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The non-pathogenic *Escherichia coli* strain W secretes SsIE via the virulence-associated type II secretion system beta

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Abstract

Background: Many pathogenic *E. coli* strains secrete virulence factors using type II secretory systems, homologs of which are widespread in Gram-negative bacteria. Recently, the enteropathogenic *Escherichia coli* strain E2348/69 was shown to secrete and surface-anchor SsIE, a biofilm-promoting virulence factor, via a type II secretion system. Genes encoding SsIE and its associated secretion system are conserved in some non-pathogenic *E. coli*, including the commonly-used W (Waksman) strain.

Results: We report here that *E. coli* W uses its type II secretion system to export a cognate SsIE protein. SsIE secretion is temperature- and nutrient-dependent, being robust at 37°C in rich medium but strongly repressed by lower temperatures or nutrient limitation. Fusing either of two glycosyl hydrolases to the C-terminus of SsIE prevented it from being secreted or surface-exposed. We screened mutations that inactivated the type II secretion system for stress-related phenotypes and found that inactivation of the secretion system conferred a modest increase in tolerance to high concentrations of urea. Additionally, we note that the genes encoding this secretion system are present at a hypervariable locus and have been independently lost or gained in different lineages of *E. coli*.

Conclusions: The non-pathogenic *E. coli* W strain shares the extracellular virulence factor SsIE, and its associated secretory system, with pathogenic *E. coli* strains. The pattern of regulation of SsIE secretion we observed suggests that SsIE plays a role in colonization of mammalian hosts by non-pathogenic as well as pathogenic *E. coli*. Our work provides a non-pathogenic model system for the study of SsIE secretion, and informs future research into the function of SsIE during host colonization.

Keywords: Type II secretion, Surface display, *Escherichia coli*, Colonization factor

Background

Gram-negative bacteria use diverse type II secretion systems (T2SS) to deliver a wide variety of proteins into the extracellular milieu [1,2]. Transport is effected by a membrane-spanning complex of 12–15 structural proteins, generically termed Gsp proteins (for general secretory pathway). Secreted substrates first cross the inner membrane by the Sec or Tat pathways; the Gsp proteins then recognize substrates and transport them across the outer membrane. T2SS function requires several proteins that have homologs in type IV pilus biogenesis systems, including an oligomerized secretin, a

helical protein filament called the pseudopilus, and a prepilin peptidase essential for pseudopilus assembly [3,4].

Secreted proteins serve many purposes, from electron transport to nutrient acquisition, and some are important pathogenicity factors for plant and animal pathogens in the Enterobacteraceae [5,6]. Type II secretion has been extensively studied in pathogenic strains of *Escherichia coli*, which collectively are known to use two distinct disease-promoting T2SS: the StcE secreting system encoded by the pO157 virulence plasmid [7], and the heat-labile enterotoxin (LT) secreting system common to many pathogenic strains [8]. Recently the latter T2SS was shown for the first time to additionally secrete a non-LT protein, known as SsIE, from the enteropathogenic strain E2348/69, thereby promoting biofilm maturation and rabbit colonization by E2348/69 [9,10]. The

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ssIE gene sits immediately upstream of the T2SS-encoding secretory genes, and transcription of *ssIE* and the *gsp* genes was shown to be co-regulated in *E. coli* strain H10407 [11]. In E2348/69, SsIE exists as a lipid-anchored, surface-exposed protein in the outer membrane and is also released into the culture supernatant. Strozen et al. termed the LT- and SsIE-secreting system T2SS_β, to distinguish it from the chitinase-secreting T2SS_α that co-occurs in several *E. coli* strains [12]. Based on phylogenetic and structural analyses, Dunstan et al. recently determined that the *E. coli* T2SS_β is part of a larger group of T2SS that contain “*Vibrio*-type secretins”, making it a model for numerous type II secretion systems used to deliver toxic substrates by *Vibrio* and *Escherichia* species [10].

The SsIE-secreting T2SS_β, unlike the StcE-secreting pO157 T2SS, is conserved in several non-pathogenic “safe” strains of *E. coli* (“safe” strains may colonize hosts, but have never been known to cause disease), including wild-type B and W isolates [13]. To date, however, no report has described secretion of proteins by T2SS_β in any non-pathogenic strain. We were interested to determine whether non-pathogenic *E. coli* could also secrete the “virulence factor” SsIE. Secretion of SsIE by a safe strain would imply that SsIE itself is not capable of promoting a disease state, and would invite comparisons of SsIE function between pathogens and non-pathogens. Furthermore, if non-pathogenic *E. coli* could secrete SsIE, the T2SS_β system could be studied using a non-pathogenic model organism.

We demonstrate here that the non-pathogenic *E. coli* strain W encodes a functional T2SS_β that secretes a cognate SsIE protein. We found a strong effect of growth conditions on SsIE secretion, which is relatively robust in rich medium at 37°C and undetectable when cells are cultured at 30°C or in minimal medium. Previous work suggested that the C-terminus of SsIE might be a permissive site for sequence insertions with regards to T2SS_β recognition [9], but we found that C-terminal enzyme fusions to SsIE blocked protein secretion and surface display.

As noted above, the T2SS_β was shown to promote mature biofilm formation in *E. coli* E2348/69. We searched for additional phenotypes in *E. coli* W by phenotypic microarray analysis of a mutant lacking T2SS_β-encoding genes on Biolog stress plates. The phenotypic microarray indicated a potential fitness effect of the mutation in high concentrations of urea. Using standard culture techniques, we found that deletion of T2SS_β-encoding genes, or the *ssIE* gene, conferred a small survival advantage in medium containing high concentrations of urea.

Our findings make T2SS_β the only virulence-associated T2SS with shared functions in pathogenic and non-pathogenic *E. coli*. Considering our regulatory data and

the clear homology between the T2SS_β-encoding operons of W and E2348/69, we propose that SsIE is used by non-pathogenic as well as pathogenic strains of *E. coli* during host colonization.

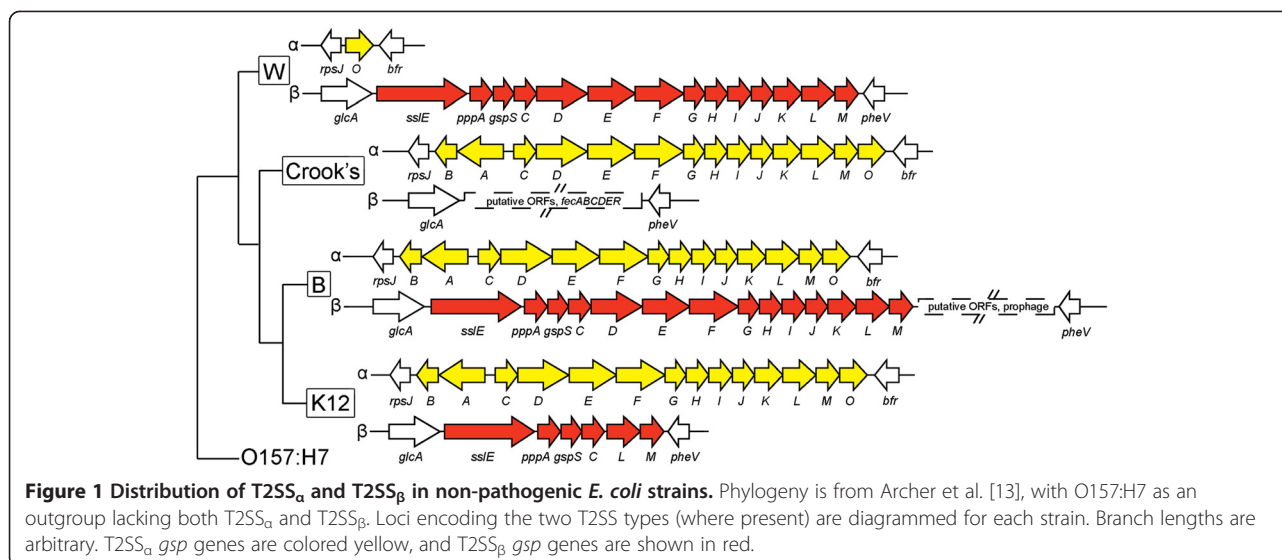
Results

E. coli W secretes SsIE using T2SS_β under specific temperature and nutrient conditions

Prior to publication of the finished *E. coli* W genome sequence [13], a draft *E. coli* W genomic sequence generated by the U.S. Department of Energy Joint Genome Institute in collaboration with the Great Lakes Bioenergy Research Center (GenBank accession NZ_AEDF00000000) revealed the presence of the entire T2SS_β gene cluster, including a copy of the gene encoding SsIE (see Figure 1 for a depiction of the locus). To determine whether *E. coli* W secreted endogenous SsIE via T2SS_β, we analyzed the proteomes of the wild-type strain (WT) and a mutant lacking the genes encoding the conserved structural proteins of T2SS_β (Δ *gspC-M*). We grew strains in liquid culture, then harvested cells by centrifugation and compared the proteins present in cell lysates and cell-free supernatants (the latter containing any secreted proteins) by SDS-PAGE. We observed a ~180 kDa protein, the expected size for SsIE, that was present in the supernatants of WT cultures but not Δ *gsp* cultures (Figure 2A). The ~180 kDa protein band was absent from supernatants and cell extracts of a Δ *ssIE* strain, but reappeared when we complemented the *ssIE* deletion with plasmid-encoded *ssIE* (Figure 2B). To further confirm that SsIE was secreted and did not play an intracellular role in activating protein secretion, we attempted to complement the Δ *ssIE* strain with a form of SsIE lacking the Sec signal peptide (SsIE-SP). Unlike wild-type SsIE, SsIE-SP could not complement the secretory defect in the Δ *ssIE* strain. Taken together, our data demonstrate that SsIE is secreted from wild-type *E. coli* W by T2SS_β.

Intracellular SsIE did not appear abundant in wild-type *E. coli* W, even under conditions where secretion of SsIE was detectable. We observed accumulation of SsIE in the cell when SsIE was expressed from a multicopy plasmid, however. We postulate that in wild-type cells, the intracellular concentration of SsIE is maintained at a relatively low level, and that SsIE release from cells over time results in accumulation in the supernatant.

Type II secretion systems require prepilin peptidases to produce the mature, functional forms of their prepilin proteins [1], and the prepilin peptidase PppA is required for secretion of LT by T2SS_β in *E. coli* H10407 [12]. To determine whether PppA is similarly required for SsIE secretion by *E. coli* W, we compared SsIE secretion in WT to a Δ *pppA* strain. SsIE secretion was not detectable in the Δ *pppA* background, and the mutation could be complemented by plasmid-encoded PppA (Figure 2C).



These results confirm that a fully-functional T2SS_β is required to secrete SslE, and indicate that expression of the *gspC-M* genes alone is not sufficient to allow SslE secretion.

We hypothesized that SslE secretion in *E. coli* W might play a role in host colonization, and that secretion might be regulated such that more SslE is secreted under conditions that resemble the mammalian gut. We assessed this conditionality by examining SslE secretion from cultures grown at different temperatures and nutrient conditions: 30°C vs. 37°C, and minimal MOPS-glycerol broth vs. rich LB (Figure 2D). We observed secretion of SslE only in cultures grown in LB at 37°C, indicating that either reduced temperature or nutrient limitations are sufficient to block SslE secretion.

C-terminal fusions to SslE prevent secretion

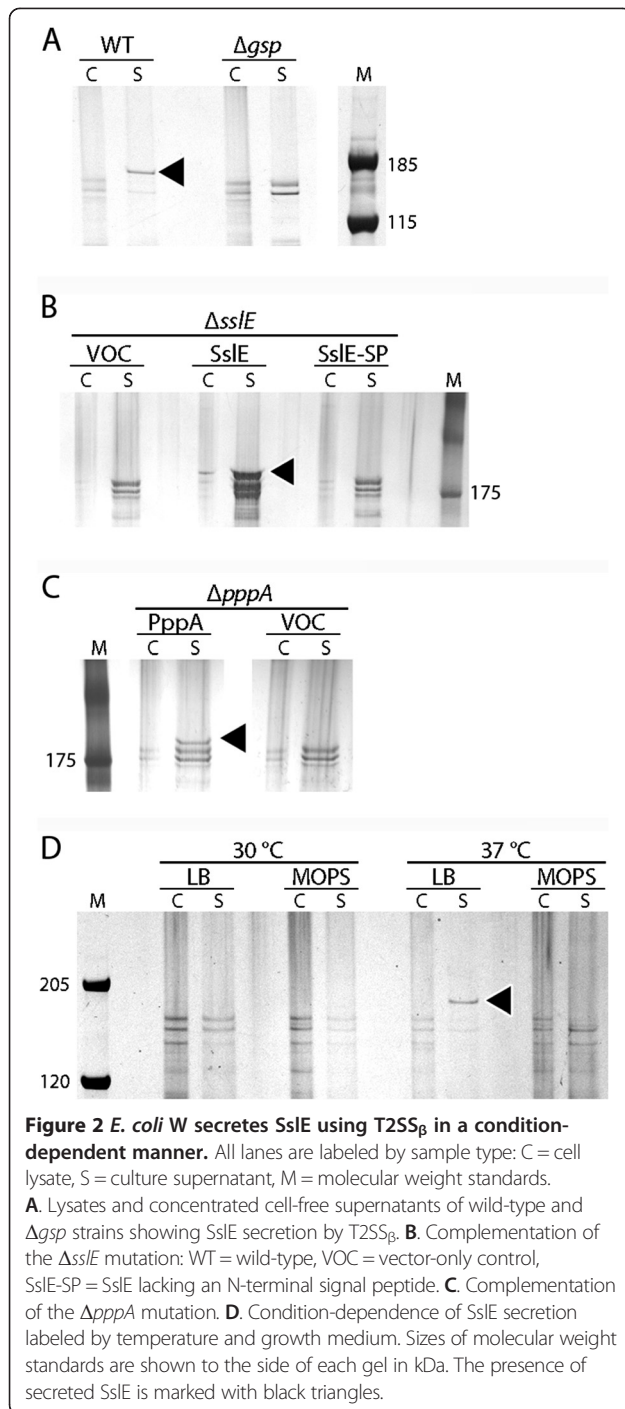
In their initial characterization of SslE surface display and secretion, Baldi et al. found that C-terminal fusion of a small tetracysteine-containing motif to SslE did not interfere with localization of SslE [9]. This result suggested that the C-terminus of SslE might not be important for the recognition of SslE by T2SS_β, and thus might be a permissive site for polypeptide fusions. We were interested in testing C-terminal permissiveness for two reasons: first, because it might provide information about the targeting of SslE for secretion (as there are no defined secretory signals for type II secretion substrates), and second, because SslE fusions might be useful to anchor other proteins to the cell surface. We therefore independently fused two plant cell wall degrading enzymes, Cel45A and Pel10A from *Cellvibrio japonicus*, to the C-terminus of *E. coli* W SslE and assessed the capacity of these fusion proteins to be secreted or displayed on the cell surface. Both fusions resulted in

stable, enzymatically active proteins when expressed in *E. coli* W. We did not generate fusions to the potentially lipidated N-terminus of SslE to avoid changes in lipidation that could affect protein localization.

We performed all secretion and display experiments side-by-side in wild-type and T2SS-deficient $\Delta pppA$ strains, and present the results in Table 1. By following activity of the enzymatic fusions, we found that neither fusion protein was released into the medium under conditions in which we found wild-type SslE to be released. Indeed, extracellular activity of SslE-Cel45A was difficult to detect, though lysed cells released highly active enzyme. Because the substrates for Cel45A (carboxymethyl cellulose) and Pel10A (polygalacturonic acid) are high molecular weight polysaccharides that cannot enter the *E. coli* cell, we were able to assess surface display of fusion proteins by measuring the enzymatic activity of intact cells as compared to cell lysates. These experiments further demonstrated that the fusion proteins were not displayed on the surface of the cell, but accumulated intracellularly.

Inactivation of T2SS_β modestly increases urea tolerance

Baldi et al. demonstrated that inactivation of T2SS_β in *E. coli* E2348/69 inhibited biofilm maturation in confocal microscopic analysis of flow cell cultures, though it had no effect on early biofilm development in stationary plate assays [9]. To uncover other phenotypes related to T2SS_β disruption, we used *E. coli* W as a non-pathogenic model system in a partial Biolog phenotypic microarray to compare wild-type and Δgsp strains grown with various stressors. The Biolog dye-reduction traces are presented in Additional file 1. Under most conditions the two strains were indistinguishable, but the screen indicated that elevated urea concentrations might differentially affect their



growth. We examined this phenomenon in 96-well plate growth experiments under conditions in which our data showed SsIE to be secreted (LB at 37°C). Compared to the wild-type control, Δgsp and $\Delta pppA$ strains maintained higher stationary-phase densities in the presence of 0.90 M and 1.15 M urea (Additional file 2: Figure S1), suggesting that inactivation of the T2SS_β system modestly increased urea tolerance even when the structural Gsp proteins were still expressed. We determined the role of

Table 1 Extracellular and surface-displayed activity of SsIE-Cel45A and SsIE-Pel10A from liquid cultures

Strain	SsIE-Cel45A activity ^a		SsIE-Pel10A activity ^a	
	Supernatant fraction ^b	Displayed fraction ^c	Supernatant fraction ^b	Displayed fraction ^c
WT pRH153	< 0.01	< 0.01	–	–
$\Delta pppA$ pRH153	< 0.01	< 0.01	–	–
WT pRH154	–	–	0.11(1)	0.08(1)
$\Delta pppA$ pRH154	–	–	0.10(4)	0.095(5)

^a Values shown are means of at least two biological replicates, with error in the last digit denoted parenthetically.

^b Extracellular activity divided by the activity from an equivalent fraction of lysed culture.

^c Activity measured using intact cells divided by the activity from an equivalent fraction of lysed culture.

SsIE in this phenotype and verified modest urea tolerance by following the growth and viability of wild-type, Δgsp , and $\Delta sslE$ strains for 48 hours with or without 1.15 M urea under the standard culture conditions we used for SsIE secretion experiments (in culture tubes on a rolling wheel for vigorous aeration). Culture absorbance readings and viable cell counts indicated that, without urea, the three strains grew equivalently up to 12 hours and slowly lost viability between 12 and 48 hours, with indistinguishable final viable counts at 48 hours (Figure 3 and Table 2). In the presence of 1.15 M urea all strains grew poorly, but Δgsp and $\Delta sslE$ strains maintained higher turbidity and viable cell counts than wild-type, with both mutants having > 60% more surviving cells than wild-type at 48 hours. We conclude that the inability to secrete SsIE confers a small survival advantage in the presence of high concentrations of urea.

Discussion and conclusions

Strains within the species *Escherichia coli* encode different combinations of type II secretion systems, each of which secrete different effectors and presumably provide specific advantageous phenotypes to their host organisms. To this point, the only T2SS shown to be functional in non-pathogenic *E. coli* strains is the chitinase-secreting T2SS_α, which is the sole T2SS encoded by *E. coli* K-12 [13,14] and whose role in natural environments is unknown. We demonstrate here that, surprisingly, the T2SS_β that promotes virulence of the enterotoxigenic strain H10407 and the enteropathogenic strain E2348/69 is conserved, and secretes a virulence factor homolog, in the non-pathogenic *E. coli* W strain. To our knowledge, this is the first time a virulence-associated type II secretion system has been shown to function in non-pathogenic *E. coli*. Deletion of *sslE* could be complemented *in trans*, indicating that an *sslE* disruption does not prevent expression or assembly of T2SS_β in *E. coli* W. We observed that *E. coli* W preferentially secretes SsIE under nutrient-rich conditions at human body temperature (37°C), which suggests that

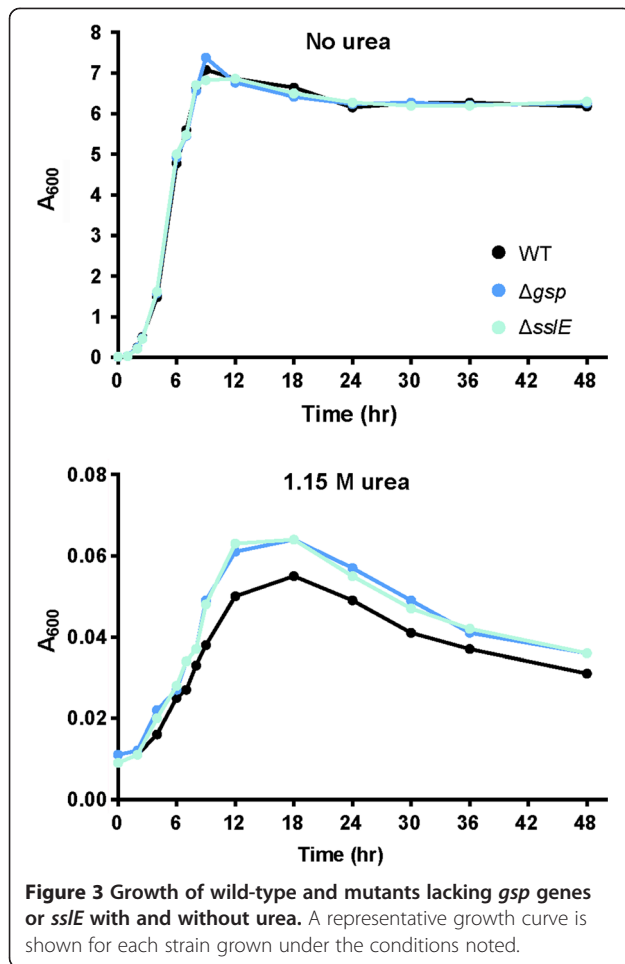


Figure 3 Growth of wild-type and mutants lacking *gsp* genes or *ssIE* with and without urea. A representative growth curve is shown for each strain grown under the conditions noted.

SsIE may be a colonization factor in non-pathogenic strains. The regulation of SsIE secretion in other strains is unclear, but expression of genes encoding the LT-secreting T2SS_β in *E. coli* H10407 was also shown to be upregulated at host-associated temperatures [11]. We hope that future experiments will elucidate the role of SsIE in host colonization by non-pathogenic *E. coli*.

If secretion of SsIE indeed aids diverse *E. coli* in gut colonization, it is perhaps surprising that some gut-derived isolates of *E. coli*, such as K-12 and O157:H7,

lack the T2SS responsible for SsIE secretion. Such strains may compensate for the loss of biofilm-forming propensity using other mechanisms; strains bearing the F plasmid (such as wild-type K-12) may rely on F pilus-mediated aggregation [15], for example. The genes encoding the SsIE-secreting T2SS_β are present adjacent to the *pheV* tRNA gene, which appears to be a hypervariable locus in *E. coli* [16-18], so they may be randomly lost at a relatively high rate. Indeed, a comparison between phylogeny and T2SS_α/T2SS_β presence suggests independent losses of T2SS_β in non-pathogenic strains (Figure 1). Notably, B and W encode the complete T2SS_β, while Crook's and K-12 do not, in spite of the fact that Crook's diverged from K-12 prior to the divergence of B. This indicates that either Crook's and K-12 lost the T2SS_β-encoding genes independently, or that an ancestor of Crook's, B, and K-12 lost the genes, which were subsequently re-acquired by strain B. An examination of the T2SS_β-encoding loci in Crook's and K-12 strongly supports the former explanation. In K-12, the T2SS_β-encoding *gsp* operon clearly experienced an internal deletion that removed the *gspD-K_β* genes, inactivating the T2SS. In Crook's, however, the homologous genomic locus appears entirely different: all *gsp* genes are absent, and in their place is the *fec* operon (encoding a ferric citrate transport system) and a variety of putative ORFs. We infer that the most parsimonious explanation of the phylogenetic distribution of T2SS_β is that K-12 and Crook's both lost the T2SS at different points in their evolutionary histories. It remains an open question what pattern of gene gains and losses best explains the distribution of T2SS_β across the diversity of *E. coli* strains not considered in our analysis.

It is of interest to note that a non-polar deletion of the *pppA* gene, encoding a prepilin peptidase, prevents secretion of SsIE by *E. coli* W. This result agrees with a similar experiment performed by Strozen et al. to assess effects of PppA on LT secretion in H10407 [12]. Both W and H10407 also encode a second prepilin peptidase (GspO) whose homolog is functional in facilitating ChiA secretion via T2SS_α in K-12 [19]. Whether the GspO peptidase is not expressed under conditions associated with SsIE secretion in both W and H10407, or whether

Table 2 Viable cell counts for cultures grown with and without urea

Strain	Urea ^a	6 hr ^b	12 hr ^b	24 hr ^b	48 hr ^b
Wild-type	-	2.8 ± 0.1 × 10 ⁹	6.9 ± 0.3 × 10 ⁹	2.0 ± 0.3 × 10 ⁹	1.2 ± 0.1 × 10 ⁹
Δ <i>gsp</i>	-	2.6 ± 0.3 × 10 ⁹	6.2 ± 0.2 × 10 ⁹	2.4 ± 0.2 × 10 ⁹	1.2 ± 0.1 × 10 ⁹
Δ <i>ssIE</i>	-	2.7 ± 0.1 × 10 ⁹	5.7 ± 0.2 × 10 ⁹	2.3 ± 0.3 × 10 ⁹	1.2 ± 0.1 × 10 ⁹
Wild-type	+	5.8 ± 0.3 × 10 ⁶	3.2 ± 0.1 × 10 ⁶	1.6 ± 0.1 × 10 ⁶	3.1 ± 0.1 × 10 ⁵
Δ <i>gsp</i>	+	7.9 ± 0.9 × 10 ⁶	4.1 ± 0.2 × 10 ⁶	2.2 ± 0.2 × 10 ⁶	5.7 ± 0.3 × 10 ⁵
Δ <i>ssIE</i>	+	6.3 ± 0.3 × 10 ⁶	4.1 ± 0.3 × 10 ⁶	2.1 ± 0.4 × 10 ⁶	5.0 ± 0.6 × 10 ⁵

^a -, no urea present; +, 1.15 M urea present.

^b Colony-forming units per ml of culture at the indicated time after inoculation, shown as means ± SEM for at least three replicate plate counts.

the two peptidases display different substrate specificities, remains to be determined.

Strikingly, in the presence of the otherwise intact *gsp* operon, deletion of *sslE* was effective in promoting modest urea tolerance. When we first observed the urea-tolerant phenotype of the Δgsp strain, we hypothesized that the mutant's advantage stemmed from lacking the transmembrane components of the T2SS, particularly the secretin pore in the outer membrane, which might be denatured by urea. The urea tolerance of the $\Delta sslE$ mutant rules out this hypothesis, however, and indicates that secretion of SsIE by T2SS _{β} renders cells modestly more sensitive to urea. Relative urea sensitivity is likely due to indirect effects on cell physiology of bearing surface-displayed SsIE or of releasing of SsIE into the culture medium.

We report here that enzymatic fusions to the C-terminus of SsIE interfere with its targeting to the T2SS, as measured by release of fusion proteins and by display of fusion proteins on the outer leaflet of the outer membrane. Previously, Baldi et al. fused a tetracysteine motif to the C-terminus of E2348/69 SsIE and saw that the fusion protein was still displayed on the cell surface [9]. We do not think these results contradict ours, due to the significant structural differences between the fusion proteins in question. We propose that the six amino acids appended to the C-terminus of SsIE in the study by Baldi et al. did not affect secretion of SsIE, but that our fusions of SsIE to large tightly-folded proteins (plant cell wall degrading enzymes from *Cellvibrio japonicus*) occluded

important targeting motifs recognized by the T2SS. The uncharacterized nature of T2SS recognition of substrates [20] unfortunately limits our ability to speculate further as to what these motifs might be. Future dissection of the SsIE protein with internal deletions and protein fusions may yield new insights into the targeting motif(s) of SsIE, and determine whether SsIE fusions can be used in the surface display of other proteins.

Methods

Growth media, strains and plasmids

E. coli strains and plasmids used in this study are summarized in Table 3, and sequences of the plasmids are provided in Additional file 3. The rich (LB) and minimal (Neidhardt MOPS minimal with 0.2% glycerol) media [21,22] contained supplements at the following concentrations: 25 μ g/ml kanamycin, 100 μ g/ml ampicillin, and 30 μ g/ml chloramphenicol. Mutant strains were constructed by replacing various loci with a FRT-*kan*-FRT cassette via the λ Red method, and *kan* cassettes were then removed by FLP excision as described [23,24]. The FRT-*kan*-FRT cassette used for gene disruptions of *gspC-M*, *pppA*, and *sslE* was amplified from Keio mutant genomic DNA [24] using the primer pairs noted in Table 4. To ensure our phenotypes did not result from second-site mutations, we generated all mutant strains twice in parallel and performed assays with two independent isolates, which behaved similarly in all cases.

All synthetic DNA for plasmid constructions described below was provided by Geneart (Regensburg, Germany).

Table 3 Strains and plasmids used in this study

<i>E. coli</i> strain or plasmid	Description ^a	Reference or source ^b
Strains		
W	Wild-type <i>E. coli</i> W	ATCC 9637
W $\Delta gspC$::Kan	W $\Delta gspC$ -M::FRT- <i>kan</i> -FRT	This work
W $\Delta gspC$::FRT	W $\Delta gspC$ -M::FRT, derived by FLP recombination from W $\Delta gspC$::Kan	This work
W $\Delta pppA$::Kan	W $\Delta pppA$::FRT- <i>kan</i> -FRT	This work
W $\Delta pppA$::FRT	W $\Delta pppA$::FRT, derived by FLP recombination from W $\Delta pppA$::Kan	This work
W $\Delta sslE$::Kan	W $\Delta sslE$::FRT- <i>kan</i> -FRT	This work
W $\Delta sslE$::FRT	W $\Delta sslE$::FRT, derived by FLP recombination from W $\Delta sslE$::Kan	This work
Plasmids		
pRH21	pACYC184-derived; <i>trc</i> promoter; <i>lacI</i> ^q	This work
pRH31	pTrc99A-derived; <i>trc</i> promoter; <i>lacI</i> ^q	This work
pMSD6	pRH21 with <i>sslE</i> cloned into the MCS	This work
pMSD7	pRH21 with <i>sslE</i> lacking the signal peptide-encoding sequence cloned into the MCS	This work
pMSD8	pRH21 with <i>pppA</i> cloned into the MCS	This work
pRH153	pRH31 with an <i>sslE</i> - <i>cel45A</i> fusion cloned into the MCS	This work
pRH154	pRH31 with an <i>sslE</i> - <i>pel10A</i> fusion cloned into the MCS	This work

^a MCS, multiple cloning site.

^b ATCC, American Type Culture Collection.

Table 4 Primers used in this study

Name	Description	Application
gspKO-up	GACAATCTTTAATACAGACAAAGAGCATCTGCGAAAAATTGTACGCGGGATCCGGGGATCCGTCGACC	<i>gspC-M</i> deletion
gspKO-dn	CGCCACGTTAACGAGAGTAATTTTATTGATACTAATCTCTGATACTTTATGTAGGCTGGAGCTGCTTCG	<i>gspC-M</i> deletion
pppAKO-dn	ATACACTTGACAGGCCCGCATCCGGCAAGTTACAACAAACAACCTTTAACCATCCGGGGATCCGTCGACC	<i>pppA</i> deletion
pppAKO-dn	TTATTAATAAGAGTTAAAATGTCACCTTTGATAATGACGTTGTTATCATTATGTAGGCTGGAGCTGCTTCG	<i>pppA</i> deletion
sslEKO-up	TTTCTCCAGTTACGAATTTTTTAACATTGTTTTGTCACCTTGCGTTATAATTCCGGGGATCCGTCGACC	<i>sslE</i> deletion
sslEKO-dn	TTATTCATGCCGGATGCGGCGTGAACGCCTTATCCGGCATAACAGGATTATGTAGGCTGGAGCTGCTTCG	<i>sslE</i> deletion
pppA-up	TTATTAGGTACCATGCTTTTTGATGTTTTTCAGC	<i>pppA</i> cloning
pppA-dn	ATATTAGGATCCTTAAACAATGCCTGTAGATAAATTG	<i>pppA</i> cloning
sslE-up	TTATTAGGTACCATGAATAAGAAATTTAAATATAAGAAATCG	<i>sslE</i> cloning
sslE-noSP-up	TTATTAGGTACCATGCTTCCTCCGATACG	<i>sslE-SP</i> cloning
sslE-dn	TTATTAGGATCCTTACTCGACAGACATCTTATG	<i>sslE</i> cloning
sslE-dn-nostop	TTATTAGGATCCGCTCTCGACAGACATCTTATG	<i>sslE</i> cloning for fusions
cel45A-noSP-up	TTATTAGGATCCGAGTTTGTGAATATCGTGTACC	<i>sslE-cel45A</i> fusion
cel45A-dn	TTATTAAGCTTTTACGGGCAGGTATTACGAATATC	<i>sslE-cel45A</i> fusion
pel10A-noSP-up	TTATTAGGATCCGCTGCAGTTACAAGGTAC	<i>sslE-pel10A</i> fusion
pel10A-dn	TTATTAAGCTTTTACAGGTAACCCACTTCTGG	<i>sslE-pel10A</i> fusion

Plasmid pRH21 was constructed from pEG100 [25] by replacing the multiple cloning site (MCS) with a synthetic variant including a tandem His-FLAG tag and by adding the *rrnB*-derived terminators from pTrc99A downstream of the MCS. pRH31 was constructed from pTrc99A [26] by replacing the MCS with the same synthetic variant as in pRH21. pMSD6 was constructed using *sslE* amplified from *E. coli* W genomic DNA with the *sslE*-up and *sslE*-dn primers (Table 4). pMSD7 was constructed using *sslE* similarly amplified with the *sslE*-noSP-up and *sslE*-dn primers. pMSD8 was constructed using *pppA* similarly amplified with the *pppA*-up and *pppA*-dn primers. For construction of pMSD6, pMSD7, and pMSD8, the PCR products were digested with *Acc65I* and *BamHI* and ligated into the large *Acc65I/BamHI* fragment of pRH21.

For construction of pRH153, *sslE* was amplified from *E. coli* W genomic DNA using primers *sslE*-up and *sslE*-dn-nostop, and the PCR product was digested with *Acc65I* and *BamHI*. A gene encoding the mature form of Cel45A from *Cellvibrio japonicus* Ueda107 was synthesized and codon optimized for *E. coli* expression, then amplified using the *cel45A*-noSP-up and *cel45A*-dn primers, and the PCR product was digested with *BamHI* and *HindIII*. The two digested PCR products (*sslE* and *cel45A*) were ligated into the large *Acc65I/HindIII* fragment of pRH31. pRH154 was constructed as pRH153, with a synthetic gene encoding mature Pel10A from *C. japonicus* Ueda107 (with altered codon usage for expression in *E. coli*) being amplified using the *pel10A*-noSP-up and *pel10A*-dn primers prior to digestion and ligation.

Protein expression and detection

For assessing secretion of wild-type SslE, cultures of indicated strains (mutants were all *kan*-marked, except Δ *pppA* mutants, which were unmarked) were grown in liquid media (LB at 37°C unless otherwise noted) with aeration for 16–20 hours. For complementation of the Δ *sslE* mutation, gene expression from plasmids was induced with 1 μ M isopropyl- β -D-galactopyranoside (IPTG). Cells were harvested by centrifugation and resuspended in SDS sample buffer (SSB) [21] according to the following formula: resuspension volume (in μ l) = $100 \times A_{600} \times \text{vol harvested (in ml)}$. These concentrated cell lysates were diluted 1:100 in SSB for SDS-PAGE. Cell-free supernatants were concentrated ~10-fold by filtration using Centricon spin columns (Millipore, Billerica, MA, USA), and added to concentrated SSB for SDS-PAGE. Samples were separated on 4-12% SDS-polyacrylamide gels and stained with silver to visualize protein bands [21]. SslE secretion experiments were repeated 2–4 times, and single representative gels are shown.

To produce the images in Figure 2, the stained gels were digitally photographed and gel images were enhanced using Adobe Photoshop software. Linear transformations (contrast and brightness adjustments) were applied to the images for clarity; such transformations were applied uniformly across any given gel image.

Fusion protein localization by enzyme activity

To measure secretion and surface display of SslE-enzyme fusions, cultures of WT and Δ *pppA*::FRT strains bearing the indicated plasmids were grown in LB at 37°C with aeration for 16–20 hours. Cells were harvested by

centrifugation, and cell-free supernatants were removed; an aliquot of collected cells was removed and lysed using the PopCulture reagent from Novagen (Madison, WI, USA). Enzymatic activities associated with intact cells, lysed cells, and cell-free supernatants were then immediately measured. SslE-Cel45A activity was measured using the CRACC assay [27], and SslE-Pel10A activity was measured using the pectate lyase assay described by Collmer [28].

Growth comparisons

Phenotypic microarray experiments were performed using an OmniLog reader (Biolog, Hayward, CA, USA) as per the manufacturer's instructions using plate types PM-9 and PM-10. Cultures were grown at 37°C for 48 hours, and respiration data were analyzed using the PM software provided with the OmniLog reader. Strains used were wild-type *W* and $\Delta gsp::FRT$ (unmarked deletion of *gspC-M*).

To compare urea tolerances in 96-well plates, wild-type, $\Delta gsp::FRT$, and $\Delta pppA::FRT$ strains were cultured in 200 μ l aliquots of LB containing 0, 0.9 M, or 1.15 M urea in 96-well plates (inoculated as 1:100 dilutions from LB overnight cultures). Plates were grown with shaking at 37°C in a Tecan M1000 plate reader (Durham, NC, USA). Growth and survival were followed by regular measurement of A_{595} for each culture.

To compare urea tolerances in glass culture tubes, wild-type, $\Delta gsp::FRT$, and $\Delta sslE::FRT$ strains were cultured in 8 ml volumes of LB containing no urea or 1.15 M urea on a rolling wheel at 37°C. Biological duplicate cultures of each strain were inoculated with 1:1000 dilutions from LB overnight cultures after verification that all overnight cultures grew to equivalent A_{600} turbidity readings. Turbidity in growing cultures was measured by reading A_{600} using a Spectronic 20D digital spectrophotometer; for cultures with high densities ($A_{600} > 1.5$), aliquots of the culture were diluted 1:10 or 1:20 prior to measurement of A_{600} . Viable cells were enumerated by 10-fold serial dilution of cultures into sterile 0.9% NaCl followed by plating of dilutions on non-selective media and colony counting.

Availability of supporting data

Biolog cultivation data are included as Additional file 1. Data from microtiter plate growth experiments of cells under urea stress are included as in Additional file 2: Figure S1. The sequences of all plasmids described in this study are included as Additional file 3.

Additional files

Additional file 1: Dye reduction traces for Biolog experiments.

Additional file 2: Figure S1. Growth of wild-type and mutant strains with and without urea in 96-well plate experiments.

Additional file 3: Sequences of plasmids used in this study.

Abbreviations

T2SS: Type II secretion systems; LT: Heat-labile enterotoxin; Gsp: General secretory pathway; WT: Wild-type; MCS: Multiple cloning site.

Competing interests

The authors declare no competing interests.

Authors' contributions

MD, RL, and RH designed experiments and contributed to writing the manuscript. MD and RH performed experiments and analyzed data. All authors read and approved the final manuscript.

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