The bifunctional cell wall hydrolase CwlT is needed for conjugation of the integrative and conjugative element ICE*Bs1* in *Bacillus subtilis* and *B. anthracis*

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Abstract

The mobile genetic element ICE*Bs1* is an integrative and conjugative element found in *Bacillus subtilis*. One of the ICE*Bs1* genes, *cwlT*, encodes a cell wall hydrolase with two catalytic domains, a muramidase and a peptidase. We found that *cwlT* is required for ICE*Bs1* conjugation. We examined the role of each of the two catalytic domains, and found that the muramidase is essential, whereas the peptidase is partially dispensable for transfer of ICE*Bs1*. We also found that the putative signal peptide in CwlT is required for CwlT to function in conjugation, consistent with the notion that CwlT is normally secreted from the cytoplasm. We found that alteration of the putative lipid attachment site on CwlT had no effect on its role in conjugation, indicating that if CwlT is a lipoprotein, the lipid attachment is not required for conjugation. Finally, we found conditions supporting efficient transfer of ICE*Bs1* into and out of *B. anthracis* and that *cwlT* was needed for ICE*Bs1* to function in *B. anthracis*. The mature cell wall of *B. anthracis* is resistant to digestion by CwlT, indicating that CwlT might act during cell wall synthesis, before modifications of the peptidoglycan are complete.

Introduction

Integrative and conjugative elements (ICEs) are mobile genetic elements that are found stably integrated into a bacterial chromosome. Under certain conditions, an ICE can excise from the chromosome, circularize, and transfer to a recipient cell via the ICE-encoded conjugation machinery (reviewed in 1, 2). ICEs are found in a wide variety of bacterial species, both Gram positive and Gram negative (3), and they often bestow physiologically and clinically relevant traits, including nitrogen fixation, biofilm formation, virulence, and antibiotic resistance.

ICE*Bs1* is a mobile genetic element found in many isolates of *Bacillus subtilis* (4-6). It is approximately 21 kb in length with 24 open reading frames (Fig. 1A). ICE*Bs1* is found integrated in *trnS-leu2*, the gene for a leucine-tRNA, and it remains stably integrated as long as its major operon is repressed. Derepression of ICE*Bs1* gene expression and subsequent excision occur in response to DNA damage, or when the cell-cell signaling regulator RapI is produced and becomes active, usually when cells are crowded by potential recipients that do not have ICE*Bs1* (4, 7, 8).

The ICE*Bs1* gene *cwlT* (cell wall lytic; previously *yddH*) encodes a bifunctional cell wall hydrolase (Fig. 2) capable of degrading peptidoglycan (9). Peptidoglycan is the major component of the bacterial cell wall and is composed of long carbohydrate chains of alternating amino sugars, N-acetylglucosamine and N-acetylmuramic acid, crosslinked by short peptide chains (10-12). In *B. subtilis*, the cell wall is approximately 40-50 nm thick (13, 14), and the genome encodes a complement of >30 hydrolases that digest the various covalent bonds in the cell wall peptidoglycan to facilitate processes such as growth, separation of cells after division, and mother cell lysis during sporulation (12, 13).

Peptidoglycan hydrolases are widespread in mobile genetic elements and are often found associated with Type IV secretion systems (T4SS) involved in conjugation (15-19). One of the best characterized of the Type IV secretion systems is the VirB/D4 system from *Agrobacterium tumefaciens*. This system is composed of a large multiprotein channel that spans the cell envelope and mediates the secretion of conjugative DNA and associated proteins. It is generally assumed that the hydrolases cause localized degradation of the cell wall to allow the assembly of the large secretion apparatus. However, relatively little is known about their function in conjugation. Hydrolases in Gram negative organisms tend to have one hydrolytic domain and are usually not essential for conjugation (20-22). Hydrolases from conjugative systems in Gram positive organisms typically have two or more catalytic domains, and conjugation is significantly reduced or eliminated in mutants (23-25).

Cell wall hydrolases from *B. subtilis* phage and conjugative elements typically have multiple domains (9, 26). CwlT has two domains for peptidoglycan hydrolysis and each has been characterized biochemically (9). The N-terminal domain is an *N*-acetylmuramidase (muramidase) that cleaves the linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine. The C-terminal endopeptidase (peptidase) domain cleaves the bond between D- γ -glutamate and *meso*-diaminopimelic acid (9).

We found that *cwlT* is required for conjugation of ICE*Bs1*. Using mutants in each of the two domains (Fig. 2), we found that the muramidase function is essential, and that the peptidase function is important but partially dispensable, for ICE*Bs1* conjugation. We found that the signal sequence involved in secretion of CwlT is critical for its function in conjugation. It was previously predicted that CwlT might be a lipoprotein (9, 27). We found that alteration of the putative lipid anchor site in CwlT had no affect on conjugation, indicting that if CwlT is a

lipoprotein, lipid attachment is likely not required for CwlT function. We also analyzed whether CwlT functions were needed for ICE*Bs1* to function in *B. anthracis*, whose cell wall is modified and resistant to hydrolysis by CwlT (and lysozyme). Our results indicate that CwlT activity is essential for ICE*Bs1* to transfer into and/or out of *B. subtilis* and *B. anthracis*.

Materials and Methods

Strains and alleles. *B. subtilis* and *B. anthracis* strains used are listed in Table 1. Standard techniques were used for cloning and strain construction (28). Some alleles related to ICE*Bs1* were previously described and are summarized below. Donor strains contained a derivative of ICE*Bs1* that contains a deletion of *rapI-phrI* and insertion of a *kan* cassette, Δ(*rapI-phrI*)::*kan* (4, 29-31) (Fig. 1B). *rapI* was overexpressed from Pxyl-*rapI* integrated into *amyE*, *amyE*::{(Pxyl-*rapI*) *spc*} (29) to induce ICE*Bs1* gene expression and excision in donor cells. ICE*Bs1*^{0} indicates that the strain is cured of ICE*Bs1*. *B. subtilis* recipients were streptomycin-resistant due to the spontaneous streptomycin-resistant allele *str84*, most likely in *rpsL* (30), and this was used as a counter-selective marker in mating experiments.

Deletion of *cwlT*. Δ*cwlT19* is unmarked deletion that removes *cwlT* entirely and fuses the stop codon of *conG* (upstream of *cwlT*) to the intergenic region upstream of *yddI*. A 2.1 kb fragment of ICE*Bs1* DNA with the Δ*cwlT19* allele was obtained by the splice-overlap-extension PCR method (32, 33) and cloned into the EcoRI and BamHI sites of pEX44 (34), a chloramphenicol-resistant vector containing *E. coli lacZ*. The resulting plasmid, pTD6, was used to introduce Δ*cwlT19* into the chromosome of MMB970 by first integrating by single crossover

and then screening for loss of the plasmid by virtue of loss of *lacZ*, and then testing by PCR for introduction of the indicated allele, essentially as described (31).

Modification of muramidase and peptidase domains. Mutations in the muramidase and peptidase domains were created using a strategy similar to that for Δ*cwlT*. *cwlT-E87Q* contains a missense mutation at position 87 of *cwlT*, converting a glutamate to a glutamine codon. *cwlT-C237A* contains a missense mutation at position 237, converting a cysteine to an alanine codon. *cwlT-E87Q-C237A* contains both of these mutations. *cwlT*Δ(*207-329*) is a deletion of the entire peptidase domain, consisting of a fusion of the first 206 codons of *cwlT* to its stop codon (Fig. 2). DNA fragments (~1.2 kb) containing one or both of these mutations were constructed and cloned into pCAL1422 by isothermal assembly (35) to yield pTD8 (*cwlT-E87Q*), pTD9 (*cwlT-C237A*), pTD10 (*cwlT-E87Q-C237A*), and pTD310 (*cwlT*Δ*207-329*). These plasmids were used to introduce their respective alleles into the chromosome of Δ*cwlT19* strain TD19 as described for pEX44 above. Mutations in *cwlT* were then confirmed by sequencing appropriate PCR products from genomic DNA.

Modifications to *cwlT* signal sequence. *cwlT*Δ*1-29* contains a deletion of the first 29 codons of *cwlT* and introduces a start codon at the beginning of the truncated gene (Fig. 2**)**. *cwlT-C23A* contains a missense mutation that removes the putative lipoprotein anchoring site by converting the cysteine codon at position 23 to an alanine codon. These mutations were introduced into MMB970 with pCAL1422-derived plasmids pTD95 (*cwlT*Δ*1-29*), pTD99 (*cwlTspoVD1-32*), and pTD116 (*cwlT-C23A*) as described above.

Construction of ICE*Bs1-cwlT* at *thrC*. To test for complementation of various *cwlT* mutants, we provided wild type *cwlT* from an ectopic copy of ICE*Bs1* integrated at *thrC* (29, 31). We found that complementation required expression of *cwlT* along with the upstream genes, similar

to findings with complementation of other ICE*Bs1* mutants (29). As discussed previously, we suspect that this has to do with some type of coupling, perhaps translational, between expression of many of the ICE*Bs1* genes (29). A complementation construct, *thrC11*::{*mls* ICE*Bs1*Δ(*yddI-attR*::*tet*)} (Fig. 1C), was created by starting with CAL229, which contains the entire ICE*Bs1* integrated into an attachment site (*attB*) placed at *thrC* and marked with macrolide-lincosamide-streptogramin (*mls*) resistance. Genes downstream from *cwlT* were deleted and a tetracycline-resistance cassette inserted, analogous to previously described alleles (31), yielding strain TD11. Transformation with chromosomal DNA from TD11 was used to introduce the complementation construct to other strains.

Construction of donor and recipient *B. anthracis* strains. In mating experiments, counter selection for *B. anthracis* recipients was with either chloramphenicol or nalidixic acid. Chloramphenicol resistance in *B. anthracis* was from the plasmid pBS42, introduced into *B. anthracis* strain UM44-1C9 (AG1924) (4, 36) by ICE*Bs1*-mediated mobilization from *B. subtilis* strain CAL1394 (37). Nalidixic acid resistance was due to a spontaneous mutation (4). ICE*Bs1* elements with mutations in *cwlT* were introduced into *B. anthracis* via conjugation from *B. subtilis* donors harboring a wild type *cwlT* allele at an exogenous chromosomal locus, complementing the loss of *cwlT* function and allowing transfer. *B. anthracis* strains TD322 (*cwlT-E87Q*), TD324 (*cwlT-C237A*), and TD326 (*cwlT-E87Q-C237A*) were created by conjugation of ICE*Bs1* from *B. subtilis* strains TD62, TD52, and TD57 respectively.

Construction of *cwlT* overexpression plasmids. Plasmids for the overproduction of CwlT, and both mutant and wild type versions of the peptidase domain of CwlT were constructed similarly to those previously described (9). Overproduction of full length CwlT in *E. coli* caused rapid cell lysis. However, deletion of the N-terminal 29 amino acids prevented lysis in *E. coli*, and this

159 deletion was used to express CwlT for purification. In contrast, overproduction of full length
160 CwlT in *B. subtilis* had no obvious effect on cell growth or viability, perhaps indicating that
161 activation of CwlT might be regulated. A fragment of *cwlT* containing codons 30–329 (with an
162 N-terminal initiation codon) was amplified by PCR and cloned into pET21b (Novagen) digested
163 with NdeI and HindIII, placing a hexa-histidine tag (his6) at the C-terminus of the protein. This
164 yielded pTD3, which was used for overexpression of CwlT-his6. For expression of the peptidase
165 domain, a fragment encoding amino acids 207 to 329 of *cwlT* was amplified by PCR either from
166 AG174 (wild type *cwlT*) or TD48 (*cwlT-C237A*), and cloned into pET28a (Novagen) digested
167 with NdeI and HindIII, placing a hexa-histidine tag at the N-terminus of the protein. This yielded
168 plasmids pTD106 (his6-CwlT-Pep) and pTD107 (his6-CwlT-PepC237A).

169 **Media and growth conditions.** Cells were grown at 37°C with agitation in LB medium (28,
170 38) as indicated. Antibiotics were used at the following concentrations: ampicillin (100 μg/ml),
171 chloramphenicol (5 μg/ml), kanamycin (5 μg/ml for *B. subtilis*, 25 μg/ml for *E. coli*),
172 spectinomycin (100 μg/ml), streptomycin (100 μg/ml), and nalidixic acid (40 μg/ml).
173 Erythromycin and lincomycin were used together (0.5 and 12/5 μg/ml, respectively) to select for
174 macrolide-lincosamide-streptogramin B (MLS) resistance. Isopropyl-β-D-thiogalactopyranoside
175 (IPTG, Sigma) was used at a final concentration of 1 mM.

176 **Mating assays.** Matings were performed essentially as previously described (4). Briefly,
177 donor and recipient cells were grown in LB. Expression of ICE*Bs1* genes was achieved in one of
178 two ways: either by production of the activator RapI from the xylose-inducible promoter Pxyl, or
179 by activation of the SOS response by addition of the DNA damaging agent mitomycin C (MMC)
180 (4). For activation of the Pxyl promoter, Xylose (1%) was added to donor cells in mid-
181 exponential growth (OD_{600} ~0.2) to induce expression of Pxyl-*rapI*. For mitomycin C induction,

1 µg/ml mitomycin C (MMC, Sigma) was added to donor cells in mid-exponential growth (OD_{600} ~ 0.5). After one hour of induction, approximately equal numbers of donor and recipient cells were mixed and filtered onto sterile nitrocellulose filters. When cultures were induced with MMC, the filter was then washed with 25 ml of LB to minimize exposure of recipients to MMC in the donor culture.

The filters were placed on plates comprised of Spizizen minimal salts (28) and 1.5% agar for 3 hr. Cells were collected from the filter and spread on selective plates. Transconjugants were identified and mating frequencies were calculated per donor cell. The reported transfer frequencies are the mean (± the standard error of mean) of at least two independent biological replicates. In mating experiments induced by MMC, donor CFU was determined prior to addition of MMC, as it can cause a drop in cell viability.

Purification of CwlT Proteins. Plasmids pTD3 (CwlT-his6), pTD106 (his6-CwlT-Pep), and pTD107 (his6-CwlT-PepC237A) were introduced into *E. coli* strain BL21-A1 (Invitrogen), generating strains TD103, TD106, and TD107 for expression of the different alleles of *cwlT*. Cells were grown in LB containing 100 µg/ml ampicillin (pTD3) or 25 µg/ml kanamycin (pTD106 and pTD107), shaking at 37º C. At OD_{600} ~0.7 to 0.9, L-arabinose (final concentration of 0.2%) and IPTG (final concentration of 1 mM) were added to induce expression of the T7 polymerase and derepress expression of *cwlT*. Cells were collected after 2 hours of induction and pelleted by centrifugation. Cell pellets were stored at -80ºC until needed.

For purification of CwlT, the cell pellet was thawed on ice, re-suspended in 0.2 volumes lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed by addition (final concentration of 1x) of CelLytic B (Sigma) and by sonication (microtip, 50% power) on ice 4 x 20 s. The lysate was incubated with DNase I (10 µg/ml) for 30 minutes on ice, and the

supernatant was separated by centrifugation at 14,000xg at 4ºC for 20 minutes. CwlT-his6, his6-CwlT-Pep, and his6-CwlT-PepC237A were purified by Ni-NTA column chromotography (Qiagen) according to the manufacturer's protocol for batch purification under native conditions.

Elution fractions were analyzed by SDS-PAGE. Those containing more than ~95% CwlT were pooled and exchanged into storage buffer (50 mM NaH_2PO_4, 300 mM NaCl, 1 mM DTT, pH 7.4) using PD-10 desalting columns (GE Healthcare). Protein concentration was determined by Bradford assay (Bio-Rad), glycerol was added to 25%, and protein was stored at -80ºC. CwlT was often unstable (degraded) after storage, so most assays were done with freshly purified protein.

Activity of CwlT against *B. subtilis* and *B. anthracis*. Cells (*B. anthracis* or *B. subtilis*) were grown to mid-exponential phase in LB liquid medium at 37ºC with shaking. Purified CwlT-his was added to the growing culture in final concentrations ranging from 1 μg/ml to 1 mg/ml. Cultures were incubated for 20 minutes and then cells were plated to determine colony forming units. Survival percentage was determined by comparison to a culture for which no CwlT was added.

Preparation of cell walls. Cell walls from *B. subtilis* and *B. anthracis* were prepared essentially as described previously (9, 39, 40). Briefly, cells were harvested from cultures (2 liters) in mid-exponential growth phase, resuspended in cold phosphate-buffered saline (PBS) (40 ml), and disrupted by sonication (microtip, 50% power) 15 x 30 s. After low-speed centrifugation (1500xg, 10 min) to remove unbroken cells, the crude cell wall was pelleted at 27,000xg for 5 min at 4ºC, suspended in 20 ml of a 4% (w/v) sodium dodecyl sulphate solution (SDS) and put in a boiling water bath for 20 min. Pellets were washed three times with warm deionized water (to prevent precipitation of SDS), two times with 1 M NaCl, and again four

228 times with deionized water. After each of the last four washes, the sample was first spun at low
229 speed (1500xg, 5 min) to separate whole cells and other contaminating material from the cell
230 wall fraction, which was then pelleted by spinning at 27,000 x g for 5 min.

231 **Determination of hydrolytic activities of CwlT proteins.** Hydrolytic activities were
232 determined essentially as described (9). Reactions were performed in 50 mM MOPS-NaOH
233 buffer, pH 6.5 at 32ºC, with 1 mg/ml *B. subtilis* or *B. anthracis* cell wall preparations. Proteins
234 were added to a final concentration of 10 µg/ml (CwlT-his6) or 5 µg/ml (his6-CwlT-Pep and
235 his6-CwlT-PepC237A), and the reaction mixture was agitated constantly to maintain the cell
236 walls in suspension. Turbidity of the reaction was monitored at 540 nm using a
237 spectrophotometer (Genesys 10 Bio, Thermo Corporation).

238 **Polyacrylamide gels and zymography.** Sodium dodecyl sulfate-polyacrylamide gel
239 electrophoresis (SDS-PAGE) and zymography were performed as previously described (38, 41).
240 For zymography, approximately 1 µg of various purified CwlT proteins were electrophoresed
241 through a 12% polyacrylamide gel containing cell wall preparations (~1 mg/ml) from *B. subtilis*
242 or *B. anthracis*. Following electrophoresis, gels were soaked in deionized water for 30 minutes
243 and then transferred into renaturation buffer (25 mM Tris-HCl, 1% Triton X-100, pH 7.2) at
244 30ºC overnight with gentle agitation. After incubation, the gels were rinsed with deionized water,
245 stained with 0.1% methylene blue in 0.01% KOH for 3 hr, and destained with deionized water.
246 Hydrolytic activity appeared as zones of clearing in the blue background of the stained cell walls.

247 **Western blot analysis.** Samples were collected from cultures after 3 hr of induction of
248 ICE*Bs1* expression. Cells were pelleted and stored at -80ºC. Pellets were thawed and
249 resuspended in buffer (10 mM Tris, 10 mM EDTA, pH 7) containing 0.1 mg/ml lysozyme and
250 the protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) at 1

mM. The volume of buffer used to resuspend each sample of cells was adjusted to the optical density at 600 nm (OD_{600}) in order to normalize the concentration of proteins in each sample. Resuspended cells were incubated at 37°C for 30 min, SDS sample buffer was added, and samples were heated at 100°C for 10 min followed by centrifugation to remove insoluble material.

Proteins were separated by SDS-PAGE on 12% gels and transferred to an Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore) using a Trans-blot semidry electroblot transfer apparatus (Bio-Rad). Membranes were blocked in Odyssey Block (Li-Cor Biosciences) for 1 hr, and then incubated in a 1:5,000 dilution of anti-CwlT rabbit polyclonal antisera (made commercially by Covance using CwlT-his6 protein purified from *E. coli*) in Odyssey Block with 0.2% Tween for 1 hr, and washed several times in phosphate-buffered saline, pH 7.8, with 0.1% Tween. Membranes were then incubated with 1:5,000 goat anti-rabbit IRDye 800 CW conjugate (Li-Cor) in Odyssey Block, 0.2% Tween, and 0.01% SDS for 1 hour, and washed several times in PBST. Signals were detected using the Odyssey Infrared Imaging System (Li-Cor) according to manufacturer protocols, and gel image was desaturated and inverted using Adobe Photoshop.

Results and Discussion

CwlT is required for horizontal transfer of ICE*Bs1*

We constructed a deletion of *cwlT* (Δ*cwlT19*) in ICE*Bs1* (Materials and Methods) and tested for the ability of ICE*Bs1*Δ*cwlT* to function in conjugation. The conjugation efficiency of wild type (*cwlT*+) ICE*Bs1* was ~5% transconjugants per donor (Table 2, line 1), similar to frequencies described previously (4). In contrast, there was no detectable transfer ($\leq 5 \times 10^{-5}$ %) of

ICE*Bs1*Δ*cwlT* (Table 2, line 2). The mutant phenotype was largely complemented by expression of wild type *cwlT* and all upstream ICE*Bs1* genes (Fig. 1) from an ectopic locus (Table 2, line 3). We were unable to complement ICE*Bs1*Δ*cwlT* by expressing *cwlT* alone at an exogenous locus (data not shown). We suspect that proper expression of *cwlT* requires coupling to expression of the upstream genes, similar to what has been observed with other ICE*Bs1* mutants (29). The complementation results indicate that the defect in conjugation was due predominantly to loss of *cwlT* and not an unexpected effect on downstream genes or a site in ICE*Bs1*, and that the ICE*Bs1*-encoded cell wall hydrolase CwlT is indispensable for conjugation.

Our results with *cwlT* contrast those for cell wall hydrolases from Gram negative conjugative elements, and are consistent with recent findings in Gram positive organisms. In Gram negative bacteria, loss of the element-encoded hydrolase reduces, but does not eliminate, conjugative transfer. For example, deletion of *virB1* from the *A. tumefaciens* Ti plasmid (20), gene *19* of R1 (21), and *traL* of pKM101 (22) results in an approximately 10- to 100-fold reduction in conjugative transfer. In contrast, loss of the hydrolase from Gram positive conjugative elements causes either a complete elimination in transfer, or a more severe reduction than that observed for conjugative elements from Gram negative bacteria. For example, loss of the hydrolase TcpG from pCW3 in *Clostridium perfringens* causes an approximately 1,000-fold decrease in conjugation (25), and loss of TraG from pIP501 (23) or PrgK from pCF10 (24) in *E. faecalis* causes complete elimination ($>10^5$-fold) of transfer.

We suspect that the apparently greater contribution to conjugation by the element-encoded hydrolases in Gram positive bacteria than that in Gram negative bacteria is partly due to the thicker cell wall. Consistent with the increased hydrolytic requirement, many hydrolases associated with Gram positive mobile elements have multiple hydrolytic domains. Like CwlT,

297 TraG (from pIP501) is predicted to contain both muramidase and peptidase function (23). PrgK
298 (from pCF10) contains three hydrolytic domains: two muramidases, and one peptidase (24). It
299 has been suggested that the peptidase domains are important in assisting digestion of highly-
300 crosslinked Gram positive cell wall (19, 42, 43).

301 Partial requirement for some hydrolases in conjugation might be due to redundant functions
302 in the host. Many hydrolases have a high degree of cross-functionality (13, 18, 44). That is,
303 there can be redundancy and the loss of one hydrolase is masked by the presence of others. For
304 the conjugative elements, we suspect that the partial requirement for hydrolases, in either Gram
305 positive or Gram negative bacteria, could be due to the activities of host hydrolases or hydrolases
306 from other resident mobile elements (25, 45-47). For CwlT of ICE*Bs1* and the essential
307 hydrolases from other mobile elements, it appears that the host hydrolases are not capable of
308 providing sufficient function to allow any detectable conjugative transfer.

309 **Different effects of muramidase and peptidase mutants of CwlT**

310 CwlT contains two peptidoglycan hydrolytic domains, a muramidase and a peptidase (9). To
311 determine their respective contributions to ICE*Bs1* transfer, we made mutations in each of the
312 two domains of CwlT and assayed for effects on the conjugation efficiency of ICE*Bs1*. Our
313 findings indicate that the muramadase is essential and the peptidase partly dispensable for the
314 function of CwlT in conjugation.

315 Muramidase activity is abolished by a previously characterized *cwlT-E87Q* mutation that
316 alters the catalytic site of the muramidase domain (9). We introduced this mutation into *cwlT* in
317 ICE*Bs1*. There was no detectable transfer of the ICE*Bs1 cwlT-E87Q* mutant (Table 2, line 4),
318 indicating that muramidase activity is required for transfer of ICE*Bs1*. Levels of CwlT-E87Q
319 protein accumulation appear comparable to those of wild type CwlT, as measured by Western

320 blots (Fig. 3), indicating that the mutant protein was accumulating to normal levels. The defect in
321 conjugation was due to the *cwlT-E87Q* mutation and not to an unexpected effect on downstream
322 genes because the mutant phenotype was fully complemented by exogenous expression of wild
323 type *cwlT* and the upstream ICE*Bs1* genes (Table 2, line 5).

324 To investigate the role of the peptidase domain, we constructed a point mutation in *cwlT* that
325 changes its putative catalytic cysteine (48, 49), *cwlT-C237A*. We used two assays to verify that
326 the mutant protein was defective in enzymatic function: a quantitative kinetic assay to measure
327 the rate at which CwlT degraded purified peptidoglycan, and a zymography assay to detect
328 hydrolase activity in purified proteins or cell lysates (41). We purified both wild type and mutant
329 peptidase fragments of CwlT, separate from the muramidase domain (9). There was no
330 detectable hydrolytic activity in the C237A mutant peptidase fragment by either kinetic assay or
331 by zymography (data not shown).

332 We introduced the *cwlT-C237A* mutation into ICE*Bs1* and tested for effects on conjugation.
333 This mutant had a conjugation efficiency of ~5 x 10^{-3} % transconjugants per donor (Table 2, line
334 6), approximately 1,000-fold less than that of wild type. Levels of CwlT-C237A protein
335 accumulation were comparable to those of wild type CwlT, as measured by Western blots (Fig.
336 3), again indicating that the mutant fragment was accumulating to normal levels. The defect in
337 conjugation was due to the *cwlT* mutation and not an unexpected effect on downstream genes
338 because the mutant phenotype was fully complemented by exogenous expression of wild type
339 *cwlT* and the upstream ICE*Bs1* genes (Table 2, line 7).

340 The conjugation efficiency of the *cwlT-C237A* peptidase mutant (~5 x 10^{-3} %) was
341 significantly and reproducibly greater than that of the muramidase mutant (< 5 x 10^{-5} %). We
342 were concerned that the *cwlT-C237A* mutation might not fully eliminate the peptidase activity in

vivo, and that the detectable conjugation could be a result of residual peptidase activity. To test this, we constructed an allele that deletes the peptidase domain, *cwlT*Δ(*207-329*), leaving the signal sequence and the muramidase domain (Fig. 2). The muramidase and peptidase domains have been shown to maintain robust enzymatic function when separated and purified as fragments (9). The deletion of the peptidase domain was introduced into *cwlT* in ICE*Bs1*. The conjugation efficiency of ICE*Bs1 cwlT*Δ*207-329* was ~3.0 x 10^{-3} % (Table 2, line 8), approximately 1,000-fold below that of wild type ICE*Bs1*, and similar to that of the *cwlT-C237A* mutant (Table 2, line 6). Again, the conjugation defect was fully complemented by expression of wild type *cwlT* and the upstream ICE*Bs1* genes (Table 2, line 9). These results indicate that the peptidase is partly dispensable for conjugation efficiency. They further indicate that the cysteine at amino acid 237 is required for peptidase activity. Based on comparisons to other peptidases, C237 is likely in the active site, and histidine at amino acid 290 and the asparagine at amino acid 302 are also likely required for peptidase activity (48, 49). Together, our results indicate that the muramidase function is absolutely required, and the peptidase function is partially required for transfer of ICE*Bs1*.

CwlT is similar to other hydrolases from well-characterized conjugative elements in Gram positive bacteria (Tn*916*, pIP501, pCW3, pCF10). The cell wall hydrolases from these elements have or are predicted to have two catalytic domains, a muramidase and a peptidase. Many other putative two-domain hydrolases are found in uncharacterized mobile elements from Gram positive hosts. Hydrolases in Gram negative conjugative elements appear to have only a single muramidase domain and the peptidase domain appears to be a unique addition to hydrolases from Gram positive systems. Some phage enzymes from Gram positives share a similar domain structure, and it has been suggested that the peptidase domains are important in assisting

digestion of highly-crosslinked Gram positive cell wall (42, 43). Our results with ICE*Bs1* are consistent with this suggestion. In ICE*Bs1*, the muramidase function of CwlT is essential, which is consistent with the observation that such activity is conserved in conjugative systems in both Gram negative and Gram positive organisms. The peptidase is partly dispensable, perhaps due to partial redundancy with host peptidases (see above).

The putative N-terminal signal sequence is, but the putative lipid attachment site is not needed for CwlT function

Subcellular localization plays an essential role in the regulation of many hydrolases. CwlT contains a putative N-terminal signal sequence, residues 1-29 (Fig. 2) that may determine its localization, though predictions of this region's function are discrepant. Different methods have predicted it to be either a lipoprotein signal sequence (9, 27), or a stable transmembrane domain (50, 51).

To determine if the putative signal sequence of CwlT in *B. subtilis*, is required for conjugation, we deleted codons 1-29 of *cwlT* {*cwlT*Δ(*1-29*)}, removing the putative signal sequence. There was no detectable transfer of ICE*Bs1 cwlT*Δ*1-29* (Table 2, line 10), indicating that this region of CwlT is important for function. These results are consistent with the notion that CwlT is a secreted protein.

The *cwlT* gene product contains an FVLC motif at amino acids 20-23, which was identified as a putative lipobox, a conserved sequence in lipoproteins (27). The cysteine in this motif is required for lipid attachment in bona fide lipoproteins. We changed the cysteine at amino acid 23 to alanine (*cwlT-C23A*) and found that there was no detectable change in conjugation efficiency (Table 2, line 11). This result indicates that if CwlT is a lipoprotein, then a lipid attachment at

388 cysteine 23 is not required for CwlT function. Alternatively, and more likely, CwlT is not a
389 lipoprotein, although we have not tested this directly.
390 The amount of each of the mutant proteins was analyzed by Western blot and was
391 indistinguishable from that of wild type CwlT (Fig. 3). Together, our results indicate that the
392 putative signal sequence of CwlT is needed for CwlT function, but that the putative lipid
393 attachment site is not. Preliminary results indicate that CwlT accumulates in culture supernatant
394 (data not shown), and that some of it is found associated with the cell (Fig. 3).
395 **CwlT can hydrolyze *B. subtilis* but not *B. anthracis* peptidoglycan in vitro**
396 ICE*Bs1* is capable of transferring from *B. subtilis* to *B. anthracis* (4). However, the cell wall
397 of *B. anthracis* is different from that of *B. subtilis* and we found that CwlT cannot degrade
398 purified *B. anthracis* peptidoglycan. The glycan strands from the cell wall of *B. anthracis* differ
399 from those of *B. subtilis* in two ways: *B. anthracis* glycan chains are *O*-acetylated and *N*-
400 deacetylated. Both of these modifications confer lysozyme resistance to *B. anthracis*, and might
401 also cause resistance to the muramidase activity of CwlT. In addition, although the peptides of *B.*
402 *subtilis* and *B. anthracis* peptidoglycan have the same amino acid sequence, in *B. subtilis*, the
403 carboxyl group of *meso*-diaminopimelic acid (m-DAP) is amidated (52). This modification is
404 not found in *B. anthracis* (11).
405 We purified CwlT and tested for degradation of cell wall material from *B. subtilis* and *B.*
406 *anthracis*. As expected, CwlT was able to degrade cell wall from *B. subtilis*, but not that from *B.*
407 *anthracis* (Fig. 4). We mixed 1.5 nmol of CwlT with 5 mg of purified *B. subtilis* cell wall and
408 monitored the change in turbidity of the solution with time (Fig. 4). There was a rapid drop in
409 turbidity within 5 minutes, indicating that the *B. subtilis* cell wall was degraded. In a similar
410 reaction with the *B. anthracis* cell wall, there was little or no change in turbidity in 20 minutes

411 (Fig. 4), indicating that the *B. anthracis* cell wall was resistant to degradation by CwlT. To be
412 sure that the preparation of peptidoglycan from *B. anthracis* did not contain an inhibitor of CwlT
413 activity, we mixed the peptidoglycan from *B. anthracis* with that from *B. subtilis*. In this mixed
414 peptidoglycan, CwlT was able to degrade about half of the material present (Fig. 4), indicating
415 that CwlT activity is not inhibited by anything in the peptidoglycan preparation from *B.*
416 *anthracis*. We also found that there was no detectable degradation of the *B. anthracis* cell wall
417 by CwlT in a polyacrylamide gel using zymography (data not shown), consistent with the results
418 in solution.

419 **CwlT is required for ICE*Bs1* mating from *B. subtilis* into *B. anthracis***

420 *B. anthracis* was a very effective recipient of ICE*Bs1*, even though its cell wall was not
421 degraded by CwlT. ICE*Bs1* was able to transfer from *B. subtilis* into *B. anthracis* with an
422 efficiency of ~3% transconjugants per donor, virtually indistinguishable from that of transfer
423 from *B. subtilis* to *B. subtilis* (Table 3). Like transfer of ICE*Bs1* from *B. subtilis* to *B. subtilis*,
424 transfer to *B. anthracis* was also dependent on *cwlT*. Both the muramidase mutant and the
425 peptidase mutant were defective in transfer from *B. subtilis* to *B. anthracis* (Table 3). Because
426 the peptidoglycan of *B. anthracis* is different from that of *B. subtilis* and was not digested by
427 CwlT, these results could indicate that CwlT is needed to act on the cell wall of the donor, in this
428 case *B. subtilis*, and not that of the recipient. However, subsequent experiments showed that
429 CwlT is also needed for ICE*Bs1* to transfer from *B. anthracis* donors.

430 **ICE*Bs1* can transfer out of *B. anthracis* into *B. subtilis* and *B. anthracis***

431 We found that ICE*Bs1* could transfer out of *B. anthracis* into both *B. subtilis* and *B.*
432 *anthracis* with similar efficiencies (Table 4). We used the DNA damaging agent mitomycin C to
433 induce ICE*Bs1* in *B. anthracis*. Mitomycin C induces ICE*Bs1* in *B. subtilis*, although less

efficiently than overproduction of RapI (4). Addition of mitomycin C to either *B. subtilis* or *B. anthracis* donors caused induction of ICE*Bs1* and enabled transfer to either *B. subtilis* or *B. anthracis* (Table 4). These results were somewhat surprising since CwlT appeared incapable of degrading the *B. anthracis* cell wall (Fig. 4).

CwlT is required for ICE*Bs1* mating from *B. anthracis* into *B. subtilis* and *B. anthracis*

It seemed possible that CwlT was not needed for ICE*Bs1* function in *B. anthracis*, and that other factors (perhaps cell wall hydrolases) in the *B. anthracis* donor strain might bypass the need for *cwlT*. For example, mitomycin C treatment induces a DNA damage response and the induction of many genes, some of which are in phage or prophage elements that contain their own hydrolytic enzymes that could substitute for CwlT (53).

We found that *cwlT* was needed for transfer of ICE*Bs1* from *B. anthracis* even after treatment with mitomycin C. We transferred ICE*Bs1 cwlT* mutants from *B. subtilis* into *B. anthracis*. This was done by complementing the *cwlT* mutants with a wild type *cwlT* in trans in the *B. subtilis* donor strains (Materials and methods). We then used the *B. anthracis* strains with the ICE*Bs1 cwlT* mutants as donors in conjugation experiments with either *B. subtilis* or *B. anthracis* as recipients (Table 4). When ICE*Bs1* was induced with mitomycin C, no transfer was detected from either *B. subtilis* or *B. anthracis* donors containing the *cwlT-E87Q*, *cwlT-C237A*, or *cwlT-E87Q-C237A* allele (Table 4). These results demonstrate that *cwlT* is needed for transfer from *B. anthracis*, that both enzymatic activities are required for transfer, and that the requirement for *cwlT* is not bypassed by treatment with mitomycin C.

Exogenous CwlT causes lysis of *B. subtilis* and *B. anthracis*

We found it puzzling that *cwlT* appeared to be required for transfer out of an organism with cell wall peptidoglycan that was resistant to its activity. We were interested in examining

457 whether CwlT might exhibit different activity on growing cell walls in vivo, than what we
458 observed on purified peptidoglycan in vitro. To test this, we added purified CwlT to *B. anthracis*
459 and *B. subtilis* cells growing in LB medium and measured effects on cell viability. Despite their
460 differences in cell wall composition and lysozyme resistance, both species were killed by CwlT.
461 Addition of 100 μg/ml of CwlT for 20 min caused an approximately 500 - 1,000 fold drop in
462 colony forming units of both *B. anthracis* and *B. subtilis*. These results indicate that CwlT was
463 able to kill both *B. subtilis* and *B. anthracis*, most likely by causing at least minimal degradation
464 of the cell wall. The amount of peptidoglycan hydrolysis by CwlT that is needed for cell lysis is
465 probably much less than that needed for detection of hydrolysis in vitro. In *B. anthracis*, cell
466 wall is first assembled in an unmodified form that resembles that of *B. subtilis*. Following the
467 initial synthesis, *N*-deacetylases and *O*-acetylases introduce modifications during peptidoglycan
468 maturation (10, 11). Our results indicate that CwlT may act on newly-synthesized peptidoglycan
469 before it is fully modified.

470 **Summary and model for CwlT Activity**

471 We found that the putative signal sequence on CwlT is essential for ICE*Bs1* conjugation, but
472 the putative lipid attachment site (cysteine at residue 23) is not. More importantly, we found that
473 the peptidase activity of CwlT is important but not essential, whereas the muramidase activity is
474 essential for conjugation. Surprisingly, we found that CwlT was needed for ICE*Bs1* to function
475 in *B. anthracis*, whose mature cell wall is resistant to degradation by CwlT. We interpret these
476 results to indicate that CwlT can act before full maturation of the cell wall, and this expands the
477 range of organisms in which ICE*Bs1* can function. We suspect that analogous cell wall
478 hydrolyases from other conjugative elements function similarly.

Our findings that CwlT is required for conjugation of ICE*Bs1* are consistent with recent results on cell wall hydrolases encoded by Gram positive conjugative plasmids (23-25). CwlT-mediated digestion likely causes local alteration of the peptidoglycan meshwork to allow assembly of the conjugation machinery. It is unknown what other ICE*Bs1*-encoded proteins associate with CwlT, though in the Gram positive conjugative plasmid pIP501, the cell wall hydrolase associates with the coupling protein, a putative ATPase, and a membrane-associated conjugation protein, indicating that it may be playing a role in recruitment of these proteins, and in the assembly of the conjugation machinery (19). CwlT may play a similar role, and it would be interesting to determine if CwlT affects localization or assembly of components of the ICE*Bs1* conjugation machinery.

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Table 1. *Bacillus* strains used.

B. subtilis	**Relevant Genotype[a] (reference)**
JH642	*trpC2 pheA1* (contains wild type ICE*Bs1*)
CAL85	ICE*Bs1*^{0} (cured of ICE*Bs1*) *str84* (30)
CAL229	*thrC*::{*mls*, ICE*Bs1* Δ(*rapI-phrI*)::*kan*}
MMB970	Δ(*rapI-phrI*)*342*::*kan amyE*::{(Pxyl-*rapI*) *spc*}
TD19	Δ(*rapI-phrI*)*342*::*kan* Δ*cwlT19 amyE*::{(Pxyl-*rapI*) *spc*}
TD37	Δ(*rapI-phrI*)*342*::*kan* Δ*cwlT19*, *thrC11*::{*mls* ICE*Bs1*Δ(*yddI-attR*::*tet*)} *amyE*::{(Pxyl-*rapI*) *spc*}
TD46	Δ(*rapI-phrI*)*342*::*kan cwlT-E87Q amyE*::{(Pxyl-*rapI*) *spc*}
TD48	Δ(*rapI-phrI*)*342*::*kan cwlT-C237A amyE*::{(Pxyl-*rapI*) *spc*}
TD50	Δ(*rapI-phrI*)*342*::*kan cwlT-E87Q-C237A amyE*::{(Pxyl-*rapI*) *spc*}
TD52	Δ(*rapI-phrI*)*342*::*kan cwlT-C237A amyE*::{(Pxyl-*rapI*) *spc*} *thrC11*::{*mls* ICE*Bs1* Δ(*yddI-attR*)::*tet*}
TD57	Δ(*rapI-phrI*)*342*::*kan cwlT-E87Q-C237A amyE*::{(Pxyl-*rapI*) *spc*} *thrC11*::{*mls* ICE*Bs1* Δ(*yddI-attR*)::*tet*}
TD62	Δ(*rapI-phrI*)*342*::*kan cwlT-E87Q amyE*::{(Pxyl-*rapI*) *spc*} *thrC11*::{*mls* ICE*Bs1*Δ(*yddI-attR*)::*tet*}
TD123	Δ(*rapI-phrI*)*342*::*kan cwlT*Δ(*1-29*) *amyE*::{(Pxyl-*rapI*) *spc*}
TD221	Δ(*rapI-phrI*)*342*::*kan cwlT-C27A amyE*::{(Pxyl-*rapI*) *spc*}
TD319	Δ(*rapI-phrI*)*342*::*kan cwlT*Δ(*207-327*) *amyE*::{(Pxyl-*rapI*) *spc*}
TD321	Δ(*rapI-phrI*)*342*::*kan cwlT*Δ(*207-327*) *amyE*::{(Pxyl-*rapI*) *spc*} *thrC11*::{*mls* ICE*Bs1* Δ(*yddI-attR*)::*tet*}
B. anthracis	**Relevant Genotype[b] (reference)**
UM44-1C9	(AG1924) *str ind* (plasmid-free strain) (4, 36)
JMA921	*str ind nal*
CAL2257	*str ind nal* pBS42 (CmR)
TD230	ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan str ind nal*
TD322	ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan cwlT-E87Q str ind nal*
TD324	ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan cwlT-C237A str ind nal*
TD326	ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan cwlT-E87Q-C237A str ind nal*

[a] All *B. subtilis* strains are derived from JH642 (54) and contain *trpC2* and *pheA1* (not shown). Unless otherwise indicated, all *B. subtilis* strains contain ICE*Bs1* integrated at its normal attachment site in *trnS-leu2*.

[b] *B. anthracis* plasmid free strain UM44-19C (55) is streptomycin resistant (*str*), requires indole (*ind*) or tryptophan for growth, and was the parent for other *B. anthracis* strains. *B. anthracis* strains do not contain ICE*Bs1* unless otherwise indicated.

667 **Table 2. Effects of *cwlT* mutations on transfer of ICE*Bs1* from *B. subtilis*.**

668

	Donor[a] (strain number)	**Mating efficiency[b]**
1.	WT *cwlT* (MMB970)	$5.9 \times 10^{-2} \pm 1.2 \times 10^{-2}$
2.	Δ*cwlT19* (TD19)	$<5 \times 10^{-7}$
3.	Δ*cwlT19 thrC11*::ICE*Bs1*Δ*yddI-attR* (TD37)	$6.6 \times 10^{-2} \pm 6.4 \times 10^{-2}$
4.	*cwlT-E87Q* (muramidase mutant) (TD46)	$<5 \times 10^{-7}$
5.	*cwlT-E87Q thrC11*::ICE*Bs1*Δ(*yddI-attR*) (TD62)	$6.0 \times 10^{-2} \pm 1.1 \times 10^{-2}$
6.	*cwlT-C237A* (peptidase mutant) (TD48)	$5.3 \times 10^{-5} \pm 3.0 \times 10^{-5}$
7.	*cwlT-C237A thrC11*::ICE*Bs1*Δ(*yddI-attR*) (TD52)	$4.4 \times 10^{-2} \pm 6.0 \times 10^{-3}$
8.	*cwlT*Δ(207-*329*) (deletion of peptidase domain) (TD319)	$3.0 \times 10^{-5} \pm 7.6 \times 10^{-6}$
9.	*cwlT*Δ(207-*329*) *thrC11*::ICE*Bs1*Δ(*yddI-attR*) (TD321)	$1.8 \times 10^{-2} \pm 2.1 \times 10^{-2}$
10.	*cwlT*Δ(*1-29*) (TD123)	$<6 \times 10^{-7}$
11.	*cwlT-C23A* (TD221)	$6.1 \times 10^{-2} \pm 2.8 \times 10^{-2}$

669

670 [a]All donor strains contain Δ(*rapI-phrI*)::*kan* in ICE*Bs1* and Pxyl-*rapI* (not shown) and the
671 indicated *cwlT* allele.

672 [b]The recipient in each conjugation experiment was CAL85 (streptomycin-resistant). Mating
673 efficiencies were calculated from the number of kanamycin-resistant, streptomycin-resistant
674 transconjugants per initial donor (± standard error of the mean). Cells were grown in LB
675 medium at 37°C and expression of RapI (Pxyl-*rapI*) in donors was induced by addition of xylose
676 for 1 hr. Mating mixtures were incubated at 37°C for 3 hr on filters (Materials and Methods).

677

678

Table 3. *cwlT* is required for ICE*Bs1* transfer from *B. subtilis* into *B. anthracis*.

Donor[a]	**Recipient**; **Mating Efficiency**[b]	
	B. subtilis (CAL85)	*B. anthracis* (JMA921)
Wild type (MMB970)	$5.5 \times 10^{-2} \pm 1.2 \times 10^{-2}$	$3.2 \times 10^{-2} \pm 5.9 \times 10^{-3}$
cwlT-E87Q (muramidase mutant) (TD46)	$< 6 \times 10^{-7}$	$< 6 \times 10^{-7}$
cwlT-C237A (peptidase mutant) (TD48)	$2.9 \times 10^{-5} \pm 9.2 \times 10^{-4}$	$4.3 \times 10^{-5} \pm 1.0 \times 10^{-5}$

[a]All donor strains are *B. subtilis* and contain ICE*Bs1* with Δ(*rapI-phrI*)::*kan* and Pxyl-*rapI* (not shown) and the indicated *cwlT* allele.

[b]Efficiencies of transfer of ICE*Bs1* from the indicated donor strains into either recipient CAL85 (*B. subtilis*) or JMA921 (*B. anthracis*) were calculated from the number of transconjugants per initial donor. Mating mixtures were incubated on filters at 37ºC for 3 hr.

690 **Table 4. *cwlT* is required for mitomycin C-induced transfer of ICE*Bs1* from *B. anthracis*.**

691

Donor[a]	**Recipient; Mating Efficiency**[b]	
	B. subtilis (CAL419)	*B. anthracis* (CAL2257)
B. subtilis; *cwlT*$^{+}$ (IRN342)	$1.2 \times 10^{-3} \pm 7.1 \times 10^{-4}$	$6.8 \times 10^{-3} \pm 1.1 \times 10^{-4}$
B. anthracis; *cwlT*$^{+}$ (TD230)	$7.0 \times 10^{-4} \pm 4.2 \times 10^{-4}$	$1.5 \times 10^{-4} \pm 8.0 \times 10^{-5}$
B. anthracis; *cwlT-E87Q* (TD322)	$< 3.6 \times 10^{-7}$	$< 5.0 \times 10^{-7}$
B. anthracis; *cwlT-C237A* (TD324)	$< 3.8 \times 10^{-7}$	$< 5.0 \times 10^{-7}$
B. anthracis; *cwlT-E87Q-C237A* (TD326)	$< 8.4 \times 10^{-7}$	$< 5.1 \times 10^{-7}$

692

693 [a]All donor strains contained ICE*Bs1* with Δ(*rapI-phrI*)::*kan* (not shown) and the indicated
694 *cwlT* allele. ICE*Bs1* was induced by addition of mitomycin C for 1 hr.

695 [b]Mating efficiencies from the indicated donor strains into either CAL419 (*B. subtilis*) or
696 CAL2257 (*B. anthracis*) were calculated from the number of transconjugants per initial donor.
697 Cells were grown in LB medium at 37ºC and ICE*Bs1* was induced by addition of mitomycin C
698 for 1 hr (Materials and Methods). Mating mixtures were incubated on filters at 37ºC for 3 hr.

699

700

Figure legends

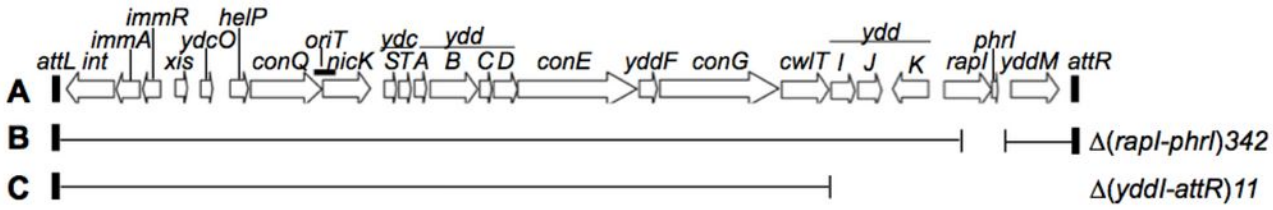
Figure 1. Map of ICE*Bs1* and derivatives.

A. Linear genetic map of ICE*Bs1* integrated in the chromosome. Open arrows indicate open reading frames and direction of transcription. Gene names are indicated above the arrows. The small rectangles at the ends of the element represent the 60 bp direct repeats that contain the site-specific recombination sites in the left and right attachment sites, *attL* and *attR*.

B and C. Various deletions of ICE*Bs1* used in this study. Thin horizontal lines below the map of ICE*Bs1* represent regions that are present, and open spaces represent regions that are missing. **B.** Δ(*rapI-phrI*) contains an insertion of *kan* (not shown). **C.** This construct is contained at *thrC* and was used to complement various *cwlT* mutations in ICE*Bs1* in the normal attachment site. Δ(*yddI-attR*) deletes all ICE*Bs1* genes to the right of *cwlT* and contains an insertion of *tet* (not shown). Figure adapted from (56).

Figure 2. Features of CwlT. CwlT is 328 amino acids. The putative signal sequence (amino acids 1 - 29), the muramidase domain (amino acids 62 - 164), and the peptidase domain (amino acids 216 - 328) are indicated. The putative lipid attachment residue is a cysteine at amino acid 23 (not shown. Figure adapted from (9).

723 **Figure 3. Accumulation of wild type and mutant CwlT proteins.** Western blots of cell
724 extracts 3 hr after induction of ICE*Bs1* by overproduction of RapI. The relevant *cwlT* allele is
725 indicated above each lane. The arrow on the right indicates full length CwlT. Alleles and
726 strains: wt, *cwlT*+ (MMB970); Δ*cwlT* (TD19); *cwlT-E87Q* (TD46); *cwlT-C237A* (TD48); *cwlT-*
727 *E87Q-C237A* (TD50); *cwlT*Δ(*207-329*) (TD319); *cwlT*Δ(*1-29*) (TD123); and *cwlT-C23A*
728 (TD221). Blots were probed with anti-CwlT anti-serum (Materials and Methods).
729
730
731 **Figure 4. CwlT degrades purified cell wall peptidoglycan from *B. subtilis* but not *B.***
732 ***anthracis*.** Cell wall lytic activity of CwlT on peptidoglycan from *B. subtilis* (triangles), *B.*
733 *anthracis* (diamonds), or a 1:1 mix of both types (circles). CwlT-his (10 μg/ml) was mixed with
734 approximately 1.0 mg/ml of purified peptidoglycan, and the turbidity of the reaction was
735 monitored at 540 nm (Materials and Methods).
736
737
738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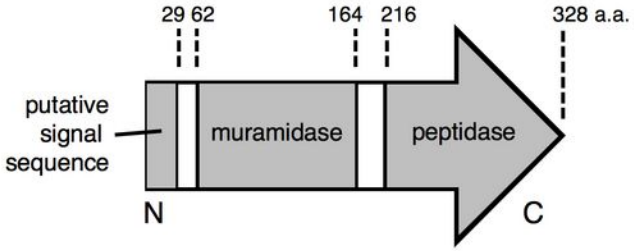
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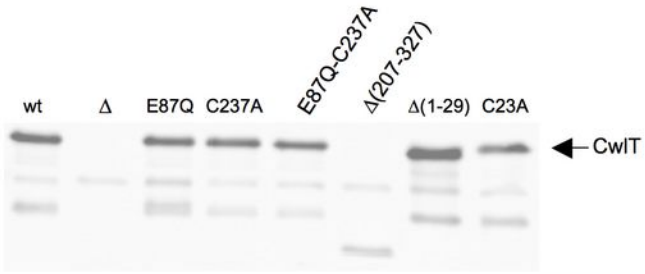
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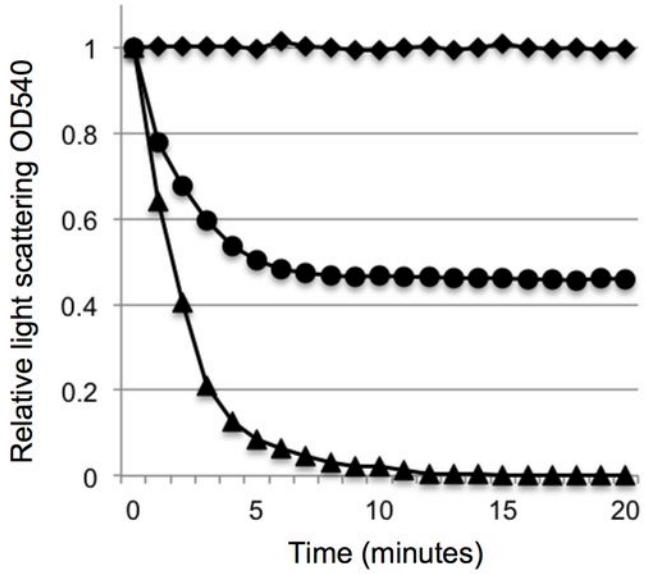
CwlT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