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In vivo cross-linking of EpsG to EpsL suggests a role for EpsL as an ATPase-pseudopilin coupling protein in the Type II secretion system of *Vibrio cholerae*

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Summary

The type II secretion system is a multi-protein complex that spans the cell envelope of gram-negative bacteria and promotes the secretion of proteins, including several virulence factors. This system is homologous to the type IV pilus biogenesis machinery and contains five proteins, EpsG-K, termed the pseudopilins that are structurally homologous to the type IV pilins. The major pseudopilin EpsG has been proposed to form a pilus-like structure in an energy-dependent process that requires the ATPase, EpsE. A key remaining question is how the membrane-bound EpsG interacts with the cytoplasmic ATPase, and if this is a direct or indirect interaction. Previous studies have established an interaction between the bitopic inner membrane protein EpsL and EpsE; therefore, in this study we used *in vivo* cross-linking to test the hypothesis that EpsG interacts with EpsL. Our findings suggest that EpsL may function as a scaffold to link EpsG and EpsE and thereby transduce the energy generated by ATP hydrolysis to support secretion. The recent discovery of structural homology between EpsL and a protein in the type IV pilus system implies that this interaction may be conserved and represent an important functional interaction for both the type II secretion and type IV pilus systems.

Keywords

type IV pilus; prepilin peptidase; PilD

Introduction

The type II secretion (T2S) system is found widely distributed among gram-negative bacteria and is considered a primary virulence system (Cianciotto, 2005, Sandkvist, 2001b). In *Vibrio cholerae*, this system is encoded by the extracellular protein secretion (*eps*) operon and is used to secrete several proteins, including the main virulence factor cholera toxin, across the cell envelope (Sandkvist *et al.*, 1997). The complex is composed of at least 12 different proteins, each individually essential for secretion to occur, which form a structure that spans the entire cell envelope (Filloux, 2004). The T2S apparatus is highly homologous to the type IV pilus biogenesis machinery which produces cell surface filaments that are

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involved in multiple processes, including attachment and colonization, biofilm formation, DNA uptake, twitching motility, and virulence (Craig & Li, 2008).

Specifically, the assembled T2S system consists of the cytoplasmic ATPase, EpsE, an inner membrane platform composed of EpsC, F, L, and M, and an outer membrane secretin pore formed by EpsD (Johnson *et al*., 2006). The T2S machinery also contains five additional gene products termed the pseudopilins, EpsG, H, I, J and K, that are structurally homologous to the type IV pilins (Alphonse *et al.*, 2010, Kohler *et al.*, 2004, Korotkov *et al.*, 2009, Korotkov & Hol, 2008, Yanez *et al*., 2008). Additionally, both the T2S pseudopilins and the type IV pilins contain a conserved N-terminus which is recognized and processed by the prepilin peptidase PilD, also referred to as VcpD (Marsh & Taylor, 1998, Strom *et al.*, 1993, Fullner & Mekalanos, 1999). Processing of the pilin subunits is a prerequisite for their incorporation into functional pili (Fullner & Mekalanos, 1999, Marsh & Taylor, 1998, Nunn *et al*., 1990).

Due to the similarities between the pseudopilins and the type IV pilins, EpsG-K have been suggested to form a pilus-like structure, or pseudopilus, where EpsG is the major and EpsH-K are minor pseudopilins (Campos *et al.*, 2010, Johnson *et al*., 2006). Over-expression of the EpsG homologs XcpT and PulG in *Pseudomonas aeruginosa* and *Klebsiella oxytoca*, respectively, confirmed that the major pseudopilin is indeed capable of assembling into a pilus-like structure that can be detected on the surface of the bacteria (Durand *et al.*, 2003, Vignon *et al.*, 2003). Detection of surface pseudopili required the ATPase and inner membrane platform proteins, and was only observed under over-expression conditions when all other components of the T2S system were held constant. However, during native expression of the complex surface appendages were not observed. Instead, the pseudopilus is hypothesized to span the periplasm and promote secretion by functioning as a piston that can push secreted proteins through the outer membrane pore, or alternatively, by acting as a retractable plug (Filloux, 2004, Hobbs & Mattick, 1993, Sandkvist, 2001a).

The type IV pilins and T2S pseudopilins require the function of an ATPase to polymerize (Camberg & Sandkvist, 2005, Durand *et al.*, 2005, Pelicic, 2008); however, a key question is how the cytoplasmic ATPase of these systems is able to interact with the pilin or pseudopilin subunits that are located within the inner membrane and exposed to the periplasm. A study using *P. aeruginosa* identified a point mutation in the EpsG homolog, XcpT, which inhibited secretion and could be suppressed by a second point mutation in the EpsE homolog, XcpR (Kagami *et al.*, 1998). Based on this result it has been proposed that EpsG and EpsE are capable of interacting directly, however, molecular mapping of EpsE using subcellular fractionation, domain swapping, and x-ray crystallography have strongly suggested that the region of the suppressor mutation in the EpsE homolog is involved in an interaction with the bitopic inner membrane protein XcpY, the EpsL homolog (Abendroth *et al.*, 2005, Sandkvist *et al.*, 1995). Therefore, EpsL is a plausible candidate to bridge EpsG and EpsE and transduce the energy provided by ATP hydrolysis in order to promote secretion.

In this study we used *in vivo* cross-linking and co-immunoprecipitation to establish an interaction between the major pseudopilin subunit, EpsG, and EpsL. This interaction occurs in the absence of all other structural components of the apparatus but does require the prepilin peptidase, PilD. Furthermore, when the point mutation originally isolated in XcpT was made in EpsG it severely reduced the amount of cross-linked EpsG-EpsL complex. Based on our findings we propose that EpsL is the linker that allows for association of the ATPase with EpsG, and may function as a mechanical lever to transfer the energy required to support either assembly or disassembly of the pseudopilus.

Results

EpsG and EpsL interact in *V. cholerae*

Based on our previous work detailing the interaction between EpsE and the cytoplasmic domain of the membrane protein EpsL (Abendroth *et al*., 2005, Sandkvist *et al*., 2000, Sandkvist *et al.*, 1995), we sought to determine if EpsL interacts with EpsG and thus provides a link between the pseudopilus and the ATPase. Previous reports on EpsG homologs have focused on the interactions amongst the five pseudopilins (Douzi *et al.*, 2009, Hu *et al.*, 2002, Kuo *et al.*, 2005), however, a definitive interaction between the major pseudopilin and other components of the T2S apparatus has yet to be revealed. This is likely due to the dynamic nature of the secretion machinery in which transient or weak interactions may be difficult to capture; therefore, we chose to use the membrane permeable cross-linker dithiobis (succinimidyl propionate), DSP, to preserve potential interactions that involve EpsG. Specifically, whole cells were treated with several concentrations of DSP, lysed, and subjected to SDS-PAGE and immunoblotting with anti-EpsG antiserum. Treatment with DSP produced higher molecular weight species in a concentration dependent manner in a wild type strain that were specific for EpsG, as these complexes were absent in a Δ*epsG* mutant strain (Figure 1A, compare lanes 4–6 with 7–9). The two most prominent cross-linked species were approximately 35 and 60 kDa. The 60 kDa complex was of the appropriate molecular weight to represent a heterodimer of EpsG and EpsL, whose molecular masses are 15 kDa and 45 kDa, respectively. Furthermore, this species was shown to be absent in a Δ*epsL* mutant strain (Figure 1A). The complex at 35 kDa is presumed to be a dimer of EpsG as it has been well documented that EpsG and its homologs can form dimers even in the absence of other T2S components (Kohler *et al*., 2004, Korotkov *et al*., 2009).

In order to increase the detection of the specific EpsG complexes we also examined the cross-linking profile in a strain where the native *eps* promoter has been replaced with the P_{BAD} arabinose-inducible promoter, referred to as P_{BAD}::*eps* (Sikora *et al.*, 2007). This strain allows for specific upregulation of the entire *eps* operon, resulting in increased levels of all T2S components and thereby maintaining the stochiometry of the secretion complex. Growth of the P_{BAD}::*eps* strain in the presence of 0.01% arabinose enhanced the detection of cross-linked EpsG complexes while preserving the cross-linking profile that was observed in wild type cells (Figure 1A lanes 1–3); thus, we chose to use the P_{BAD} promoter background to further elucidate the EpsG-EpsL interaction. First, we confirmed that deletions of *epsG* and *epsL* in the P_{BAD}::*eps* background inhibited secretion and complementation with plasmid-encoded *epsG* and *epsL* restored secretion in the deletion mutants to near wild-type levels (Figure 2). As expected, we found that deletion of either the *epsG* or *epsL* genes in the P_{BAD}::*eps* strain prevented detection of the 60 kDa cross-linked species with anti-EpsG antibodies (Figure 1B lanes 7 and 9). Furthermore, complementation of the deletion strains with *epsG* or *epsL* encoded upon a plasmid restored the detection of the 60 kDa complex (Figure 1B lanes 8 and 10).

To further verify that the 60 kDa species corresponds to an EpsG-EpsL complex we subjected cross-linked samples to co-immunoprecipitation. Whole cells of P_{BAD}::*eps*, P_{BAD}::Δ*epsG*, P_{BAD}::Δ*epsL*, and their respective complemented strains were cross-linked with 0.25 mM DSP, and cell lysates were prepared and incubated with antibodies specific for either EpsG or EpsL bound to protein G sepharose beads. Following extensive washing the precipitated material was analyzed by SDS-PAGE, and immunoblotted with biotinylated anti-EpsG or anti-EpsL antibodies. The results from the co-immunoprecipitation supported the whole cell cross-linking data by demonstrating that the 60 kDa band was only precipitated from the P_{BAD}::*eps* and complemented strains. The 60 kDa complex was not pulled down from the *epsG* and *epsL* deletion strains when the cell extracts were

precipitated with either EpsG or EpsL anti-sera (Figure 3). Precipitating cell extracts with anti-EpsG antibodies followed by immunoblotting for EpsG enriched for monomeric EpsG as well as cross-linked EpsG species, including the 60 kDa complex in wild type and complemented strains (Figure 3A lanes 4 and 6; Figure 3D lanes 4 and 6). Likewise, precipitating samples with anti-EpsL antibodies followed by immunoblotting for EpsL enriched for the EpsL monomer and the 60 kDa protein species (Figure 3B lanes 7 and 9; Figure 3C, lanes 7 and 9). Most intriguingly, when samples were pulled down with anti-EpsG antibodies and immunoblotted for EpsL the only band that was detected was the 60 kDa species (Figure 3B lanes 4 and 6; Figure 3C lanes 4 and 6). Similarly, when samples were immunoprecipitated with anti-EpsL antibodies and immunoblotted for EpsG (Figure 3A lanes 7 and 9; Figure 3D lanes 7 and 9), the only band that was detected was the 60 kDa species. These results confirm that the 60 kDa band represents a cross-linked complex between EpsG and EpsL.

EpsG processing is required for the interaction with EpsL

We next wanted to determine if processing of EpsG by the prepilin peptidase, PilD, is required for the interaction with EpsL. The precursors of the T2S pseudopilins and type IV pilins are known to be processed by PilD, a unique peptidase that is responsible for both cleavage and methylation of the newly formed N-terminus (Nunn & Lory, 1993, Pugsley, 1993, Strom *et al*., 1993). In the absence of *pilD* secretion through the T2S apparatus is inhibited, indicating that processing of the pseudopilins is necessary for these proteins to be functional (Fullner & Mekalanos, 1999, Marsh & Taylor, 1998). In order to address if PilD is required for EpsG and EpsL to associate we performed *in vivo* cross-linking using whole cells of wild-type and a *pilD* deficient strain of *V. cholerae*, followed by SDS-PAGE and immunoblotting for EpsG. The Δ*pilD* mutant strain did not exhibit the 60 kDa cross-linked complex observed in the wild-type strain although a very faint band slightly larger than the EpsG-EpsL complex was detected that may be indicative of unprocessed EpsG associating with EpsL (Figure 4A). However, the intensity of this band was greatly reduced in comparison to the band comprising EpsL and the mature, processed form of EpsG suggesting that the affiliation between unprocessed EpsG and EpsL was much less efficient. Furthermore, upon complementation of the Δ*pilD* strain the position and intensity of the EpsG-EpsL cross-linked species was restored to the levels observed in the wild-type cells.

To further verify that processing of EpsG is required for cross-linking with EpsL, we also examined an EpsG mutant that cannot be processed by PilD. Previous studies on type IV pilins and the EpsG homolog, PulG, from *K. oxytoca* demonstrated that substitution of the glycine residue (referred to as G-1) at the cleavage site G-F prevented cleavage by PilD, and inhibited pilus formation and secretion, respectively (Pugsley, 1993, Strom & Lory, 1991). We substituted EpsG residue G-1 for valine and expressed the mutant gene from a plasmid in the P_{BAD}::Δ*epsG* strain. Similar to the finding in *K. oxytoca*, $EpsG_{G\text{-}1V}$ was not processed (Figure 4B) and unable to support secretion of protease through the T2S apparatus (Figure 2). We next performed *in vivo* cross-linking with P_{BAD}::Δ*epsG* expressing wild type EpsG or $EpsG_{G\text{-}1V}$, and subjected samples to SDS-PAGE and immunoblotting for EpsG. As was observed for the *pilD* deficient strain, the $EpsG_{G\text{-}1V}$ mutant did not exhibit the 60 kDa cross-linked band indicative of an EpsG-EpsL interaction (Figure 4B). From these data we concluded that processing of EpsG by the prepilin peptidase is necessary for the association with EpsL.

EpsG and EpsL interact in the absence of all other T2S components

Since our data signified that processing by the prepilin peptidase is a prerequisite for EpsG to interact with EpsL, we wanted to examine whether any other components of the T2S machinery are essential for the interaction to occur. We first determined the *in vivo* cross-

linking pattern of EpsG in strains deficient for each individual gene encoded by the *eps* operon and found that EpsG and EpsL still interacted, indicating that no specific component was required for their association (data not shown). To further explore the requirements necessary for EpsG and EpsL to assemble we expressed either *epsG* alone (pEpsG), or in conjunction with *epsL* (pEpsGL), in a *V. cholerae* strain where the entire *eps* operon has been deleted (Δ*eps*) but still encodes the prepilin peptidase, PilD (Sikora *et a*l., 2007). Whole cells were incubated with DSP, subjected to SDS-PAGE, immunoblotted for EpsG, and the cross-linking profiles were compared to that of wild-type cells. As shown in Figure 5A, EpsG in the presence of only EpsL was sufficient for the 60 kDa complex to be restored and the band migrated in accordance to the EpsG-EpsL protein species observed in the wild-type strain. Expression of *epsG* alone did not result in the 60 kDa cross-linked band; however, when EpsG was produced alone or with EpsL we did observe other EpsG protein species, including the intense band around 35 kDa which is presumed to be an EpsG dimer. Despite the presence of these additional EpsG specific bands, the 60 kDa band was only observed in the presence of EpsL.

Although we were capable of establishing an EpsG-EpsL interaction in the absence of other Eps components in *V. cholerae*, we wanted to additionally determine if any other proteins specific to *V. cholerae* are required for EpsG and EpsL to form a complex by performing the cross-linking experiments in *E. coli*. Because processing of EpsG by PilD was required for the EpsG-EpsL association, we tested *E. coli* expressing *pilD* from a plasmid as well as an additional vector containing either *epsG* (pEpsG), *epsL* (pEpsL), or both *epsG* and *epsL* (pEpsGL). Whole cells were cross-linked, and analyzed by SDS-PAGE and immunoblotting with either EpsG or EpsL antisera. As was observed in *V. cholerae*, the 60 kDa protein species was only detected when both EpsG and EpsL were present, suggesting that no other protein unique to *V. cholerae* is required for their interaction (Figure 5B). In *E. coli* we also saw other EpsG specific protein species that were present in the absence of EpsL, including the proposed dimer at 35 kDa. Interestingly, the 35 kDa band and another EpsG complex located at approximately 80kDa were reduced when *epsL* was expressed with *epsG*. This may imply that EpsG preferentially associates with EpsL and that the addition of EpsL titrated EpsG away from other protein complexes.

In *V. cholerae* we were unable to determine the cross-linking profile for EpsL due to cross-reactive bands recognized by our polyclonal EpsL antibodies, unless the cross-linking procedure was followed by immunoprecipitation and immunoblotting. However, in *E. coli* we were able to address whether an EpsG and EpsL interaction could be detected by immunoblotting with anti-EpsL antibodies. When comparing cells expressing EpsL either alone or in the presence of EpsG the only additional species that was detected when EpsG and EpsL were co-produced migrated to approximately 60 kDa, implying a specific EpsG-EpsL association (Figure 5C). Our findings that expression of *epsG* and *epsL* concomitantly in either *V. cholerae* Δ*eps* or *E. coli* results in an EpsG-EpsL cross-linked complex suggests that they do not require any additional members of the T2S machinery in order to associate. Furthermore, these data establish that the cross-linked EpsG species observed at 60 kDa in wild type *V. cholerae* is indicative of an EpsG-EpsL complex.

Mutation of EpsG residue T112 alters the interaction with EpsL

To further explain the original finding by Kagami *et al*. that showed that the threonine to leucine mutation isolated in XcpT, the *P. aeruginosa* homolog of EpsG, was suppressed by a second mutation in the EpsE homolog, XcpR, we created the equivalent residue change, $EpsG_{T112L}$, as was reported for XcpT (Kagami *et al*., 1998). The recently solved structure for EpsG indicated that the side chain of residue T112 is within hydrogen bond distance to residue D91 and mutation of either residue should have a similar effect on EpsG (Figure 6A) (Korotkov *et al*., 2009); therefore, we also substituted D91 for glutamic acid. The mutant

genes were expressed from a plasmid in the P_{BAD}::Δ*epsG* strain and we addressed whether the mutant proteins would interfere with secretion through the T2S apparatus by testing for the presence of a secreted protease in the culture supernatant. As shown in Figure 2, neither $EpsG_{T112L}$ nor $EpsG_{D91E}$ were able to support secretion of the protease, indicating that mutation of either residue resulted in a non-functional protein. The residue changes reduced the detection of EpsG protein seen by immunoblotting, implying that the mutant proteins may be less stable (data not shown).

In order to examine if replacement of residues T112 or D91 affected the ability of EpsG to cross-link with EpsL we needed to produce the mutant proteins at the same level as wild type EpsG in P_{BAD}::Δ*epsG*. We first determined the amount of IPTG required to produce the equivalent amount of all EpsG variants. Whole cells were then incubated with or without 0.1 mM DSP, and subjected to SDS-PAGE and immunoblotting with anti-EpsG antiserum. Neither mutant protein was able to cross-link with EpsL at the level observed in the complemented strain with both $EpsG_{T112L}$ and $EpsG_{D91E}$ exhibiting significantly reduced amounts of the EpsG-EpsL 60 kDa band (Figure 7). While wild type EpsG was found in the 60 kDa complex with EpsL the non-functional mutant proteins accumulated in a 45 kDa complex, similar to the cross-linking profile observed in an *epsL* deficient strain. Taken in conjunction with the protease secretion data for the point mutations, our cross-linking result implies that the changes in EpsG residues T112 or D91 prevent or alter the EpsG-EpsL interaction. However, since the side chains of T112 and D91 are barely surface exposed and occur at the bottom of a deep crevice (Figure 6B) they may not mediate a direct contact with EpsL. Therefore, replacement of these residues likely has an indirect effect on the EpsG-EpsL interaction.

Discussion

In order to better understand how the T2S apparatus assembles and promotes secretion through the gram-negative cell envelope, we have continued to examine the specific protein interactions that occur within this secretion complex. The T2S machinery is likely dynamic, therefore, in this study we elected to use *in vivo* cross-linking in whole cells in order to capture interactions within their native environment. Using this methodology, we have established a protein interaction between the major pseudopilin, EpsG, and the inner membrane component, EpsL. Additionally, we have determined the T2S components necessary for the EpsG-EpsL interaction, thus allowing us to begin to dissect the sequential order of events that leads to the incorporation of EpsG into the T2S complex, or pseudopilus. Previous studies have shown that homologs of EpsG are cotranslationally inserted into the inner membrane using the Signal Recognition Particle and SEC pathway, and do not require any components of the T2S inner membrane platform in order to be targeted to the inner membrane (Arts *et al.*, 2007, Francetic *et al.*, 2007). Following insertion into the inner membrane, our results indicate that EpsG must be processed by the prepilin peptidase before it can interact with EpsL. The processing results in the removal of 7 cytoplasmically exposed N-terminal residues of EpsG (Fullner & Mekalanos, 1999, Marsh & Taylor, 1998). The processing and methylation events, both performed by PilD, have been speculated to cause a conformational change that alters the way that EpsG is presented within the inner membrane, thus enabling EpsG to interact with other components of the T2S apparatus (Nunn & Lory, 1993, Strom *et al.*, 1993). This hypothesis is supported by our finding that processing is a prerequisite for EpsG to cross-link with EpsL, suggesting that it is the mature form of EpsG that is recognized by EpsL. Furthermore, we show that no other T2S component is required for the interaction between EpsG and EpsL. This suggests that EpsL may be the first protein that EpsG interacts with as it is incorporated into the T2S machinery, and that EpsL may be responsible for recruiting EpsG to this complex.

One implication as to the importance of the interaction between EpsG and EpsL is that EpsL may function to link the cytoplasmic ATPase, EpsE, with EpsG. The interaction between EpsE and the cytoplasmic domain of EpsL is necessary and sufficient to recruit EpsE to the inner membrane (Abendroth *et al*., 2005, Sandkvist *et al*., 1995). In addition, this interaction allows EpsE to adopt an active oligomeric state and *in vitro* studies have demonstrated that EpsL stimulates the ATPase activity of EpsE (Camberg *et al.*, 2007). Recent findings from our laboratory and others have shown that members of the type II/IV secretion ATPase family to which EpsE belongs undergo dynamic changes as they bind and hydrolyze ATP (Patrick *et al*., submitted, Misic *et al*., 2010, Planet *et al*., 2001, Robien *et al*., 2003, Satyshur *et al.*, 2007, Savvides *et al.*, 2003, Yamagata & Tainer, 2007). Due to the presence of a flexible linker, the entire N-terminal domain (NTD) of EpsE undergoes a large movement relative to the nucleotide-binding C-terminal domain. The ensuing conformational changes do not only have substantial effects on the arrangement of subunits within the EpsE hexamer, but may also affect EpsL, which is interacting with a sub-domain of the NTD (Abendroth *et al.*, 2005). The conversion of chemical energy to mechanical work, translated through EpsL, may in turn promote assembly of the pseudopilus. In support of this suggestion, Py *et al*. have shown that the interaction between the cytoplasmic domain of the EpsL and EpsE homologs, OutL and OutE, in *Dickeya dadantii* (previously classified as *Erwinia chrysanthemi*) results in a conformational change in the periplasmic domain of OutL (Py *et al*., 2001).

We found that the T112L and D91E substitutions in EpsG severely reduced the amount of cross-linked EpsG-EpsL complex and negatively affected the secretion of protease via the T2S machinery. Sequence comparison of EpsG with other T2S homologs showed that T112 is highly conserved and is found as either a threonine or serine, while D91 is conserved as an aspartic acid in all homologs examined (Korotkov *et al*., 2009). The structure of EpsG revealed that the side chain hydroxyl of T112 forms a solvent exposed hydrogen bond with one of the carboxylate oxygens from D91 at the bottom of a deep crevice (Figure 6) (Korotkov *et al*., 2009). The second carboxylate atom of D91 is engaged in a hydrogen bond with the main chain NH group of Asn95. The mutation T112L disrupts the hydrogen bond between T112 and D91. Due to close packing of residues at this site the substitution D91E could also affect this hydrogen bond as well as the one between D91 and Asn95 and thus alter the conformation of loop 90–96. The resultant conformational change may explain why introducing an additional atom through the conservative replacement D91E is sufficient to have a negative effect on EpsG. In summary, the T112L and D91E mutations may cause a perturbation of the native fold and thus indirectly affect the EpsG-EpsL interaction. In preliminary attempts to identify the site(s) of interaction between EpsG and EpsL we have replaced every individual lysine residue in EpsG (data not shown). Only in one of the mutants ($EpsG_{K70A}$) was the EpsG-EpsL cross-linking reduced, but not abolished (data not shown). Therefore, future studies will be needed to determine the precise site of interaction between EpsG and EpsL, which could occur either between the exposed periplasmic globular domains or within the membrane spanning portion of the proteins.

Due to the structural similarities between the pseudopilins, several intriguing questions remain regarding the minor pseudopilins, EpsH-K, which are proposed to form a cap on the tip of the pseudopilus (Douzi *et al*., 2009, Korotkov & Hol, 2008). It has yet to be determined if the minor pseudopilins also interact with EpsL; however, if the role of EpsL is to recruit EpsG to the T2S complex then it may also serve as a recruitment factor for one or more of the structurally related minor pseudopilins. Support for this suggestion comes from a yeast two hybrid study of T2S proteins from *D. dadantii* that detected an interaction between the EpsJ and EpsL homologs, OutJ and OutL (Douet *et al.*, 2004). It is possible that the minor pseudopilins also require an interaction with EpsL and energy provided by EpsE in order to become incorporated into the pseudopilus. Energy transduced by EpsL may be

required to extract the N-terminal hydrophobic alpha-helix of both the major and minor pseudopilins from the inner membrane as the pseudopilus is formed in the periplasmic compartment.

A related mechanism to link the pilin subunits to the ATPase is likely to exist for the type IV pilus biogenesis machinery. Recently, the structure of *P. aeruginosa* PilN was found to be homologous to that of the periplasmic domain of EpsL (Sampaleanu *et al.*, 2009). PilN was suggested to interact with the cytoplasmic protein PilM, which has a predicted actin-like fold similar to the cytoplasmic domain of EpsL, in order to form a complex that is functionally equivalent to EpsL (Ayers *et al.*, 2009, Abendroth *et al.*, 2004). The findings that PilM and PilN, as well as homologs for EpsF and EpsM are required to form the type IV pilus suggests that the inner membrane complex of the type IV pilus machinery may be analogous to the one formed by the T2S apparatus (Ayers *et al*., 2009, Carbonnelle *et al*., 2006). Misic *et al*. have speculated that the type IV pilins may either directly interact with the ATPase or that their interaction may be mediated by the inner membrane complex composed of PilN, and the EpsF and EpsM homologs (Misic *et al*., 2010). Based on our findings, we propose that the PilM/PilN complex interacts with the pilin subunits and links them to the ATPase to support pilus assembly. At this point it is not understood why the EpsL equivalent is split into two proteins in the type IV pilus biogenesis system in *P. aeruginosa*, but it may be related to the requirement for several different ATPases to support pilus assembly and disassembly that lead to twitching motility (Pelicic, 2008). However, comparable to the T2S system, non-retractile type IV pili such as the toxin co-regulated pilus (TCP) in *V. cholerae* may only require the function of one ATPase. The cytoplasmic ATPase TcpT of the TCP system has been shown to interact with TcpR, a bitopic inner membrane protein that has suggested homology to EpsL (Tripathi & Taylor, 2007). Similar to the relationship between EpsE and EpsL, the interaction between TcpT and TcpR is responsible for bringing the ATPase to the inner membrane (Tripathi & Taylor, 2007). Intriguingly, the first 100 amino acids of TcpT are involved in the interaction with TcpR which is in agreement to the region of EpsE that interacts with EpsL, thus, it is likely that TcpR may interact directly with the pilin to support assembly.

In this study we have furthered our understanding of the T2S system by establishing a protein interaction between the major pseudopilin EpsG and the inner membrane component EpsL. Interestingly, every condition examined that disrupted or altered the interaction between EpsG and EpsL also prevented secretion, validating the importance of their association within the T2S complex. Future work will be needed to completely understand the implications of this interaction; however, we propose that EpsL functions to link EpsG to the ATPase, providing a means by which energy is transduced between the two compartments in order for the pseudopilus to assemble.

Experimental procedures

Bacterial strains and growth conditions

All strains and plasmids used in this study are listed in Table 1. Strains were grown in Luria-Bertani (LB) broth at 37°C. *V. cholerae* TRH7000 and its derivatives were cultured in the presence of 100 μg/ml thymine. *V. cholerae* P_{BAD}::*eps* strains were grown in the presence of 0.01% arabinose. Antibiotics (Sigma-Aldrich) were used at the following concentrations: ampicillin at 100 μg/ml, carbenicillin at 200 μg/ml, chlormanphenicol at 2 μg/ml, and kanamycin at 50 μg/ml. Expression of *eps* genes was induced with isopropanyl-β-D-thiogalactopyranoside (IPTG) at the specified concentrations.

Construction of deletion strains

P_{BAD}::Δ*epsG* was constructed by replacing chromosomal *epsG* with the *cat* gene conferring chloramphenicol resistance in P_{BAD}::*eps* using the conditions previously described for creating TRH7000 Δ*epsG* (Lybarger *et al.*, 2009). Simlarly, P_{BAD}::Δ*epsL* was constructed in P_{BAD}::*eps* by replacing chromosomal *epsL* with *aph*-3 conferring kanamycin resistance using the conditions previously described for creating TRH7000 Δ*epsL* (Sikora *et al*., 2007).

Plasmid construction and conjugation

pEpsGL was constructed by excising *epsG* cloned in PCR-script (Stratagene) using the restriction sites PstI and SalI. The excised *epsG* fragment was cloned into similarly digested pMS44 using T4 DNA Ligase (New England BioLabs) in order to concomitantly express full length EpsG upstream of full length EpsL.

T112L mutation of *epsG* was introduced with QuickChange II site-directed mutagenesis kit (Stratagene) using previously constructed pEpsG as a template (Lybarger *et al*., 2009). Primers used for the site change were 5′-ggcacgattgatgtgttcctgttaggtgcggacggtcaag-3′ and 5′-cttgaccgtccgcacctaacaggaacacatcaatcgtgcc-3′. G-1V mutation of *epsG* was made similarly using the primers 5′-tgcgtaaacaaacggtctttaccctgctcgaagtaatg-3′ and 5′-cattacttcgagcagggtaaagaccgtttgtttacgca-3′.

pMMB872 was created for downstream subcloning by sequential removal of the BstEII sites from pMMB67 using the mutagenesis technique described previously (Allemandou *et al.*, 2003). The primer pairs used to remove the site from lacIQ were 5′-cagcgcgatttgctggtggcccaatgcgaccaga-3′ and 5′-gggctggacggtaactgagttcgcggcgggca-3′ (pMMBO109-1), and 5′-P-tgctccacgcccagtcgcgtaccgtcttcatgg-3′ and 5′-cgagacaggccctgcggggctgcacacgcgcc-3′ (pMMBO109-2). The primer pairs used to remove the site from repC were 5′-cttgcccgccgcgaactcagttaccgtccagcccag-3′ and pMMBO109-1 and 5′P-cgcgaccagctccggcaacgcctcgcgcac-3′ and pMMBO109-2. pMMB917 was generated by inserting *epsG* as a EcoRI-SalI fragment into pMMB872.

D91E was introduced with the primers 5′-aagcgtctgcctaaagaaccttggggtaacgac-3′ and 5′-gtcgttaccccaaggttctttaggcagacgctt-3′ by the same Stratagene technology described for T112L using truncated *epsG* encoding residues G25 through Q127 and the stop codon inserted in the vector pET28b (Novagen, Madison WI) as a template. A *Bst*EII – *Kpn*I fragment of the mutated gene was then excised from pET28b and used to replace the similar fragment in pMMB917. Sequences were verified at the University of Michigan DNA Sequencing Core.

All plasmid constructs were introduced into their bacterial strains by using the conjugative helper strain MM294/pRK2013 (Meselson & Yuan, 1968). Transconjugants were selected for antibiotic resistance encoded by the vector. All parental strains also received empty vectors except for C6706 Δ*pilD* which would not grow in the presence of vector only.

In vivo cross-linking

Overnight cultures were diluted in fresh media. The following concentrations of IPTG were used to induce the plasmid constructs equivalent to the P_{BAD}::*eps* chromosomal EpsG or EpsL levels: P_{BAD}::Δ*epsG* with pEpsG, pEpsG$_{G-1V}$, pEpsG$_{D91E}$, or pEpsG$_{T112L}$ at 15, 40, 40, and 20 μM, respectively, and P_{BAD}::Δ*epsL* with pEpsL at 10 μM. *E. coli* MC1061 containing pEpsG, pEpsL, or pEpsGL were induced with 10 μM IPTG. TRH7000 Δ*eps* containing pEpsG or pEpsGL were induced with 10 and 100 μM IPTG, respectively.

Diluted cultures were grown to an optical density at 600 nm between 1.5 and 2.0. Cells were harvested by centrifugation at 3,500 × g for 5 minutes and concentrated 8 fold by suspension

in 500 µl of phosphate-buffered saline. Samples were cross-linked using dithiobis (succinimdyl propionate) (Pierce) for 1 hour at room temperature. The reactions were quenched for 10 minutes with 25 µl of 1 M Tris pH 8.0. Samples were centrifuged 8,000 × g for 5 minutes and suspended in 500 µl 50 mM Tris pH 8.0. To lyse, cells were incubated with 10 µl of 10 mg/ml lysozyme and 10 µl of 1 mg/ml DNase for 10 minutes followed by sonication at 30% amplitude for 10 seconds using one second pulses using the Vibra Cell Ultrasonic Processor (Sonics and Materials, Inc.). Samples were boiled for 5 minutes in SDS loading buffer and analyzed on NuPAGE 4 to 12% Bis-Tris polyacrylamide gels (Invitrogen) at the following concentrations: TRH7000 wild type and MC1061 were loaded at 10 µl of OD_{600}=2.0, all P_{BAD}::*eps* strains, Δ*eps*, and C6706 wild type and the Δ*pilD* complemented strain were loaded at 10 µl of OD_{600}=1.5, TRH7000 Δ*epsG* and Δ*epsL*, and C6706 Δ*pilD* were loaded at 10 µl of OD_{600}=1.0. Gels were transferred to nitrocellulose membranes in NuPAGE transfer buffer and blocked in phosphate buffered-saline containing 3% BSA. Membranes were incubated with polyclonal antisera against EpsG (1:150,000 in Tris-buffered saline with 0.1% Tween-20) or EpsL (1:40,000 in Tris buffered saline with 0.1% Tween-20), followed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (BioRad) diluted 1:15,000 in Tris-buffered saline containing 0.1% Tween-20. Blots were developed with ECL Plus Western blotting detection reagent (GE Healthcare) and protein was visualized using a Typhoon Trio variable mode imager system and Image Quant software.

Triton X-100 extraction and co-immunoprecipitation

Cultures were grown, cross-linked, and quenched as described above. Following quenching samples were subjected to Triton X-100 extraction as previously described (Johnson *et al.*, 2007). 100 µl of OD_{600}=2.0 of the Triton X-100 extracted material was immunoprecipitated with anti-EpsL or anti-EpsG antiserum bound to protein G-sepharose as previously detailed (Sandkvist *et al*., 1999). Antiserum labeled protein G sepharose beads bound to the cell extract were washed twice in high salt Triton X-100 buffer (Tris-buffered saline containing 500 mM NaCl, 0.01% Triton X-100, and 1M EDTA). Samples were then washed once in low salt buffer Triton X-100 buffer (Tris-buffered saline containing 150 mM NaCl, 0.01% Triton X-100, and 1M EDTA), followed by a final wash in Tris-buffered saline. 25 µl of Laemmli buffer was added to the washed beads and boiled for 10 minutes prior to centrifugation. Immunoprecipitated samples were divided equally and analyzed on NuPAGE 4–12% Bis-Tris polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in Tris-buffered saline containing 5% milk, incubated with either biotinylated EpsG (1:5,000) or biotinylated EpsL antibodies (1:10,000) in Tris-buffered saline with 0.1% Tween-20, followed by incubation with horseradish peroxidase-conjugated streptavidin at 1:25,000 in Tris-buffered saline with 0.1% Tween-20. Blots were developed with ECL Plus Western blotting detection reagent (GE Healthcare) and protein was visualized using a Typhoon Trio variable mode imager system and Image Quant software.

Protease secretion assay

Secretion of extracellular protease was determined as previously described (Sikora *et al.*, 2007). The amount of fluorescence was compared to a standard curve of cleaved substrate in order to determine the nanograms of protease secreted.

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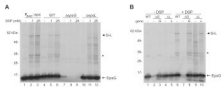

Figure 1. *In vivo* cross-linking of EpsG and EpsL

(A) Whole cells of *V. cholerae* TRH7000 wild-type, Δ*epsG*, Δ*epsL*, and P_{BAD}::*eps* (native *eps* promoter replaced by arabinose inducible promoter and grown in the presence of 0.01% arabinose) were cross-linked using the indicated concentrations of DSP as described in the experimental procedures, and immunoblotted for EpsG. (B) Whole cells of P_{BAD}::*eps* wild-type containing pMMB67 vector, P_{BAD}::Δ*epsG* with empty vector or vector encoded *epsG*, and P_{BAD}::Δ*epsL* with empty vector or vector encoded *epsL* were cross-linked with 0.1 mM DSP and immunoblotted for EpsG. The position of EpsG is indicated. An arrow head designates the EpsG-EpsL complex, and an asterisk represents the putative EpsG dimer. The band directly below the putative EpsG dimer is presumed to be cross-reactive as it is also observed in the absence of DSP. The molecular weight markers are shown in kilo Daltons and lane numbers are indicated.

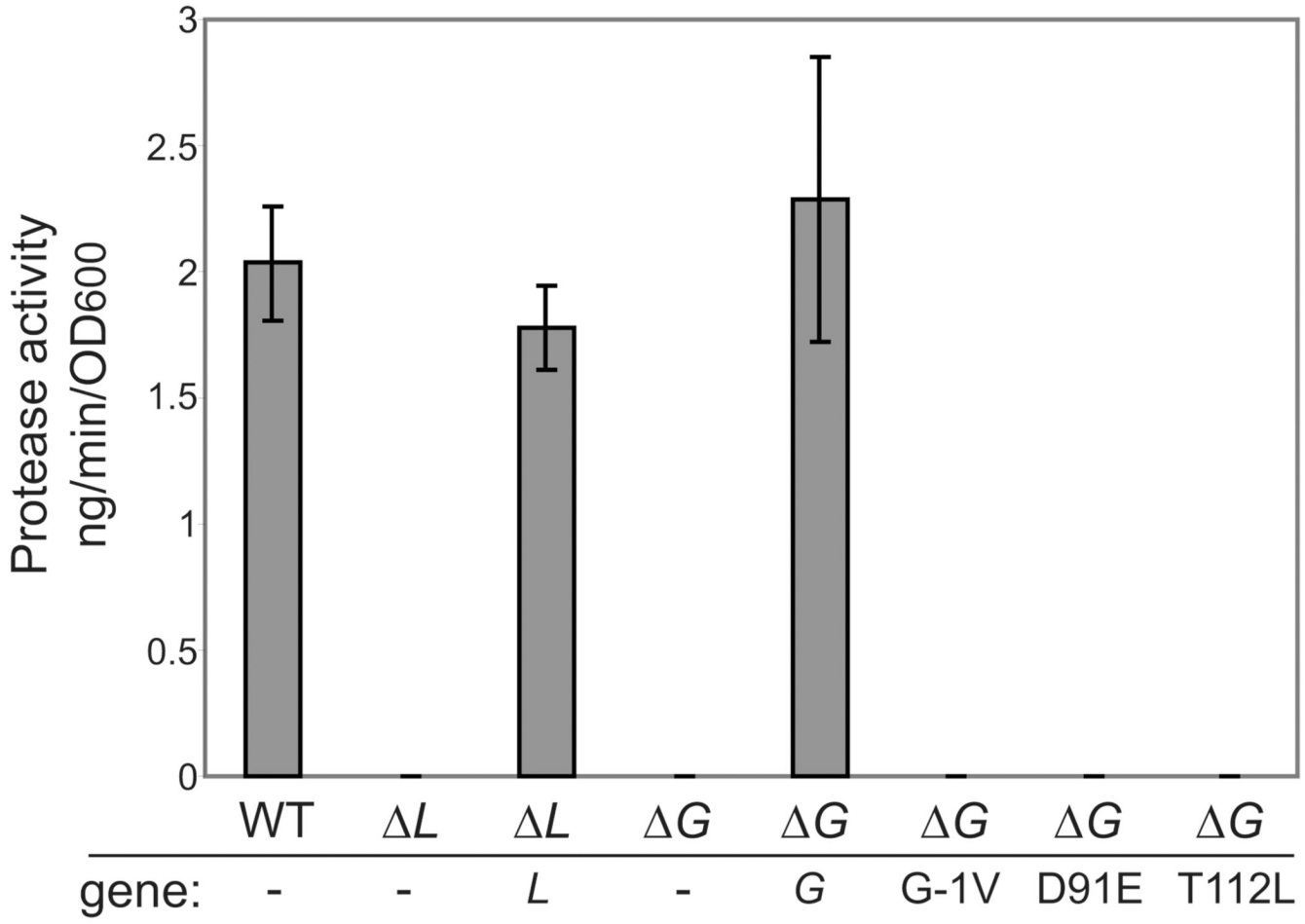

Figure 2. Mutations in EpsG prevent secretion

Supernatants of over-night cultures of *V. cholerae* TRH7000 P_{BAD}::*eps* wild-type, P_{BAD}::Δ*epsL* containing empty vector or vector encoding wild-type *eps*L, P_{BAD}::Δ*epsG* with empty vector, and vector encoded wild-type *epsG* or mutants were grown over-night at 37°C. Supernatants were separated from the cells and analyzed for the presence of an extracellular protease using the cleavable fluorogenic substrate *N*-tert-butoxy-canbonyl-Gln-Ala-Arg-7-amido-4-methyl-coumarin as described in the materials and methods. Each sample was assayed in triplicate and the standard error is indicated.

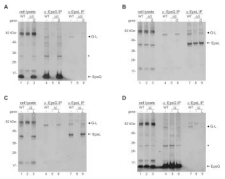

Figure 3. Co-immunoprecipitation of EpsG and EpsL

Triton X-100 cell extracts were prepared from P_{BAD}::*eps* wild-type, P_{BAD}::Δ*epsG* and complemented strain (Panels A and B), and P_{BAD}::Δ*epsL* and complemented strain (Panels C and D) after cross-linking with 0.25 mM DSP. Cleared cell extracts were immunoprecipitated with either anti-EpsG or anti-EpsL antibodies and subjected to SDS-PAGE and immunoblotting with biotinylated anti-EpsG (Panels A and D) or biotinylated anti-EpsL (Panels B and C) antibodies. In all panels the monomer for EpsG or EpsL is indicated with an arrow and the 60 kDa EpsG-EpsL complex is denoted by an arrow head. In panels immunoblotted for EpsG, the putative EpsG dimer is labeled with an asterisk. The molecular weight markers are shown in kilo Daltons. Lane numbers are indicated.

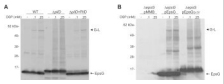

Figure 4. Processing of EpsG is necessary for EpsG-EpsL cross-linking

(A) Whole cells of *V. cholerae* C6706 wild-type, Δ*pilD*, and Δ*pilD* complemented with pACYC184 expressing *pilD* were cross-linked with the indicated concentrations of DSP, and immunoblotted with antibodies specific for EpsG. (B) Whole cells of P_{BAD}::Δ*epsG* expressing wild-type *epsG*, or *epsG* with mutation to residue G-1 were cross-linked and analyzed as described above. The processed monomer of EpsG is represented by an arrow, and the EpsG-EpsL complex is labeled with an arrow head. Position of molecular weight markers is shown.

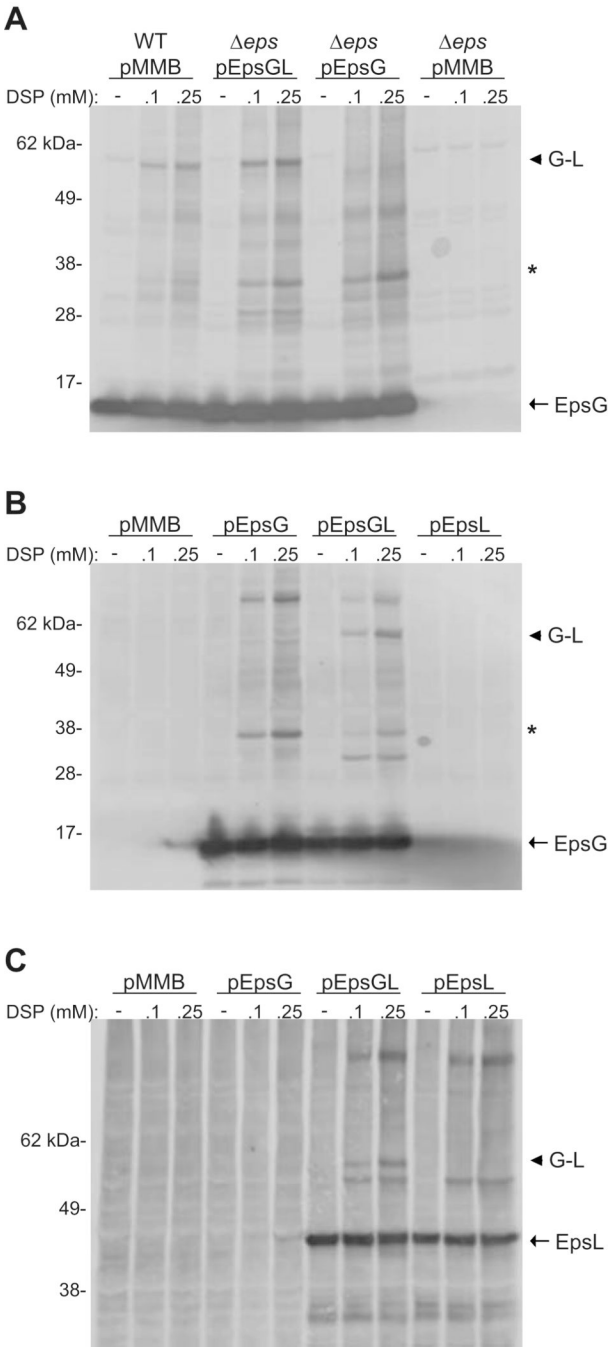

Figure 5. EpsG and EpsL interact in the absence of other T2S components

(A) Whole cells of *V. cholerae* TRH7000 wild-type, and TRH7000 with the entire *eps* operon removed (Δ*eps*) expressing plasmid-encoded *epsG*, or *epsG* and *epsL* (pEpsGL) were cross-linked with the indicated concentrations of DSP and immunoblotted for EpsG. (B and C) Whole cells of *E. coli* MC1061 with pACYC184-*pilD* and pMMB67 encoding *epsG*, *epsL*, or *epsG* and *epsL* concomitantly were cross-linked with the indicated concentrations of DSP and probed with antibodies specific for EpsG (B) or EpsL (C). The monomer for EpsG (Panels A and B) and EpsL (Panel C) is indicated by an arrow. An arrow head designates the EpsG-EpsL complex, and an asterisk represents the putative EpsG dimer in Panels A and B. Positions of molecular weight markers are indicated.

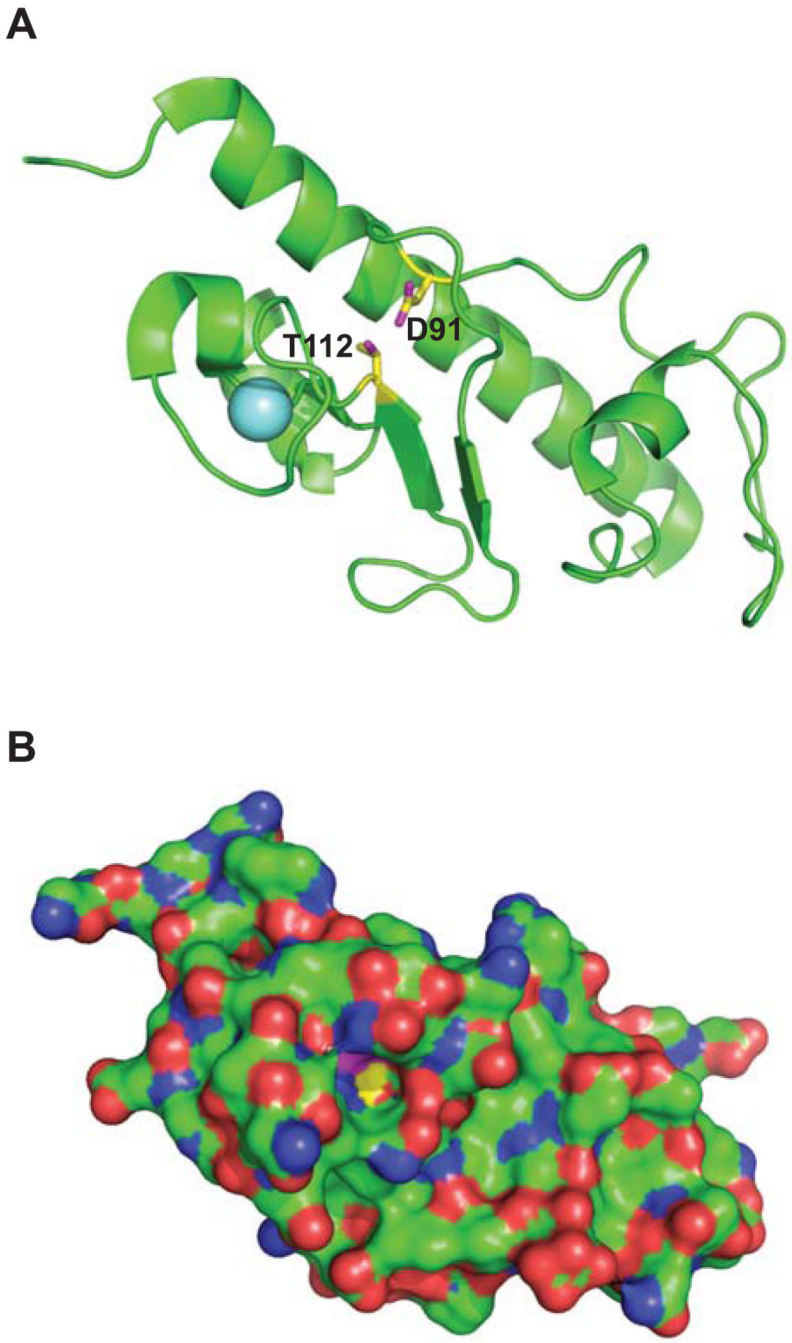

Figure 6. Location and interaction between residues Asp91 and Thr112 in EpsG

V. cholerae EpsG (Korotkov *et al*., 2009; PDB code 3FU1) depicted with the side chains of D91 and T112 colored with yellow carbons and magenta oxygens. The light blue sphere is a bound calcium ion. (A) View approximately perpendicular to the EpsG helix axis with the backbone cartoon in green and the side chains of D91 and T112 as sticks. (B) Surface representation of the same view with the side chains of D91 and T112 with the same color code as in Panel A, the other atoms are colored green for carbons, blue for nitrogens and red for oxygens. A deep cavity is apparent where only a few atoms of D91 and T112 are visible. Figure prepared with PyMOL (DeLano, 2002).

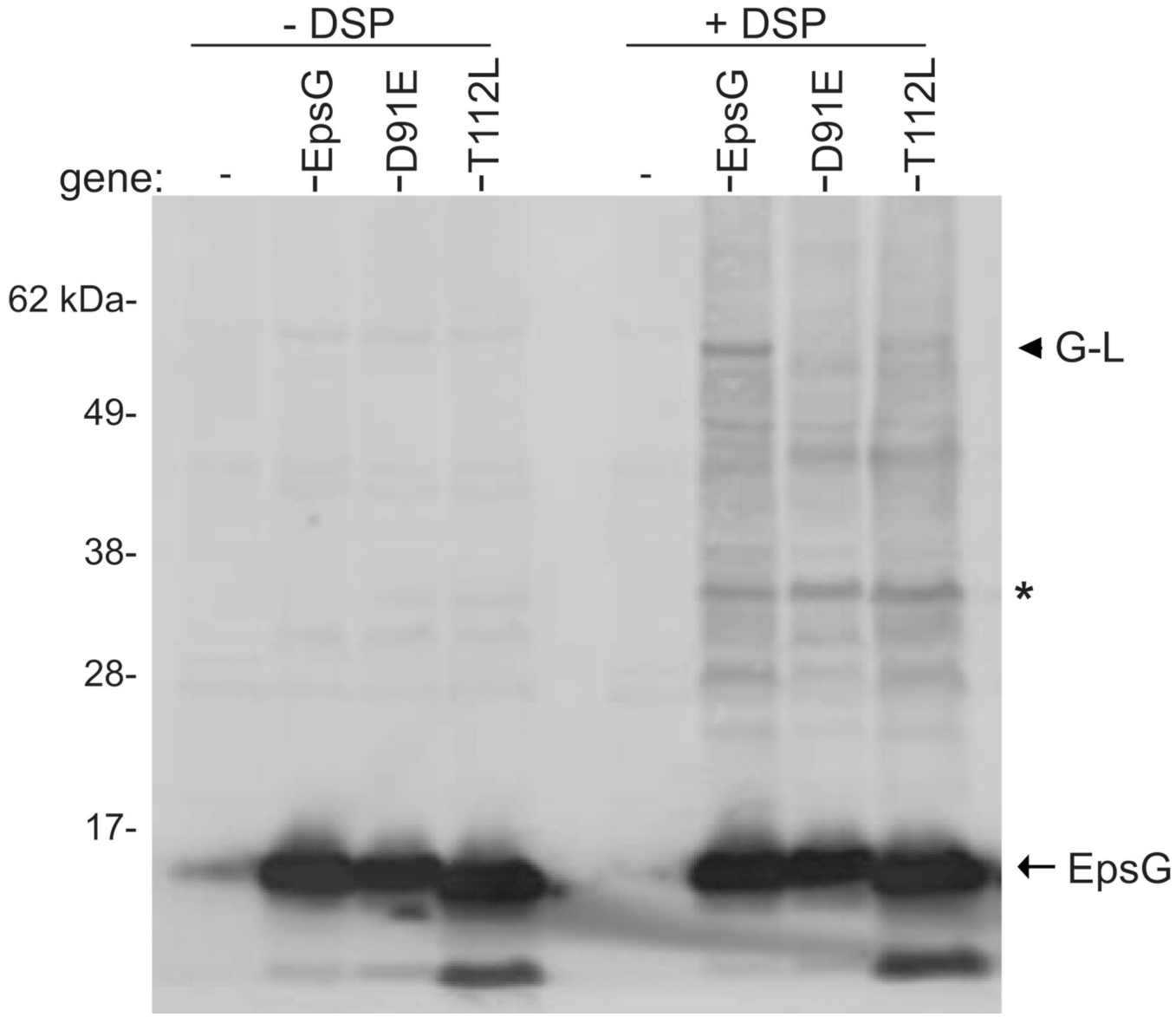

Figure 7. Replacement of residues D91 or T112 alters the cross-linking of EpsG with EpsL

Whole cells of P_{BAD}::Δ*epsG* expressing wild-type *epsG*, or *epsG* with mutation to either residue D91 or T112 were incubated in the absence or presence of 0.1 mM DSP and subjected to SDS-PAGE and immunoblotting with anti-EpsG antibodies. The EpsG monomer is designated by an arrow, the EpsG-EpsL complex is indicated by an arrow head, and a predicted EpsG dimer is labeled with an asterisk. Molecular weight markers are indicated.

Table 1

Strains and plasmids used in this study.

Strain or plasmid	Features	Reference or source
Strains		
V. cholerae		
TRH7000	El Tor strain, wild-type for type II secretion	Hirst *et al.*, 1984
Δ*epsG*	in frame replacement of *epsG* with Cm^R	Lybarger *et al*., 2009
Δ*epsL*	in frame replacement of *epsL* with *aph-3* (Km^R)	Lybarger *et al*., 2009
P_{BAD}::*eps*	TRH7000 P_{BAD}::*eps*	Sikora *et al*., 2007
P_{BAD}::Δ*epsG*	TRH7000 P_{BAD}::*eps* with in frame replacement of *epsG* with Cm^R	This study
P_{BAD}::Δ*epsL*	TRH7000 P_{BAD}::*eps* with in frame replacement of *epsL* with *aph-3* (Km^R)	This study
Δ*eps*	TRH7000 in frame replacement of *epsC-N* with Cm^R	Sikora *et al*., 2007
C6706str2	El Tor o1, Inaba, Sm^R	Thelin & Taylor, 1996
Δ*pilD* (JM313)	C6706str with in frame deletion of *pilD* (Cm^R)	Marsh & Taylor, 1998
E. coli		
MC1061	*F- lac-* K-12 laboratory strain	Casadaban & Cohen, 1980
MM294/pRK2013	helper strain for conjugations	Meselson & Yuan, 1968
Plasmids		
pMMB67	low copy number IPTG inducible vector (Ap^R)	Furste *et al.*, 1986
pMMB872	pMMB67 with *Bst*EII sites in lacIQ and repC removed	This study
pMMB917	*epsG* in pMMB872	This study
pEpsG	*epsG* in pMMB67	Lybarger *et al*., 2009
$pEpsG_{D91E}$	substitution of residue D91 to glutamic acid in pMMB917	This study
$pEpsG_{T112L}$	substitution of residue T112 to leucine in pEpsG	This study
$pEpsG_{G-1V}$	substitution of residue G-1 to valine in pEpsG	This study
pEpsL (pMS44)	*epsL* in pMMB67	Sandkvist *et al*., 1995
pEpsGL	*epsGepsL* in pMMB67	This study
pACYC184	low copy number vector (Cm^R, Tc^R)	Chang & Cohen, 1978
pPilD (pJM294)	*pilD* in pACYC184	Marsh & Taylor, 1998