaguchi H, Ouyang Q, Furuhashi K, Yamamoto H, Shida T, Sekiguchi J. 2008. Identification and characterization of novel cell wall hydrolase CwlT: a two-domain autolysin exhibiting n-acetylmuramidase and DL-endopeptidase activities. *J. Biol. Chem.* 283:11117–11125. <http://dx.doi.org/10.1074/jbc.M706626200>.
- Bantwal R, Bannam TL, Porter CJ, Quinsey NS, Lyras D, Adams V, Rood JJ. 2012. The peptidoglycan hydrolase TcpG is required for efficient conjugative transfer of pCW3 in *Clostridium perfringens*. *Plasmid* 67:139–147. <http://dx.doi.org/10.1016/j.plasmid.2011.12.016>.
- Arends K, Celik EK, Probst I, Goessweiner-Mohr N, Fercher C, Grumet L, Soellue C, Abajy MY, Sakin T, Broszat M, Schiwon K, Koraimann G, Keller W, Grohmann E. 2013. TraG encoded by the pIP501 type IV secretion system is a two domain peptidoglycan degrading enzyme essential for conjugative transfer. *J. Bacteriol.* 195:4436–4444. <http://dx.doi.org/10.1128/JB.02263-12>.
- Laverde Gomez JA, van Schaik W, Freitas AR, Coque TM, Weaver KE, Francia MV, Witte W, Werner G. 2011. A multiresistance megaplasmid pLGI bearing a *hlyEfm* genomic island in hospital *Enterococcus faecium* isolates. *Int. J. Med. Microbiol.* 301:165–175. <http://dx.doi.org/10.1016/j.ijmm.2010.08.015>.
- Christie PJ, Korman RZ, Zahler SA, Adsit JC, Dunny GM. 1987. Two conjugation systems associated with *Streptococcus faecalis* plasmid pCF10: identification of a conjugative transposon that transfers between *S. faecalis* and *Bacillus subtilis*. *J. Bacteriol.* 169:2529–2536.
- Roberts AP, Mullany P. 2009. A modular master on the move: the Tn916 family of mobile genetic elements. *Trends Microbiol.* 17:251–258. <http://dx.doi.org/10.1016/j.tim.2009.03.002>.
- Hirt H, Manias DA, Bryan EM, Klein JR, Marklund JK, Staddon JH, Paustian ML, Kapur V, Dunny GM. 2005. Characterization of the pheromone response of the *Enterococcus faecalis* conjugative plasmid pCF10: complete sequence and comparative analysis of the transcriptional and phenotypic responses of pCF10-containing cells to pheromone induction. *J. Bacteriol.* 187:1044–1054. <http://dx.doi.org/10.1128/JB.187.3.1044-1054.2005>.
- Stentz R, Wegmann U, Parker M, Bongaerts R, Lesaint L, Gasson M, Shearman C. 2009. CsiA is a bacterial cell wall synthesis inhibitor contributing to DNA translocation through the cell envelope. *Mol. Microbiol.* 72:779–794. <http://dx.doi.org/10.1111/j.1365-2958.2009.06683.x>.
- Leenhouts K, Buist G, Bolhuis A, ten Berge A, Kiel J, Mierau I,

- Dabrowska M, Venema G, Kok J. 1996. A general system for generating unlabelled gene replacements in bacterial chromosomes. *Mol. Gen. Genet.* 253:217–224. <http://dx.doi.org/10.1007/s004380050315>.
27. Kristich CJ, Chandler JR, Dunne GM. 2007. Development of a host-genotype-independent counterselectable marker and a high-frequency conjugative delivery system and their use in genetic analysis of *Enterococcus faecalis*. *Plasmid* 57:131–144. <http://dx.doi.org/10.1016/j.plasmid.2006.08.003>.
 28. Bryan EM, Bae T, Kleerebezem M, Dunne GM. 2000. Improved vectors for nisin-controlled expression in gram-positive bacteria. *Plasmid* 44: 183–190. <http://dx.doi.org/10.1006/plas.2000.1484>.
 29. Chen Y, Zhang X, Manias D, Yeo HJ, Dunne GM, Christie PJ. 2008. *Enterococcus faecalis* PcfC, a spatially localized substrate receptor for type IV secretion of the pCF10 transfer intermediate. *J. Bacteriol.* 190:3632–3645. <http://dx.doi.org/10.1128/JB.01999-07>.
 30. Skerra A, Schmidt TG. 2000. Use of the Strep-tag and streptavidin for detection and purification of recombinant proteins. *Methods Enzymol.* 326:271–304. [http://dx.doi.org/10.1016/S0076-6879\(00\)26060-6](http://dx.doi.org/10.1016/S0076-6879(00)26060-6).
 31. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254. [http://dx.doi.org/10.1016/0003-2697\(76\)90527-3](http://dx.doi.org/10.1016/0003-2697(76)90527-3).
 32. Li G, Howard SP. 2010. ExeA binds to peptidoglycan and forms a multimer for assembly of the type II secretion apparatus in *Aeromonas hydrophila*. *Mol. Microbiol.* 76:772–781. <http://dx.doi.org/10.1111/j.1365-2958.2010.07138.x>.
 33. Leclerc D, Asselin A. 1989. Detection of bacterial cell wall hydrolases after denaturing polyacrylamide gel electrophoresis. *Can. J. Microbiol.* 35:749–753. <http://dx.doi.org/10.1139/m89-125>.
 34. Potvin C, Leclerc D, Tremblay G, Asselin A, Bellemare G. 1988. Cloning, sequencing and expression of a *Bacillus bacteriolytic* enzyme in *Escherichia coli*. *Mol. Gen. Genet.* 214:241–248. <http://dx.doi.org/10.1007/BF00337717>.
 35. Stojkovic EA, Rothman-Denes LB. 2007. Coliphage N4 N-acetylmuramidase defines a new family of murein hydrolases. *J. Mol. Biol.* 366:406–419. <http://dx.doi.org/10.1016/j.jmb.2006.11.028>.
 36. Helal R, Melzig MF. 2008. Determination of lysozyme activity by a fluorescence technique in comparison with the classical turbidity assay. *Pharmazie* 63:415–419.
 37. Kelley LA, Sternberg MJ. 2009. Protein structure prediction on the Web: a case study using the Phyre server. *Nat. Protoc.* 4:363–371. <http://dx.doi.org/10.1038/nprot.2009.2>.
 38. Schultz J, Copley RR, Doerks T, Ponting CP, Bork P. 2000. SMART: a web-based tool for the study of genetically mobile domains. *Nucleic Acids Res.* 28:231–234. <http://dx.doi.org/10.1093/nar/28.1.231>.
 39. Letunic I, Doerks T, Bork P. 2012. SMART 7: recent updates to the protein domain annotation resource. *Nucleic Acids Res.* 40:D302–D305. <http://dx.doi.org/10.1093/nar/gkr931>.
 40. Petersen TN, Brunak S, von Heijne G, Nielsen H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* 8:785–786. <http://dx.doi.org/10.1038/nmeth.1701>.
 41. Ramadurai L, Jayaswal RK. 1997. Molecular cloning, sequencing, and expression of *lytM*, a unique autolytic gene of *Staphylococcus aureus*. *J. Bacteriol.* 179:3625–3631.
 42. Firczuk M, Mucha A, Bochtler M. 2005. Crystal structures of active LytM. *J. Mol. Biol.* 354:578–590. <http://dx.doi.org/10.1016/j.jmb.2005.09.082>.
 43. Cohen DN, Sham YY, Haugstad GD, Xiang Y, Rossmann MG, Anderson DL, Popham DL. 2009. Shared catalysis in virus entry and bacterial cell wall depolymerization. *J. Mol. Biol.* 387:607–618. <http://dx.doi.org/10.1016/j.jmb.2009.02.001>.
 44. Xiang Y, Morais MC, Cohen DN, Bowman VD, Anderson DL, Rossmann MG. 2008. Crystal and cryoEM structural studies of a cell wall degrading enzyme in the bacteriophage phi29 tail. *Proc. Natl. Acad. Sci. U. S. A.* 105:9552–9557. <http://dx.doi.org/10.1073/pnas.0803787105>.
 45. Rossi P, Aramini JM, Xiao R, Chen CX, Nwosu C, Owens LA, Maglaqui M, Nair R, Fischer M, Acton TB, Honig B, Rost B, Montelione GT. 2009. Structural elucidation of the Cys-His-Glu-Asn proteolytic relay in the secreted CHAP domain enzyme from the human pathogen *Staphylococcus saprophyticus*. *Proteins* 74:515–519. <http://dx.doi.org/10.1002/prot.22267>.
 46. Bateman A, Rawlings ND. 2003. The CHAP domain: a large family of amidases including GSP amidase and peptidoglycan hydrolases. *Trends Biochem. Sci.* 28:234–237. [http://dx.doi.org/10.1016/S0968-0004\(03\)00061-6](http://dx.doi.org/10.1016/S0968-0004(03)00061-6).
 47. Layec S, Decaris B, Leblond-Bourget N. 2008. Diversity of Firmicutes peptidoglycan hydrolases and specificities of those involved in daughter cell separation. *Res. Microbiol.* 159:507–515. <http://dx.doi.org/10.1016/j.resmic.2008.06.008>.
 48. Lopez R, Gonzalez MP, Garcia E, Garcia JL, Garcia P. 2000. Biological roles of two new murein hydrolases of *Streptococcus pneumoniae* representing examples of module shuffling. *Res. Microbiol.* 151:437–443. [http://dx.doi.org/10.1016/S0923-2508\(00\)00172-8](http://dx.doi.org/10.1016/S0923-2508(00)00172-8).
 49. Li M, Shen X, Yan J, Han H, Zheng B, Liu D, Cheng H, Zhao Y, Rao X, Wang C, Tang J, Hu F, Gao GF. 2011. GI-type T4SS-mediated horizontal transfer of the 89K pathogenicity island in epidemic *Streptococcus suis* serotype 2. *Mol. Microbiol.* 79:1670–1683. <http://dx.doi.org/10.1111/j.1365-2958.2011.07553.x>.
 50. Peters NT, Morlot C, Yang DC, Uehara T, Vernet T, Bernhardt TG. 2013. Structure-function analysis of the LytM domain of EnvC, an activator of cell wall remodelling at the *Escherichia coli* division site. *Mol. Microbiol.* 89:690–701. <http://dx.doi.org/10.1111/mmi.12304>.
 51. Meisner J, Maehigashi T, Andre I, Dunham CM, Moran CP, Jr. 2012. Structure of the basal components of a bacterial transporter. *Proc. Natl. Acad. Sci. U. S. A.* 109:5446–5451. <http://dx.doi.org/10.1073/pnas.1120113109>.
 52. Meisner J, Moran CP, Jr. 2011. A LytM domain dictates the localization of proteins to the mother cell-forespore interface during bacterial endospore formation. *J. Bacteriol.* 193:591–598. <http://dx.doi.org/10.1128/JB.01270-10>.
 53. Stentz R, Bongaerts RJ, Gunning AP, Gasson M, Shearman C. 2010. Controlled release of protein from viable *Lactococcus lactis* cells. *Appl. Environ. Microbiol.* 76:3026–3031. <http://dx.doi.org/10.1128/AEM.00021-10>.
 54. Rigden DJ, Jedrzejewski MJ, Galperin MY. 2003. Amidase domains from bacterial and phage autolysins define a family of gamma-D,L-glutamate-specific amidohydrolases. *Trends Biochem. Sci.* 28:230–234. [http://dx.doi.org/10.1016/S0968-0004\(03\)00062-8](http://dx.doi.org/10.1016/S0968-0004(03)00062-8).
 55. Lawley TD, Klimke WA, Gubbins MJ, Frost LS. 2003. F factor conjugation is a true type IV secretion system. *FEMS Microbiol. Lett.* 224:1–15. [http://dx.doi.org/10.1016/S0378-1097\(03\)00430-0](http://dx.doi.org/10.1016/S0378-1097(03)00430-0).
 56. Christie PJ. 2004. Type IV secretion: the *Agrobacterium* VirB/D4 and related conjugation systems. *Biochim. Biophys. Acta* 1694:219–234. <http://dx.doi.org/10.1016/j.bbamcr.2004.02.013>.
 57. Clarke M, Maddera L, Harris RL, Silverman PM. 2008. F-pili dynamics by live-cell imaging. *Proc. Natl. Acad. Sci. U. S. A.* 105:17978–17981. <http://dx.doi.org/10.1073/pnas.0806786105>.
 58. Abajy MY, Kopec J, Schiwon K, Burzynski M, Doring M, Bohn C, Grohmann E. 2007. A type IV-secretion-like system is required for conjugative DNA transport of broad-host-range plasmid pIP501 in gram-positive bacteria. *J. Bacteriol.* 189:2487–2496. <http://dx.doi.org/10.1128/JB.01491-06>.
 59. Zhao Y, Liu G, Li S, Wang M, Song J, Wang J, Tang J, Li M, Hu F. 2011. Role of a type IV-like secretion system of *Streptococcus suis* 2 in the development of streptococcal toxic shock syndrome. *J. Infect. Dis.* 204:274–281. <http://dx.doi.org/10.1093/infdis/jir261>.
 60. Kristich CJ, Manias DA, Dunne GM. 2005. Development of a method for markerless genetic exchange in *Enterococcus faecalis* and its use in construction of a *srtA* mutant. *Appl. Environ. Microbiol.* 71:5837–5849. <http://dx.doi.org/10.1128/AEM.71.10.5837-5849.2005>.
 61. Staddon JH, Bryan EM, Manias DA, Chen Y, Dunne GM. 2006. Genetic characterization of the conjugative DNA processing system of enterococcal plasmid pCF10. *Plasmid* 56:102–111. <http://dx.doi.org/10.1016/j.plasmid.2006.05.001>.
 62. Dunne GM, Brown BL, Clewell DB. 1978. Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. *Proc. Natl. Acad. Sci. U. S. A.* 75:3479–3483. <http://dx.doi.org/10.1073/pnas.75.7.3479>.
 63. Dunne G, Funk C, Adsit J. 1981. Direct stimulation of the transfer of antibiotic resistance by sex pheromones in *Streptococcus faecalis*. *Plasmid* 6:270–278. [http://dx.doi.org/10.1016/0147-619X\(81\)90035-4](http://dx.doi.org/10.1016/0147-619X(81)90035-4).
 64. Chandler JR, Flynn AR, Bryan EM, Dunne GM. 2005. Specific control of endogenous cCF10 pheromone by a conserved domain of the pCF10-encoded regulatory protein PrgY in *Enterococcus faecalis*. *J. Bacteriol.* 187: 4830–4843. <http://dx.doi.org/10.1128/JB.187.14.4830-4843.2005>.