J. Microbiol. Biotechnol. (2017), 27(4), 791–807
https://doi.org/10.4014/jmb.1611.11002

jmb

Native and Foreign Proteins Secreted by the *Cupriavidus metallidurans* Type II System and an Alternative Mechanism S

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Received: November 1, 2016
Revised: January 17, 2017
Accepted: January 24, 2017

First published online
January 24, 2017

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Supplementary data for this paper are available on-line only at http://jmb.or.kr.

pISSN 1017-7825, eISSN 1738-8872

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The type II secretion system (T2SS), which transports selected periplasmic proteins across the outer membrane, has rarely been studied in nonpathogens or in organisms classified as Betaproteobacteria. Therefore, we studied *Cupriavidus metallidurans* (*Cme*), a facultative chemilithoautotroph. Gel analysis of extracellular proteins revealed no remarkable differences between the wild type and the T2SS mutants. However, enzyme assays revealed that native extracellular alkaline phosphatase is a T2SS substrate, because activity was 10-fold greater for the wild type than a T2SS mutant. In *Cme* engineered to produce three *Ralstonia solanacearum* (*Rso*) exoenzymes, at least 95% of their total activities were extracellular, but unexpectedly high percentages of these exoenzymes remained extracellular in T2SS mutants cultured in rich broth. These conditions appear to permit an alternative secretion process, because neither cell lysis nor periplasmic leakage was observed when *Cme* produced a *Pectobacterium carotovorum* exoenzyme, and wild-type *Cme* cultured in minimal medium secreted 98% of *Rso* polygalacturonase, but 92% of this exoenzyme remained intracellular in T2SS mutants. We concluded that *Cme* has a functional T2SS despite lacking any abundant native T2SS substrates. The efficient secretion of three foreign exoenzymes by *Cme* is remarkable, but so too is the indication of an alternative secretion process in rich culture conditions. When not transiting the T2SS, we suggest that *Rso* exoenzymes are probably selectively packaged into outer membrane vesicles. Phylogenetic analysis of T2SS proteins supports the existence of at least three T2SS subfamilies, and we propose that *Cme*, as a representative of the Betaproteobacteria, could become a new useful model system for studying T2SS substrate specificity.

Keywords: Type II secretion system, protein secretion, endoglucanase, polygalacturonase, pectin methyl esterase, alkaline phosphatase, outer membrane vesicles, phylogeny

Introduction

The type II secretion system (T2SS) is one of multiple mechanisms that gram-negative bacteria use to transport proteins across the outer membrane [1–3]. Unlike most other secretion systems, where proteins move in one step from the cytoplasm to their destination, the T2SS is part of a two-step process. Like many proteins, T2SS substrates are first exported to the periplasm by the widely conserved Sec-dependent or twin-arginine translocation (Tat) pathways [4, 5]. However, unlike the many dedicated periplasmic proteins, a small percentage of proteins is subsequently secreted by the T2SS to the cell surface or into the extracellular milieu. The number of known T2SS substrates varies widely among organisms, from one to more than 20 [6–8], and we will refer to them as exoproteins even when they are present intracellularly.

The nanoscale T2SS machine, or secreton, comprises a minimum of 11 general secretion pathway (Gsp) proteins plus a prepilin peptidase [6, 7, 9, 10]. The genes encoding Gsp proteins are usually organized into one or two operons contained within a single contiguous gene cluster [6, 11].

GspC, F, L, and M form a "platform" in the inner membrane and probably transduce energy provided by GspE, a cytoplasmic ATPase. GspG, H, I, J, and K are pseudopilins, so-named due to their sequence similarity, processing by a specific prepilin peptidase, and functional similarity to pilins that form a type IV pilus [9, 10, 12]. In some bacteria, the prepilin peptidase is encoded by *gspO*, which is part of the T2SS gene cluster, whereas in other bacteria the remote *pilD* gene encodes a dual-function prepilin peptidase that processes both type IV pilins and T2SS pseudopilins [7, 10, 13]. GspG is the major pseudopilin and forms the pilus fiber [14] integral with the inner membrane platform, and GspH, I, J, and K are minor pseudopilins that decorate the pilus tip [15, 16] and/or participate in initiation of pseudopilus assembly [17]. Multimers of GspD, a member of the secretin family [18], form a gated pore in the outer membrane through which proteins exit. Parts of the periplasmic domains of GspD physically interact with one or more regions of GspC, a bitopic inner membrane protein, the bulk of which spans the periplasm [19–22]. Current models suggest that exoproteins are recognized by specific physical interactions with GspC, GspD, and the tip of the nascent pseudopilus, which then elongates to push exoproteins through the secretin's pore [6, 7, 17, 18, 23].

The T2SS is present in many gram-negative bacteria in the phylum *Proteobacteria* [7, 8, 11]. This secretion system has primarily been studied in organisms classified as Gammaproteobacteria, where model species include the plant pathogens *Dickeya dadantii* (*Dda*; ex. *Erwinia chrysanthemi*), *Pectobacterium carotovorum* (ex. *Erwinia carotovora*), and *Xanthomonas campestris*, and the human pathogens *Klebsiella oxytoca* (*Kox*), *Pseudomonas aeruginosa* (*Pae*), and *Vibrio cholerae*. The T2SS has occasionally been demonstrated in bacteria classified as Betaproteobacteria, where the most studied organism is the plant pathogen *Ralstonia solanacearum* (*Rso*) [24], a member of the Burkholderiaceae family. Seven known exoenzymes, some of which contribute to virulence, are known to be substrates of the *Rso* T2SS [25, 26], which also secretes over 20 additional exoproteins [27] that in some combination are essential for pathogenesis [25].

The presence of a T2SS in nonpathogenic bacteria has been reported less frequently, but it probably serves an important role for many such organisms [8, 28]. One such bacterium could be *Cupriavidus metallidurans* (*Cme*) strain CH34, a well-studied facultative chemilithoautotroph that is a model organism for investigating how bacteria tolerate toxic metals [29–31]. Recently, *Cme* strains have also been isolated from clinical settings [32]. Originally described as *Alcaligenes eutrophus* [33], *Cme* is a Betaproteobacteria in the Burkholderiaceae that is closely related to *Rso* [30], because their main replicons are highly syntenic [31]. Moreover, the megaplasmid in *Rso* strain GMI1000 carries some *Cme*-like metal resistance genes [34], and CH34 *phcA* can functionally replace its ortholog in the *Rso* Phc quorum sensing system [35].

We are unaware of any publications describing a T2SS in *Cme*, although its genome appears to encode one [31]. The primary objectives of this study were to determine if *Cme* CH34 has a functional T2SS and whether it will secrete *Rso* exoproteins. We did this by examining total extracellular proteins produced by CH34 and mutants lacking essential T2SS components, using gel electrophoresis and testing for secretion of selected candidate exoenxymes. CH34 appeared to secrete no abundant native proteins via its T2SS system, but secreted one of two native PhoA-like alkaline phosphatases (APases) and all three *Rso* exoenzymes tested.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

Table 1 describes the strains and plasmids used. *Escherichia coli* strains were incubated at 30°C (broth) or 37°C (plates), whereas *Cme* and *Rso* were incubated at 30°C. Rich broth media were LB [36], BG [37], and BY (which is the same as BG with 0.5% glycerol substituted for glucose). When supernatant proteins were to be analyzed on gels, the dialysate of concentrated B broth was used to prepare BY. Liquid minimal media were MM [37]; MMP, which has 10 mM K_2HPO_4, 5.5 mM NaH_2PO_4, 5 mM $(NH_4)_2SO_4$, 2.7 mM sodium citrate, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and Ho-Le trace elements [38]; and M963, which has 51 mM K_2HPO_4, 21 mM NaH_2PO_4, 15 mM $(NH_4)_2SO_4$, 17 mM NaCl, 2.7 mM Na citrate, 1 mM $MgSO_4$, and 0.1 mM $CaCl_2$. Filter-sterilized carbon sources were added to the sterile media: MMP had 0.1% (w/v) glycerol; and M963 had 0.5% (w/v) potassium gluconate. The liquid media were solidified with 1.5% agar when desired. The antibiotics used were chloramphenicol (Cm, 50 μg/ml), kanamycin (Km, 25–50 μg/ml), tetracycline (Tc, 5–15 μg/ml), or trimethoprim (Tp, 50–100 μg/ml) unless indicated otherwise.

Plasmid and Mutant Construction

Standard methods were used for DNA manipulation and transformation [25]. Derivatives of CH34 with unmarked deletions of *gspD*, *gspC*, and *phoA1/phoA2* were created as described previously [25] with the following changes. The regions flanking these genes were PCR-amplified from CH34 genomic DNA using Epicentre MasterAmp *Tfl* polymerase and the primers described in Table S1, and the products were gel purified. The Δ*gspD* "deletion allele" was created by using an In-Fusion Advantage kit (Clontech) to join both amplimers and SmaI-digested pEX18Tc DNA in one reaction. *E. coli* DH5α was transformed with the

Table 1. Strains and plasmids used in this study.

Strain, plasmid	Relevant characteristics[a]	Source
Strains		
Escherichia coli		
DH5α	Plasmid host	Invitrogen
JM107	Plasmid host, Tp^s	[98]
NEB5α	Plasmid host	New England Biolabs
Cupriavidus metallidurans		
CH34	Wild type, Tc^s Tp^s Ap^r Cm^r Gm^r Km^r Sp^r	[33]
CH34ΔD	Unmarked deletion of *gspD* (ABF10264) in CH34	This work
CH34ΔC	Unmarked deletion of *gspC* (ABF10253) in CH34	This work
Ralstonia solanacearum		
GMI-E	GMI1000 *epsB*::pCR2.1, Km^r	M.A. Schell
GMI-D	GMI1000 *gspD*::pTOK2, Tc^r	[25]
GMI-ED	GMI1000 *epsB gspD*, Km^r Tc^r	This work
Plasmids		
pLAFR3	Broad-host-range cosmid vector, *oriT* IncP, Tc^r	[73]
pBBR1MCS-2	Broad-host-range vector, pBBR1 ori, compatible with IncP plasmids, Km^r	[99]
pSCrhaB2	Broad-host-range vector, pBBR1 ori, Tp^r	[100]
pEX18Tc	Gene-replacement vector, pBR322 ori, *oriT*+ *sacB*+, Tc^r	[101]
pDONR-SacTet	Gene-replacement vector, pBR322 ori, *sacB*+, Km^r Tc^r	[102]
pTOK2	Suicide vector, pBR322 ori, Tc^r	[103]
pJE8	*R. solanacearum pglA* (and other genes) in pLAFR3	[104]
pMB2	*R. solanacearum egl* and *pme* (and other genes) in pLAFR3	M. A. Schell
pEX-ΔD	*C. metallidurans gspD* deletion allele in pEX18Tc	This work
pST-ΔC	*C. metallidurans gspC* deletion allele in pDONR-SacTet	This work
pEX-ΔphoA1/2	*C. metallidurans phoA1/A2* deletion allele in pEX18Tc	This work
pBBR-CmeC	*C. metallidurans gspC* wild-type allele in pBBR1MCS-2	[87]
pSC-GspC	*C. metallidurans gspC* wild-type allele in pSCrhaB2	This work
pSC-PehA	Promoterless *P. atrosepticum pehA* in pSCrhaB2	This work
pGA91-lacZ	pLAFR3 with *phcA95*::Tn*3*HoHo1, LacZ+, Ap^r	[105]

[a]Ap, Cm, Gm, Km, Sp, Tc, Tp = ampicillin, chloramphenicol, gentamicin, kanamycin, spectinomycin, tetracycline, and trimethoprim, respectively. *pglA* encodes an endopolygalacturonase, *egl* encodes a β-1,4-endoglucanase, *pme* encodes a pectin methylesterase, *pehA* encodes an endopolygalacturonase, and *sacB* encodes a levan sucrase.

reaction mixture and selected on LB Tc plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The Δ*gspC* "deletion allele" was created using Gateway BP Clonase II (Life Technologies Corp.) to introduce both amplimers into undigested pDONR-SacTet DNA in one reaction, which was used to transform *E. coli* DH5α followed by selection on LB Km Tc plates. The D*phoA1/phoA2* "deletion allele" was created by first digesting both amplimers with KpnI, spin-column purifying the products, and then ligating them. This reaction was used as the template for PCR amplification with primers PhoAF1 and PhoAR4, and the product was then digested with BamHI, mixed with BamHI-digested pEX18Tc DNA, and spin-column purified. After ligation, the reaction was used for transformation of NEB5α and selection on LB Tc plates. Clones with the correct constructs (pEX-ΔD, pST-ΔC, pEX-DA1-A2) were retained and the deletions were introduced by homologous recombination into the CH34 genome [25]. Mutations were confirmed by restriction endonuclease digestions and sequencing of the cloned plasmids and by PCR from genomic DNA of the mutants using diagnostic primers. The *epsB gspD* mutant of *Rso* GMI1000 was generated by transforming GMI-E, which lacks extracellular polysaccharide, with genomic DNA from GMI-D followed by selection for Tc and Km resistance.

Transformants were screened for the inability to secrete multiple exoenzymes, and one EPS-negative T2SS mutant was retained as GMI-ED.

A wild-type copy of *Cme gspC* and its native promoter was subcloned from pBBR-CmeC by digesting the plasmid with KpnI and HindIII, ligating the gel-purified product to similarly digested pSCrhaB2, transforming *E. coli* JM107, and selecting on LB Tp plates. The *P. atrosepticum pehA* gene without its native promoter was PCR-amplified from *P. atrosepticum* strain SCRI1043 genomic DNA using the primers described in Table S1. The amplicon was digested with BamHI and XbaI, gel purified, and ligated to similarly digested pSCrhaB2, which positioned *pehA* so that it is expressed from the vector's rhamnose-inducible promoter. After electroporating NEB5α, transformants were selected on LB plates containing 200 μg/ml Tp. Strains with the desired constructs (pSC-GspC and pSC-PehA) were identified using a combination of partial DNA sequencing and restriction endonuclease digestions.

Enzyme Assays

Unless noted, cells were cultured in BY broth, starting at 1 × 10^6 CFU/ml, for 40 to 48 h at 250 rpm on an orbital shaker (reaching stationary phase, about 4 × 10^9 cells/ml) and 1.0 ml of culture was centrifuged (13,000 ×*g* for 5 min at room temperature). The supernatant was retained and the cells were washed with 1 ml of 0.1 M NaCl and resuspended in 1.0 ml of 10 mM $NaKPO_4$ buffer (pH 7); samples were stored at −20°C until assayed. Cells were permeabilized immediately before assaying by adding SDS (0.003% final concentration) and chloroform (6% final concentration) and vortexing vigorously for 10 sec. This treatment is commonly used for assaying cellular β-galactosidase [36] and APases activities [39], and our preliminary tests using culture supernatants containing *Rso* PglA or Egl and Pme and *P. atrosepticum* PehA exoenzymes showed that it did not affect their activity. Total cellular protein concentration was determined using BCA reagent [40].

Endopolygalacturonase (PglA and PehA) and Egl activities were quantified by measuring the reducing sugars released from 0.5% (w/v) polygalacturonic acid and 1.5% (w/v) carboxymethyl cellulose, respectively [40]. Samples of each reaction mixture taken at time 0 were used for background subtraction, except when 0.5% rhamnose was added to the broth to induce *pehA* expression from pSC-PehA. In this case, to compensate for the background created by this reducing sugar, which increased with time, we used supernatants in which PehA was heat-inactivated (1 min at 100°C) to make "blank" time-course reactions. Sample volumes and reaction times were adjusted to maintain first-order kinetics. The Pme assay [37] was modified as follows. A solution of 0.5% (w/v) pectin, 50 mM NaCl, 2 mM each Tris and $CaCl_2$, and 0.004% (w/v) bromocresol purple was adjusted to pH 6.8 with 0.1 N NaOH, and 250 μl was added to tubes held in ice-cold water. Cold supernatant or permeabilized cells (≤50 μl) and cold water were added to give a 300 μl reaction volume. Tubes were transferred en masse to a 37°C water bath, and the minutes required for the pH of the reaction to reach 5.2, as indicated by the change from purple to yellow color, was recorded. Activity, given as pmole H^+ released per minute per microliter, was calculated with the formula [(reaction vol. ÷ supernatant vol.)(6.15 pmole H^+/μl)] ÷ minutes, and the results were corrected for the concentration of total cell protein.

Reaction mixtures for β-galactosidase activity [36] contained 0.1 ml of appropriately diluted supernatant or permeabilized cells (see above) mixed with 0.1 ml of double-strength Z-buffer containing 0.12 mg of *o*-nitrophenyl-β-galactoside in ice-cold tubes. Tubes were transferred en masse to a 37°C water bath to initiate the reaction, which was later terminated by adding 0.1 ml of 1 M Na_2CO_3 and moved to ice. Reaction mixtures for APase activity [39] contained 70 μl of appropriately diluted supernatant or frozen/thawed cells (not permeabilized) mixed with 56 μl of 1.0 M Tris-HCl, pH 8.0, plus 0.2 mM $ZnSO_4$ and 14 μl of 0.4% *p*-nitrophenyl phosphate added to start the reaction. Reactions were stopped by adding 14 μl of the standard stop buffer and placed on ice. Reaction tubes for both assays were centrifuged to pellet cell debris before reading the supernatants' A_{420}, and unit calculations used the OD_{600} values of cultures read before processing instead of using the OD_{550} reading of the reaction tube.

Gel Analysis of Total Extracellular Proteins

General methods for sample preparation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were described previously [25]. Briefly, cultures were shaken for about 48 h (to stationary phase, 2-4 × 10^9 cells/ml), and proteins in cell-free supernatants were precipitated by adding one tenth volumes of both 1.0% (w/v) sodium deoxycholate and 100% trichloroacetic acid and incubating on ice for 30 min. The precipitate was collected by centrifugation and the pellets were washed three times with ice-cold 100% acetone. The pellet was solubilized by adding a small volume of 5 mM NaOH and then, after about 1 min of gentle agitation, an equal volume of 50 mM $NaKPO_4$ buffer (pH 6.5). Samples were mixed with 3× Laemmli sample buffer (Bio-Rad), heated 5 min at 100°C, cooled, and centrifuged to remove insoluble material. Samples representing equal volumes of original supernatants were run either on 0.75-mm-thick mini-gels cast using EZ-Run 12.5% Protein Gel Solutions (Fischer BioReagents, USA) or on precast 10−20% gradient Criterion gels (Bio-Rad, USA) using the recommended buffers and running conditions, and were then stained with silver.

To localize enzyme activity within SDS-PAGE gels, supernatants were concentrated about 60-fold by ultrafiltration (Spin-X UF 500 concentrator with 30 kDa cut-off; Corning, USA) and desalted using 10 mM $NaKPO_4$ buffer (pH 7). Samples were mixed with 3× Laemmli buffer lacking β-mercaptoethanol, heated at 37°C for 15 min, and centrifuged before being resolved on an EZ-Run mini-gel. Afterwards, proteins were renatured [41] by agitating the gels in 200 ml of 10 mM Tris (pH 6.8) plus 0.4% (v/v) Triton X-100 three times for 30 min each, and then in 50 mM $NaKPO_4$ buffer (pH 7) for 30 min. An enzyme assay overlay (1 mm thick, 1% agarose containing 0.5% (w/v) of substrate, previously cast

between two glass plates), was cut to size and placed on top of a gel in a dry Petri dish. The sandwich was incubated overnight at 37°C at 100% relative humidity, after which the overlay was removed and "developed" as described by Liu *et al.* [25] to reveal zones of exoenzyme activity.

Results

Cme Has a Putative T2SS

A search of the CH34 genome (in four replicons: Accession Nos. NC_007971 to NC_007974) revealed one *gsp* gene cluster (Accession Nos. ABF10253 and 10256-10266) that should encode all the proteins for a functional T2SS, except a prepilin peptidase (Fig. 1A). The presumptive *Cme* PilD prepilin peptidase (ABF09982), which is 52% identical to the *Kox* PulO T2SS prepilin peptidase and 59% or 65% identical to the *Pae* and *Rso* PilD dual-function prepilin peptidases, respectively, is encoded at a remote site by the terminal gene in a cluster that includes the *pilC* and *pilB* genes essential for type IV pilus production.

Pairwise alignment of the putative *Cme* GspC to N proteins with well-characterized PulC to PulN proteins in *Kox*, a model gammaproteobacterium, using BLAST with default parameters [42] (Fig. 1A), revealed a wide range of sequence conservation that was generally less than expected [13]. The most conserved *Cme* proteins (41–56% identical over >88% of their length) are the GspE ATPase, GspF, which is part of the inner membrane platform, and the GspG major pseudopilin. Most other *Cme* proteins are 25–31% identical to their Pul orthologs over a majority of their sequence. In contrast, only small regions (9–30%) of *Cme* GspC, H, L, and N proteins align with their Pul orthologs.

Cme Gsp proteins were more consistently homologous (43–90% identical over >87% of their length) to those in *Rso*, a closely related betaproteobacterium (Fig. 1A). The exception to this pattern was *Cme* GspC, for which only 53% of the protein (amino acids 18–124 of 204 residues total) aligned with *Rso* GspC. Significantly, amino acids 50 to 126 of *Cme* GspC correspond to the accepted GspC protein family homology region (HR) [19, 43, 44]. A search of the Conserved Domain Database [45] showed that, as expected [9, 22], this region is classified as a member of the Pilus_PilP super family (pfam11356), and according to a Jpred secondary structure prediction [46], it should fold

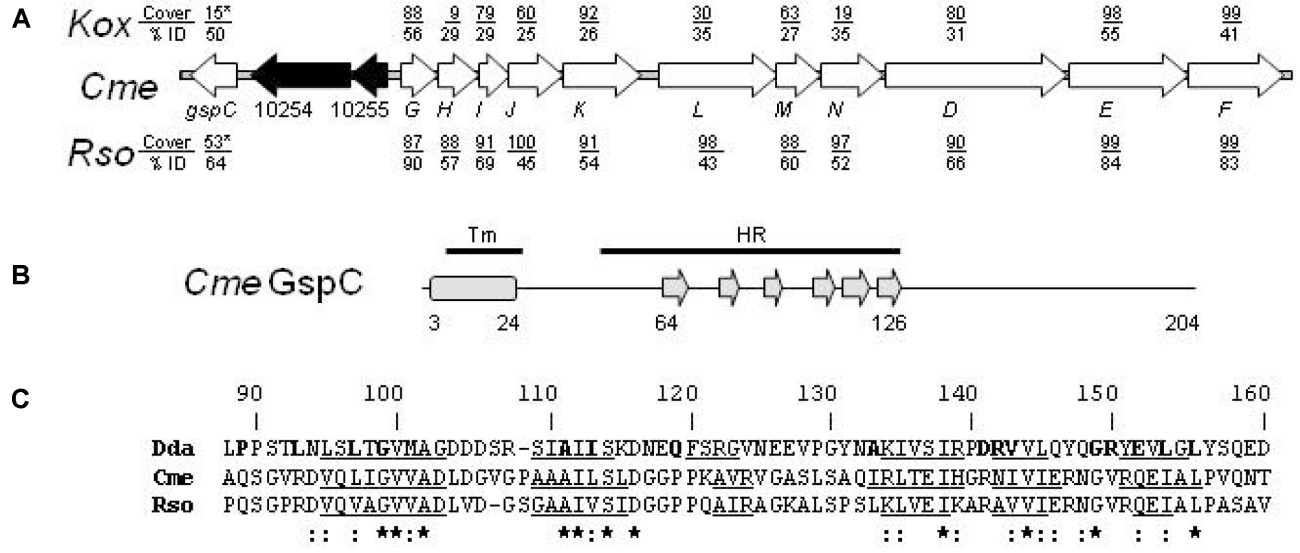

Fig. 1. Schematic showing *C. metallidurans* T2SS gene cluster, predicted *C. metallidurans* GspC secondary structures, and amino acid alignment in the homology region (HR) domain.

(**A**) The organization of T2SS genes (white arrows) in *Cme* strain CH34. Numbers are the percentage coverage (Cover) and the percentage identity (% ID) of *Cme* with either *Kox* or *Rso* proteins determined using BLAST; the asterisk indicates that homology is only in the N-terminal part of the GspC proteins (*e.g.*, *Cme* GspC amino acids 11–127). The *Cme* gene cluster (Accession No. ABF10253 to ABF10266) includes two unrelated genes (black arrows). (**B**) Predicted α helix (box) and six β strands (shaded arrows) in CH34 GspC. The probable transmembrane domain (Tm) and HR are indicated by lines above the schematic. Numbers indicate relevant amino acids. (**C**) Alignment of amino acids in the HR domain of *Dda* OutC with that of GspC in *Cme* CH34 and *Rso* GMI1000. Numbering above is for OutC and the amino acids in bold font are those best conserved in Gammaproteobacteria according to Gu *et al*. [22]. Underlined sequences are those demonstrated for OutC or predicted for *Cme* and *Rso* GspC by Jpred analysis to fold into β strands. Identical (*) and strongly conserved (:) amino acids are indicated below the alignment.

into six β strands (Fig. 1B), analogous to those in model GspC proteins [20, 22]. Crystallographic analysis of the *E. coli* ETEC GspC-GspD complex and solution structure analysis of *Dda* OutC indicated that these six β strands fold into two anti-parallel β sheets [20, 22], one of which reportedly interacts physically with GspD [19-22, 44]. In addition, amino acid alignment of the extensively studied *Dda* OutC with *Cme* and *Rso* GspC (Fig. 1C) showed that the latter two proteins have identical or conserved substitutions at four of the five amino acids most important for OutC function (Gly^{99}, Val^{100}, Ile^{113}, Val^{143}, and Val^{153}) [21, 22, 47]. The less homologous C-terminal portions of *Cme* and *Rso* GspC were predicted (using Jpred, COILS [48], and a conserved domain search) to have no secondary structures, unlike the C-terminal PDZ or coiled-coil domains typical of GspC proteins in most well-studied Gammaproteobacteria [6, 7, 12, 43, 44, 49, 50].

Gene organization in the *Cme* T2SS gene cluster (Fig. 1A) is unlike that in model Gammaproteobacteria gene clusters [6, 11], because *gspC* is transcribed divergently from other *gsp* genes rather than being located immediately upstream of and aligned with *gspD*. Instead, *Cme gspN* is located where *gspC* often is. We are confident in our gene assignments, because conserved domain searches and secondary structure predictions for *Cme* GspN and *Kox* PulN show that they are similar to each other and distinctly different from GspC and PulC (data not shown). The T2SS gene cluster in CH34 is also unusual because it has two unrelated genes between *gspC* and *gspG*; the *Rso* gene cluster has no intervening genes, but is otherwise organized the same as in *Cme*.

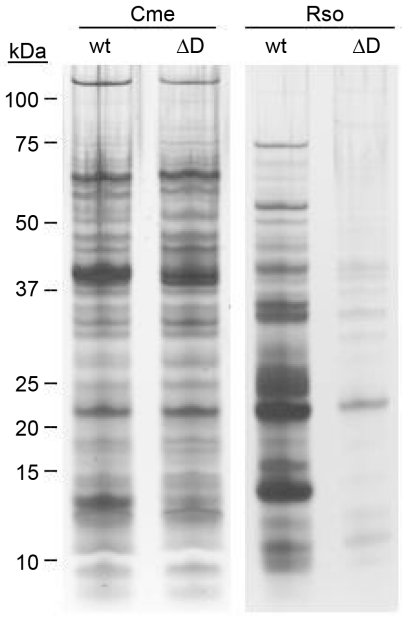

Fig. 2. Gel electrophoretic analysis of extracellular proteins from *C. metallidurans* CH34 and *R. solanacearum* GMI1000.
Wild-type (wt) and Δ*gspD* mutant (ΔD) strains were cultured in MMP liquid minimal medium for about 48 h. *Rso* strains were mutants unable to produce extracellular polysaccharide. Proteins precipitated from cell-free supernatants were resolved by SDS-PAGE (EZ-Run gels) and the gels were post-stained with silver. (Note that, unlike on this gel, the ~12 kDa *Cme* protein was not consistently less abundant in the profile of the Δ*gspD* mutant.) The locations of Bio-Rad's Precision Plus molecular mass standards are indicated on the left. Digital images were converted to gray scale and corrected to give a white background and normal contrast.

Cme Secretes No Abundant Exoproteins Using Its T2SS

Pathogenic bacteria often secrete enough of one or more exoproteins for comparative gel electrophoresis of culture supernatant preparations from wild type and T2SS mutants to reveal obvious differences [25, 51–55]. We therefore first created two *Cme* T2SS mutants, CH34ΔD and CH34ΔC, by site-specifically deleting *gspD* and *gspC*. We then cultured these mutants and their wild-type parent in MMP liquid medium and used SDS-PAGE and silver staining to examine the total proteins precipitated from cell-free supernatants. Examination of several independent protein preparations revealed no consistent differences in the many exoproteins made by CH34 and either CH34ΔD or CH34ΔC (Fig. 2 and data not shown, respectively). As a positive control [25], we also examined exoproteins from wild-type *Rso* GMI1000 and GMI-D, a Δ*gspD* mutant, both of which were modified to make them unable to produce the normally copious extracellular polysaccharide. Because *Rso* extracellular polysaccharide complicates protein recovery and SDS-PAGE, use of these strains allowed us to prepare and analyze its exoproteins the same way as those from *Cme*. As expected, we observed dramatically fewer proteins in the supernatant of the *Rso* T2SS mutant compared with its wild-type parent (Fig. 2).

Cme Secreted a Native Alkaline Phosphatase via Its T2SS

Most T2SS exoproteins have first been discovered by detecting their enzyme activities in culture supernatants, because this approach can succeed even when the amount of protein is below that easily visualized by gel electrophoresis [56, 57]. In *Kox*, *Dda*, and two *Pseudomonas* species, some exoproteins are encoded by genes adjacent to the T2SS gene cluster [55, 57–61], so we first examined the *Cme* proteins encoded by the two genes between *gspC* and *gspG* and those flanking each end of the gene cluster. However, none of these six proteins is a good candidate for

Table 2. Alkaline phosphatase activity of *C. metallidurans* wild type and a Δ*gspC* T2SS mutant lacking the *phoA1/A2* locus.

		24 h[a]				48 h			
		Units act.[b]		Percent act.		Units act.		Percent act.	
Strain	Mutation	Supt.	Cell	Supt.	Cell	Supt.	Cell	Supt.	Cell
CH34	None	5.8	46.0	11.3	88.7	7.4	27.0	21.4	78.6
	phoA1 phoA2	0.1	0.3	nc	nc	0.1	0.3	nc	nc
CH34ΔC	*gspC*	0.4	309.3	0.1	99.9	0.8	284.4	0.3	99.7
	gspC phoA1 phoA2	0.1	1.3	nc	nc	0.1	0.8	nc	nc

[a]BY broth cultures were incubated for either 24 or 48 h before cells and supernatants were harvested and stored frozen until being assayed. Cells were not permeabilized.

[b]Units of alkaline phosphatase were determined using *p*-nitrophenyl phosphate as the substrate as described in Materials and Methods. Values are averages of two biological repetitions. Supt., culture supernatant; Cell, whole cells; nc, percentage activity not calculated owing to low total activity.

a T2SS substrate, because only one (ABF10252) has a signal peptide and it is predicted to be a bitopic Ca^{2+} sensor in the EF-Hand superfamily. We next checked for secreted EddB DNase activity like that made by several *Pae* strains [62] (encoded by PA3909), because the orthologous protein in *Cme* (ABF11851) is 47% identical and predicted to have a signal peptide and the correct conserved domains. However, although we readily detected T2SS-dependent DNase activity produced by *Pae* strain PAK, no detectable extracellular DNase activity was made by *Cme* CH34 using the same growth and assay conditions (data not shown).

Several *Pae* and *Pseudomonas putida* strains secrete one or more APases via their T2SS systems [3, 55, 57, 60]. Quantification of P_i-repressible *Cme* APase activity in cells and supernatants from cultures grown in BY broth (which is naturally low in P_i) showed that only ~10% to 20% of the total APase activity was extracellular (Table 2). Interestingly, under the same conditions, the T2SS mutants CH34ΔC (and CH34ΔD, data not shown) produced over 6-fold more total APase activity. More importantly, extracellular APase made by the T2SS mutant was about 10% of the level in wild-type supernatant (Table 2). To better understand this phenomenon, we examined the CH34 genome and found two adjacent *phoA*-like genes (*phoA1*, ABF10951; and *phoA2*, ABF10952), which are transcribed in the same direction. The predicted PhoA proteins are 53% identical to each other as well as being about 30% identical (and ~42% similar, with conserved functional sites) to both the periplasmic *E. coli* PhoA (WP_000814403) and the extracellular *Pae* PhoA (PA3296) that is secreted by the conserved Xcp T2SS [3, 55]. Deletion of *phoA1* and *phoA2* virtually eliminated APase activity in both wild-type and Δ*gspC* backgrounds (Table 2). The unequal distribution of APase activity between cells and supernatants is probably because the signal peptide of PhoA1 is predicted to be processed by SPase II to create a lipoprotein, whereas that of PhoA2 should be processed by SPase I to create a soluble protein in the periplasm. Therefore, the large amount of cell-associated APase is presumably from membrane-bound lipid-modified mature PhoA1, whereas the extracellular APase activity is from PhoA2 that is secreted from the periplasm by the T2SS.

Recombinant *Cme* CH34 Efficiently Secreted Three *Rso* Exoenzymes in Part via Its T2SS

Type II secretion is often viewed as exhibiting well-defined species-specificity like that described for *Dda* and *P. carotovorum*, which do not reciprocally secrete some of each other's cellulase and pectate lyase exoenzymes [63–67]. However, there are some examples where the T2SS efficiently transports one or two foreign exoproteins [66, 68–70]. Because they are so closely related, we were hopeful that *Rso* exoenzymes might be secreted by *Cme*, and thereby help us more clearly demonstrate the function of its T2SS.

Of the many exoproteins known to be secreted by the *Rso* T2SS [25], the exoenzymes endopolygalacturonase (PglA), β-1,4-endoglucanase (Egl), and pectin methylesterase (Pme) had previously been cloned on cosmids pJE8 and pMB2. The *Cme* genome lacks genes for these enzymes, and preliminary assays of CH34 and CH34(pLAFR3) strains detected no PglA, Egl, or Pme activities. We introduced pJE8 and pMB2 into CH34 and its T2SS mutants CH34ΔC or CH34ΔD and quantified exoenzyme activities in both the supernatant and the cells recovered from BY broth cultures. In the wild-type background, almost 95% of the total PglA and Egl activities were extracellular, as was 99% of the Pme activity (Table 3). That virtually all the enzyme activities were extracellular suggests that the proteins were actively secreted. Deletion of either *gspD* or *gspC* decreased total exoenzyme activity either slightly for PglA (72% of

Table 3. Enzyme activity of *C. metallidurans* strains carrying *R. solanacearum* genes encoding exoenzymes secreted by the T2SS.

Strain	Plasmid	Enzyme act. (U/mg cell protein)[a]			Percent act.	
		Supt.	Cell	Total	Supt.	Cell
Polygalacturonase						
CH34	pJE8	157.7 ± 11.0	9.1 9 ± 2.1	166.8	94.6	5.5
CH34ΔD	pJE8	67.0 ± 5.4	49.8 ± 4.2	116.8	57.4	42.6
CH34ΔC	pJE8	73.6 ± 4.6	48.8 ± 4.9	122.5	60.1	39.9
CH34ΔC	pJE8 + pSCrhaB2	54.8 ± 8.6	42.5 ± 5.0	97.3	56.3	43.7
CH43ΔC	pJE8 + pSC-GspC	155.9 ± 18.0	13. ± 1.8	168.8	92.3	7.7
Endoglucanase						
CH34	pMB2	36.3 ± 1.0	2.1 ± 0.4	38.4	94.6	5.4
CH34ΔD	pMB2	0.8 ± 0.4	5.6 ± 1.3	6.5	12.8	87.2
CH34ΔC	pMB2	1.5 ± 0.3	8.3 ± 1.2	9.8	14.9	85.1
CH34ΔC	pMB2 + pSCrhaB2	0.7 ± 0.1	7.6 ± 0.7	8.3	8.8	91.5
CH43ΔC	pMB2 + pSC-GspC	31.9 ± 1.0	2.5 ± 0.4	34.4	92.7	7.3
Pectin methylesterase						
CH34	pMB2	28.6 ± 2.5	0.3 ± 0.0	28.9	99.0	1.0
CH34ΔD	pMB2	4.1 ± 0.2	5.7 ± 0.2	9.8	41.8	58.2
CH34ΔC	pMB2	4.2 ± 0.0	7.1 ± 0.2	11.2	36.9	63.1
CH34ΔC	pMB2 + pSCrhaB2	13.3 ± 0.7	21.0 ± 2.4	34.2	38.7	61.3
CH43ΔC	pMB2 + pSC-GspC	49.5 ± 3.4	0.9 ± 0.0	50.4	98.2	1.8

[a]BY broth cultures were incubated for 45 h. For polygalacturonase and endoglucanase activities, one unit (U) released 1 nmol reducing sugar/min. For pectin methylesterase activity, 1 U released 100 pmol H+/min. Values are averages ± SE; polygalacturonase assays had four or five biological repetitions, and endoglucanase and pectin methylesterase assays had three biological repetitions. Supt., culture supernatant; Cell, permeabilized cells.

wild type), moderately for Pme (36% of wild type), or severely for Egl (21% of wild type). The percentage of enzyme activity recovered in the supernatants of the T2SS mutants also decreased, but not to the extent that was expected, especially for PglA and Pme, because about 60% or 40%, respectively, of total activity was still extracellular.

To ensure the changes in secretion by CH34ΔC were only due to loss of *gspC*, we cloned wild-type *gspC* to create pSC-GspC, which is compatible with the pLAFR3-based cosmids, and used it to complement CH34ΔC. In control strains carrying the pSCrhaB2 vector, there was no effect on production or distribution of PglA or Egl, but much more Pme was produced without altering its distribution (Table 3). Complementation of Δ*gspC* by pSC-GspC restored total production of all three exoenzymes to wild-type levels (or higher for Pme) and distribution almost returned to normal. From these results, we concluded that wild-type CH34 has a functional T2SS that efficiently secretes *Rso* exoenzymes, but that when the T2SS is inactivated, substantial percentages of the exoenzymes (especially PglA and Pme) exit the cells cultured in BY broth by an alternative mechanism.

No Generalized Periplasmic Leakage or Cell Lysis Was Detected

The unexpectedly high percentage of *Rso* enzymes present in the supernatant of T2SS mutants is not easily explained by generalized periplasmic leakage or cell lysis, because no evidence of this was observed when we analyzed the distribution of APase activity (described above). To more specifically test for cell lysis, we introduced pGA91-lacZ, which encodes *E. coli* β-galactosidase, a cytoplasmic enzyme, into CH34, CH34ΔC, and CH34ΔD. After incubation in BY broth, these three strains had comparable intracellular enzyme activity that averaged 243 ± 14.9 (±SE) Miller units after 24 h of incubation and 324.3 ± 25.8 Miller units after 48 h, whereas <1% of the total activity was present in the supernatants, indicating negligible cell lysis (Table 4). This very low extracellular β-galactosidase activity could not be attributed to enzyme degradation in this unnatural environment, because we found that supernatants retained 86–96% of their original activity after being incubated for 5 days at 30°C.

To test for periplasmic leakage in addition to lysis, we created the plasmid pSC-PehA, which expresses the

Table 4. Distribution of *E. coli* LacZ and *P. atrosepticum* PehA enzymes in recombinant *C. metallidurans* wild type and T2SS mutants.

		24 h[a]		45 h	
		Units activity[b]		Units activity	
Enzyme[c]	Strain	Supt.	Cell	Supt.	Cell
LacZ	CH34	0.9 ± 0.3	223.4 ± 35.9 (99.6)	2.9 ± 0.8	313.0 ± 63.7 (99.3)
	CH34ΔC	0.9 ± 0.2	230.7 ± 4.1 (99.6)	2.6 ± 0.7	303.7 ± 31.9 (99.3)
	CH34ΔD	1.1 ± 0.4	275.0 ± 21.3 (99.6)	3.1 ± 0.7	356.2 ± 46.2 (99.3)
PehA	CH34	2.6 ± 0.7	468.1 ± 35.9 (99.5)	2.58 ± 1.5	334.6 ± 23.7 (99.3)
	CH34ΔC	5.2 ± 0.5	438.2 ± 41.7 (98.8)	2.13 ± 1.0	301.1 ± 10.4 (99.3)

[a]BY broth cultures (supplemented with 0.5% rhamnose in the case of PehA-expressing strains) were incubated for either 24 or 45 h before cells and supernatants were harvested and stored frozen until being assayed.

[b]Values are averages of three biological repetitions ± SE, with the percentage of the total activity present in cells shown in parentheses. Supt., culture supernatant; Cell, permeabilized cells.

[c]LacZ, β-galactosidase; PehA, endopolygalacturonase.

P. atrosepticum PehA endopolygalacturonase (CAG74005) from a rhamnose-inducible promoter. *P. atrosepticum* secretes PehA (which is 42% identical to *Rso* PglA) via its a T2SS [71, 72], and consequently this exoenzyme should at a minimum be exported to the periplasm of *Cme*. After pSC-PehA was introduced into *Cme* CH34 and CH34ΔC, we found that >99% of the activity was retained by the cells cultured in BY broth containing 0.5% rhamnose (Table 4). This result demonstrated that, in contrast to the *Rso* exoenzymes, the *Cme* T2SS did not secrete this foreign polygalacturonase, nor was there evidence of cell lysis or generalized leakage from the periplasm.

Table 5. Endopolygalacturonase activity of *C. metallidurans* strains expressing *R. solanacearum* polygalacturonase cultured in M963 liquid medium.

	Enzyme act. (U/mg cell protein)[a]			Percent act.	
Strain	Supt.	Cell	Total	Supt.	Cell
CH34	2,040.0	36.8	2,076.8	98.2	1.8
CH34ΔD	142.1	1,746.5	1,888.6	7.5	92.5
CH34ΔC	130.7	2,075.0	2,205.7	5.9	94.1

[a]One unit (U) of polygalacturonase activity released 1 nmol reducing sugar/min. Values are averages of two biological repetitions. Supt., culture supernatant; Cell, permeabilized cells.

Growth in Minimal Medium Increased Production and Secretion of PglA by Wild-Type *Cme* and Enhanced Its Retention by Cells of Δ*gspD* and Δ*gspC* Mutants

While preparing extracellular protein samples for SDS-PAGE (see below), we observed that CH34(pJE8) produced much more PglA activity when cultured in our two minimal media rather than in BY broth, but CH34(pMB2) produced less Egl and Pme. This overproduction of PglA was unexpected, because *Rso* did not behave this way (data not shown). Quantitative assays showed that total PglA activity produced by CH34(pJE8) cultured in M963 (Table 5) was at least 10-fold higher than when grown in BY, and that over 98% of the activity was extracellular. Tests with *Cme* strains carrying *pglA* subcloned into pLAFR3 in opposite orientations showed that increased PglA activity could be attributed to overexpression of *pglA* from the vector's *lacZ* promoter (data not shown), but why minimal medium had this effect on *Cme* was not determined. More importantly, however, the supernatants of CH34ΔD(pJE8) and CH34ΔC(pJE8) cultured in M963 had less than 8% of the high level of total PglA activity (Table 5), a much lower percentage than we saw for BY broth cultures (Table 3). Similar results were obtained when these strains were grown in MMP minimal medium (data not shown). Therefore, growth in minimal media suppressed the alternative mechanism of secretion observed in BY broth cultures to definitively demonstrate that PglA requires the CH34 T2SS to be secreted.

SDS-PAGE Revealed Changes in Extracellular Proteins Attributable to Insertion of pLAFR3 with and without Genes Encoding *Rso* Exoenzymes

To get a more complete picture of the effect that inactivating the *Cme* T2SS had on secretion of native and *Rso* exoproteins, we used SDS-PAGE to analyze total extracellular proteins of strains grown in several media. We first examined wild-type *Cme* CH34 and CH34(pLAFR3) grown in BY broth and, as expected, found virtually no differences (Fig. 3A). However, we observed that introduction

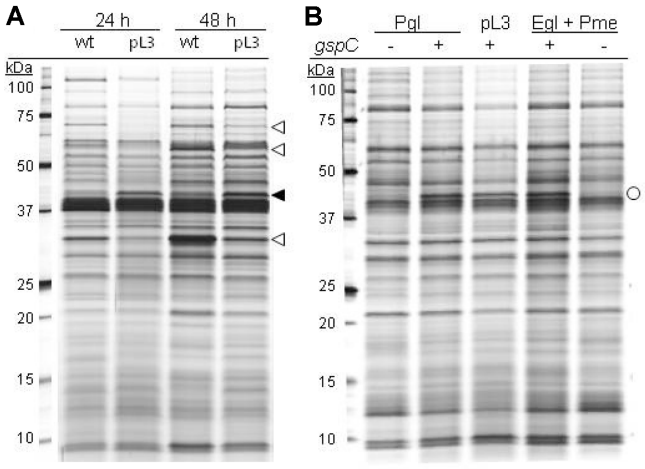

Fig. 3. Gel electrophoretic analysis of extracellular proteins from *C. metallidurans* CH34 carrying the empty vector or cosmids expressing *R. solanacearum* exoenzymes when cultured in BY broth.

(**A**) Wild-type strains that lacked (wt) or carried the pLAFR3 vector (pL3) were cultured for the two times indicated. Proteins precipitated from cell-free supernatants were resolved by SDS-PAGE (Criterion gel) and then stained with silver. The filled arrowhead indicates the ~42 kDa protein that was more abundant when strains carried the vector, whereas open arrowheads indicate proteins that were less abundant. Bio-Rad's Precision Plus standard (with indicated masses) was run in the left hand lane. Digital images were converted to gray scale and corrected to give a white background and normal contrast. (**B**) Wild type (+) or Δ*gspC* (-) strains were cultured for 48 h. and analyzed as in panel **A**. Strains carried pLAFR3 (pL3) or derivatives of pLAFR3 that produced either polygalacturonase (PglA) or both endoglucanase (Egl) and pectin methylesterase (Pme). The open circle indicates the absence of the ~42 kDa protein in the Δ*gspC* strains.

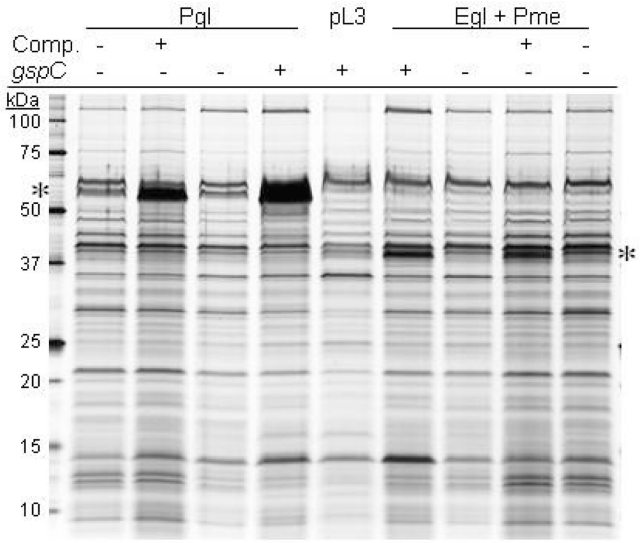

Fig. 4. Gel electrophoretic analysis of extracellular proteins from *C. metallidurans* CH34 carrying the empty vector or cosmids expressing *R. solanacearum* exoenzymes when cultured in MMP medium.

The same strains used in Fig. 3B, plus Δ*gspC* strains that were complemented by pSC-GspC (Comp. +) or carried pSCrhaB2 vector as a mock-complementation control (Comp. -), were cultured in MMP liquid minimal medium. The asterisk on the left side indicates the location of PglA. The asterisk on the right side indicates an uncharacterized ~40 kDa protein that was overabundant in the supernatant of CH34(pMB2) and CH34ΔC(pMB2) complemented by *gspC*.

of this empty vector increased the abundance of a ~42 kDa protein and decreased the abundance of at least three proteins. Production of *Rso* exoenzymes did not remarkably alter the extracellular protein profile when either wild type or T2SS mutants carrying pJE8 or pMB2 were grown in BY broth (Figs. 3B and S1, first three lanes), but the overabundant ~42 kDa protein was not present in the supernatants of the mutant strains, suggesting that it is a T2SS substrate. pLAFR3 is a large cosmid (~21 kb) derived from pRK2, a naturally occurring antibiotic resistance plasmid [73, 74] that has 23 genes encoding multiple regulatory and other DNA-binding proteins [75]. Although none of these seems likely to encode a periplasmic protein that could be a T2SS substrate, this vector appears to have somehow affected production or secretion of native *Cme* proteins.

Extracellular protein profiles for wild-type CH34 and T2SS mutants carrying either pJE8 or pMB2 were also generally very similar to each other when they were cultured in M963 (data not shown and Fig. S1, middle three lanes) or in MMP minimal media (Figs. 4 and S1, last three lanes) with two exceptions. There was an abundant ~55 kDa protein made by both CH34(pJE8) and its equivalent Δ*gspC*/*gspC*+ complemented strain that was much reduced in the Δ*gspC* mutant (and its mock-complemented strain) and absent from the vector control (Fig. 4). This band was close to the size of *Rso* PglA (52.4 kDa for the mature protein) and it co-migrated with PglA activity when the proteins in comparable gels were renatured and tested for activity by application of an enzyme substrate overlay (Fig. S2). Unlike in M963, CH34(pMB2) and its Δ*gspC*/*gspC*+ complemented strain cultured in MMP minimal medium had a unique 40 kDa band, approximately the size of Egl and/or Pme, that was absent in the Δ*gspC* mutants and vector control (Fig. 4). Unfortunately, we could not reliably demonstrate the presence of either Egl or Pme activity when comparable gels were renatured and tested with substrate overlays.

Discussion

Cme GspC Identification and T2SS Gene Organization

When T2SS gene clusters contain both *gspC* and *gspN*, there are examples in older literature [12, 50], and even now in gene and genome annotations, where GspC is misidentified as GspN. However, we are confident that our assignment of *Cme gspC* is correct, despite its not being immediately upstream of *gspD* and the C-terminal end of GspC lacking a PDZ or coil-coil domain. First, the simple fact that deleting *gspC* inactivated the T2SS indicates that it is required, whereas in the T2SS of some other bacteria *gspN* is either not present [6] or is not required [76]. Second, secondary structure prediction revealed that *Cme* GspC, but not GspN, has the six conserved β strands [20–22] that are the unifying feature defining the HR, which had originally been imprecisely identified by its weakly conserved amino acid sequence [43]. Not surprisingly, the five most important amino acids so far identified for GspC function are within those short regions encoding the β strands, and *Cme* GspC has either the same amino acids or conserved substitutions at four of these locations (Fig. 1C). Third, although model GspC proteins have PDZ or coil-coil domains, these features are not always required. There are some examples where the PDZ or coil-coil domains can be removed or exchanged with that domain from a heterologous protein without eliminating secretion of all exoproteins [19, 43, 49, 50, 66]. In addition, there are several reports of functional GspC proteins that naturally lack a PDZ or coil-coil domain because they end shortly downstream of the HR [54, 55, 77]. Indeed, "truncated" GspC proteins are not unusual, because in our phylogenetic analysis (see below) we observed that 11 of the 24 GspC proteins terminated between 5 and 50 amino acids downstream of the HR. Furthermore, GspC from 19 additional *Burkholderia* species, several of which are known to have a functional T2SS [78–80], are predicted to end just beyond the HR (data not shown).

Information from more diverse bacteria also has revealed that T2SS gene clusters can be organized in more ways than originally thought. In particular, as seen in *Cme* and *Rso*, *gspC* is not adjacent to *gspD* in the Hxc-like T2SS [55] clusters in three *Pae* strains, two *Pseudomonas fluorescens* strains, and one each of *Pseudomonas syringae* and *Pseudomonas entomophila* strains, and Durand *et al.* [81] concluded that these strains likely acquired their Hxc-like systems by horizontal gene transfer from Betaproteobacteria. It fact, because *gspC* and *gspD* are separated in many Betaproteobacteria, including multiple *Burkholderia* species [77–80], *Bordetella avium*, *Acidovorax citrulli*, and *Alcaligenes faecalis*, this may represent the normal organization in this division. Furthermore, the fact that *Legionella pneumophila* [82] and *Acinetobacter nosocomialis* [53] have their T2SS genes dispersed in four or five genomic loci, respectively, shows that genes encoding components of functional T2SS need not be clustered together.

Analysis of Genetic Relatedness Supports the Existence of at Least Three T2SS Subfamilies

Our understanding of the genetic relatedness of the T2SS proteins is also still evolving. About a decade ago, several reports proposed three subclasses of GspC family proteins based on the presence or absence of predicted C-terminal PDZ and coil-coil domains [12, 44]. However, these descriptive subclasses are artificial because they do not indicate function or genetic relatedness. Recently, Durand *et al.* [81] performed an extensive phylogenetic analysis of GspG orthologs in all divisions of Proteobacteria as part of their research demonstrating that a few specific amino acids distinguish the major pseudopilins in *Pae* Xcp and Hxc T2SS, and proposed renaming these two systems as examples of T2aSS and T2bSS subfamilies, respectively.

We extended this work with a phylogenetic analysis of GspD, GspC, GspG, and GspL proteins in 24 bacteria documented to have one or more functional T2SS that represent four of the five subdivisions of Proteobacteria (Table S2, Figs. S3, S4). The GspD secretin, which provided the most robust phylogenetic tree, has three monophyletic groups, of which clusters A and B generally support the T2aSS and T2bSS designations of Durand *et al*. [81]. Cluster A contains only species in the Gammaproteobacteria, except for *Caulobacter crescentus*, which is in the Alphaproteobacteria. Cluster B has only species in the Betaproteobacteria, except for *Pae* HxcQ, which was expected (see above). However, cluster C, which was not recognized by Durand *et al.* [81], has GspD proteins from *X. campestris*, *Xylella fastidiosa*, *P. syringae* pv. tomato, and one of the two T2SSs in *Acidovorax citrulli*. The trees for the other three Gsp proteins have many of the same organisms in these three main clusters, but in each case proteins from various organisms fall outside of the clusters. These more divergent proteins may have evolved to suit the particular demands of their cognate T2SS and substrates. We therefore propose the existence of a T2cSS subfamily, with the *X. campestris* Xps system as the model. It may be significant that in all four trees, *Geobacter sulfurreducens*, which is in the Deltaproteobacteria, is consistently outside of the three major clusters. Other bacteria that have a functional T2SS

related to that in *G. sulfurreducens* must be indentified and analyzed to determine whether there is a phylogenetically distinct fourth subfamily.

Native and Foreign Substrates of the *Cme* T2SS

Our observation that *Cme* CH34 did not secrete any abundant T2SS exoproteins when supernatants were analyzed using gel electrophoresis is not unprecedented. In 1996, de Groot *et al.* [56] similarly examined extracellular proteins made by *P. putida*, and concluded that its T2SS was inactive, because they did not find an *xcpX* homolog and also saw no difference in the protein profiles of the wild type and a T2SS mutant. However, once a *P. putida* genomic sequence became available, Putker *et al.* [57] found that there is a complete T2SS gene cluster, and that when cultured in low-P_i medium, the functional secreton transports PhoX APase, which was detected on the basis of its enzymatic activity.

There are currently at least three major classes of bacterial APases: PhoA, PhoD, and PhoX [83, 84]. The best known APase is probably *E. coli* periplasmic PhoA, because it has been widely employed in methods to monitor protein secretion across the inner membrane. However, other bacteria, especially those resident in oceans and soils [83–85], have APases that may be extracellular or located on the cell surface. It was not surprising that PhoA2, one of two potential *Cme* APases we studied, is a T2SS substrate, because *Pae* strains produces two different APases (PhoA and LapA) that are secreted by its Xcp or Hxc T2SS, respectively [3, 55]. That a *Cme* T2SS mutant has only 10% of its APase activity in the supernatant was only slightly higher than expected. For example, Bouley *et al*. [66] reported that they detected up to 5% of multiple pectate lyase exoenzymes in the supernatant of *Dda* T2SS mutants. That an APase like PhoA1 could be a membrane-bound lipoprotein is less common, but not unknown, because *P. putida* PhoX (NP_743204) is a phospolipoprotein that is secreted by its Xcp T2SS and retained on the outside of the outer membrane [57].

Unlike the T2SS species-specific secretion reported for some exoproteins [43, 63, 64, 66, 67, 86] and that we observed for *P. atrosepticum* PehA, which was not secreted by *Cme*, wild-type CH34 cultured in BY broth produced 40% to 90% as much of the three *Rso* exoenzymes and secreted them almost as efficiently as does *Rso* GMI1000 [87; and unpublished results]. It is remarkable that *Cme* secreted Egl so well, because in *Rso* this exoenzyme (unlike PglA or Pme) is initially present in the periplasm as a lipid-modified proprotein that transiently associates with the inner membrane before being proteolytically released to become a T2SS substrate [88]. Thus, *Cme* must either not lipidate the proprotein during processing of the signal peptide, or has a protease that releases the mature enzyme from the inner membrane. It should be noted, however, that secretion of *Rso* Egl is not comparable to secretion of pullanase, a starch-debranching enzyme, by *Kox*, because the latter lipoprotein is extracted intact from the inner membrane and secreted to the bacterial outer surface by the T2SS [89].

***Cme* Secretion of *Rso* Exoproteins by an Alternative Process**

Surprisingly, deletion of either *gspD* or *gspC* in CH34 resulted in approximately 60% of PglA, 40% of Pme, and 15% of Egl total activities still appearing extracellularly when *Cme* was cultured in BY broth, whereas GMI1000 T2SS mutants released only about 9% of the total PglA and less than 1% of the Pme and Egl in comparable BG broth. Our results showed that obvious possibilities of cell lysis or generalized leakage from the periplasm cannot explain the high percentage of the *Rso* enzymes still released by the *Cme* T2SS mutants, because they did not release significant amounts of β-galactosidase, PhoA2, APase, or PehA enzymes.

We considered the possibility that a secondary T2SS gene cluster or functional homologs of some secreton components might partially compensate for the loss of *gspD* or *gspC*, as seen in some bacteria [54, 55, 60, 61, 90, 91]. The CH34 genome has only the one complete T2SS gene cluster [31], but its megaplasmid has two sets of three *gspG*-like genes and one of each *gspD*-like, *gspE*-like, and *gspF*-like genes. Therefore, deletion of *gspD* might not have eliminated all production of a functional secretin. However, *gspC* is unique in the CH34 genome, so it seems unlikely that another protein could compensate for its absence. Therefore, because deletion of *gspC* and *gspD* resulted in the same high levels of extracellular PglA and Pme in BY broth cultures, this is the bona fide phenotype of a CH34 T2SS mutant in this condition.

The most likely mechanism for the substantial release of PglA and Pme by *Cme* T2SS mutants when cultured in BY broth is in outer membrane vesicles (OMV). It is now generally accepted that all gram-negative bacteria can release small (10–300 nm) vesicles from their outer membrane in the absence of cell lysis, and that during their formation the OMV trap periplasmic content, including soluble proteins [92, 93]. Significantly, OMV are not the result of random blebbing or fragmentation, but are the

result of complex, regulated biological processes that contribute to survival and pathogenesis. Although neither *Rso* nor *Cme* have been reported to produce OMV, the closely related *Burkholderia cepacia* naturally produces OMV that contain a selected subset of periplasmic proteins, including multiple enzymes that are potential virulence factors [94]. A proteomic analysis of *V. cholerae* treated to stimulate expression of virulence genes found that 11 of the 19 known T2SS substrates were present in OMV, although what percentage of these proteins was secreted by this alternative pathway was not examined [95]. Even more relevant is the report that *X. campestris* pv. *vesicatoria* secretion of two xylanases, a lipase, and a predicted protease are only partially dependent on the Xps T2SS, because some of these proteins are also released in OMV by wild-type and T2SS mutant cells [86]. Therefore, it is possible, if not likely, that in both *Cme* wild type and T2SS mutants grown in BY broth, some of the *Rso* exoenzymes are released in OMV, which due to their small size would not have been removed by our membrane filtration or low-speed centrifugation. This hypothesis, however, requires that *Rso* PglA and Pme be preferentially packaged in OMV, whereas *P. atrosepticum* PehA is left behind despite presumably accumulating in the periplasm of both *Cme* wild type and a T2SS mutant. Significantly, selective packaging of this sort was observed in a *V. cholerae* mutant lacking its DegP protease/chaperone protein, because OMV from the mutant strain lacked five of the 11 T2SS substrates present in wild-type OMV [95]. Preferential association of *Rso* PglA and Pme with a native *Cme* periplasmic chaperone might, therefore, promote selective inclusion in OMV and, more speculatively, also assist in their secretion by the T2SS. Clearly, further research is necessary to test these possibilities.

Substrate Specificity and a Proposal for *Cme* as a New T2bSS Model System

One of the least well-understood aspects of type II secretion is the process by which a small minority of periplasmic proteins preferentially transits the secreton. This substrate selectivity is currently thought to be determined by the physical interaction of GspC and GspD with each other and one or more "signaling motifs" in an exoprotein's tertiary structure [6, 7, 23, 65, 67, 70, 71, 96, 97]. A majority of research on substrate specificity have been done with *Dda*, whose T2SS OutC protein has a C-terminal PDZ domain, and although the most well-studied substrates require this domain for efficient secretion, a few do not [66, 67]. In addition, as noted above, many species of Betaproteobacteria, like *Cme* and *Rso*, have T2SS with functional GspC proteins that lack a PDZ or coil-coil domain.

These observations suggest that there are different degrees of substrate specificity that depend on how potential substrates are recognized by secreton components [6, 7]. The simplest explanation has two levels of recognition. One is basic recognition, which requires minimal numbers of interacting sites and might distinguish T2SS substrates from all other periplasmic proteins. Presumably, the same signaling motifs and recognition mechanism are involved, whether the exoprotein is native or, as shown in this study and in other examples, of foreign origin [66, 68–70]. Basic recognition would explain how *Dda* engineered to make a hybrid secreton containing GspC or GspD from *P. carotovorum* still secretes native PemA (a pectin methylesterase) [66]. A second level is enhanced recognition, which involves additional signaling motifs or motifs that have more stringent recognition criteria. In either case, however, enhanced recognition acts to distinguish between the different exoproteins, possibly as a mechanism to enhance the secretion speed or efficiency [67].

Given how difficult it has been to elucidate substrate specificity in *Dda*, which primarily uses enhanced recognition, we propose that more progress might be made by studying T2bSS (primarily in *Betaproteobacteria*) that have GspC proteins with only the HR domain, because they likely depend on basic recognition. Furthermore, we suggest that *Cme* strain CH34 would be an excellent choice for a new model system to study basic recognition because (i) it is nonpathogenic; (ii) it appears to have only one functional T2SS; (iii) it secretes few native EXP via its T2SS that might interfere with detection of foreign proteins by SDS-PAGE or enzyme assays; and (iv) exoprotein genes can be expressed at a high level from a plasmid's *lacZ* promoter simply by growth in minimal medium, which simultaneously eliminates the alternative mechanism of protein secretion observed in BY broth. Identification of foreign exoproteins that are or are not secreted by CH34 would then permit concerted studies of the tertiary structure, in combination with mutagenesis of both exoproteins and secreton components, that should better define the signaling motifs and secreton domains that determine basic substrate recognition.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant No. 31300130), Shandong Provincial Natural Science Foundation, China

(Grant No. ZR2013CQ012), an award from the Shandong Provincial Department of Education and by Georgia State and USDA Hatch funds from The University of Georgia Agricultural Experiment Station. We thank Marla Scott for technical assistance, Bryndan Durham for constructing pDONR-SacTet, and Hsien-Tzer Tseng for providing pBBR-CmeC and unpublished *R. solanacearum* exoenzyme activity data.

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