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Phage shock proteins B and C prevent lethal cytoplasmic membrane permeability in *Yersinia enterocolitica*

N. Kaye Horstman and Andrew J. Darwin*

Department of Microbiology, New York University School of Medicine, New York, NY 10016

Summary

The bacterial phage shock protein (Psp) stress response system is activated by events affecting the cytoplasmic membrane. In response, Psp protein levels increase, including PspA, which has been implicated as the master effector of stress tolerance. *Yersinia enterocolitica* and related bacteria with a defective Psp system are highly sensitive to the mislocalization of pore-forming secretin proteins. However, why secretins are toxic to *psp* null strains, whereas some other Psp-inducers are not, has not been explained. Furthermore, previous work has led to the confounding and disputable suggestion that PspA is not involved in mitigating secretin toxicity. Here we have established a correlation between the amount of secretin toxicity in a *psp* null strain and the extent of cytoplasmic membrane permeability to large molecules. This leads to a morphological change resembling cells undergoing plasmolysis. Furthermore, using novel strains with dis-regulated Psp proteins has allowed us to obtain unequivocal evidence that PspA is not required for secretin-stress tolerance. Together, our data suggest that the mechanism by which secretin multimers kill *psp* null cells is by causing a profound defect in the cytoplasmic membrane permeability barrier. This allows lethal molecular exchange with the environment, which the PspB and PspC proteins can prevent.

Keywords

Yersinia; secretin; stress response; membrane permeability

Introduction

The human pathogen *Yersinia enterocolitica* employs various virulence factors, including two type III secretion systems (T3SSs; Cornelis *et al.*, 1998, Revell and Miller, 2001). The virulence plasmid-encoded Ysc-Yop T3SS is essential for virulence due to its role in innate immune response inhibition (reviewed by Cornelis *et al.*, 1998, Cornelis, 2002, Shao, 2008). The Ysa-Ysp T3SS is encoded on the chromosome of the highly pathogenic 1B *Y. enterocolitica* biogroup (Foultier *et al.*, 2002). The precise role of the Ysa-Ysp system is unknown, but it has a small impact on virulence in mice (Haller *et al.*, 2000, Matsumoto and Young, 2006).

Outer membrane components of all T3SSs are secretin proteins, which form a channel of 12-15 homomeric subunits arranged in a ring (reviewed by Korotkov *et al.*, 2011). Secretins are also part of filamentous phage export, type IV pili, and type II secretion systems. In some cases “pilot” proteins assist secretin multimerization and/or insertion into the outer membrane (reviewed by Koo *et al.*, 2012). An example of a secretin/pilot pair is the YscC/

*Corresponding author: Department of Microbiology MSB 202, New York University School of Medicine, 550 First Avenue, New York, NY 10016, andrew.darwin@med.nyu.edu, Tel. (212) 263-3223, Fax (212) 263-8276.

YscW proteins of the Ysc-Yop T3SS. The absence of YscW increases the likelihood of YscC secretin multimers inserting into the incorrect (cytoplasmic) membrane (Koster *et al.*, 1997, Burghout *et al.*, 2004).

Secretin mislocalization to the cytoplasmic membrane induces the phage shock protein (Psp) extracytoplasmic stress response (reviewed by Darwin, 2005, Joly *et al.*, 2010, Yamaguchi and Darwin, 2012). Furthermore, in *Y. enterocolitica*, the Psp response is required for viability when the YscC secretin is produced, either alone or as part of the native Ysc-Yop T3SS (Darwin and Miller, 2001). As a result, the *Y. enterocolitica* Psp system is essential for virulence (Darwin and Miller, 1999, Darwin and Miller, 2001). The Psp system is present in many Gram-negative bacteria and has been studied extensively in *Y. enterocolitica* and *E. coli*. Homologs of some of its components are also found in Gram-positive bacteria, archaea and plants (Brissette *et al.*, 1990, Darwin, 2005, Huvet *et al.*, 2011). Other inducers of the Psp response in *E. coli* include heat, high osmolarity, ethanol, and proteins trapped in the cytoplasmic membrane translocation machinery (e.g. Brissette *et al.*, 1990, Kleerebezem and Tommassen, 1993, Weiner and Model, 1994, Jones *et al.*, 2003, Wang *et al.*, 2010). From this list of inducers, it seems likely that the Psp system is activated in response to problems with the cytoplasmic membrane.

The Psp system is encoded by *pspF-pspABCDycjXF* and *pspG* in *Y. enterocolitica* and by *pspF-pspABCDE* and *pspG* in *E. coli*. Only *pspF*, -A, -B, and -C have been linked to robust phenotypes. PspF is a DNA-binding protein that activates the σ^{54} -dependent *pspA* and *pspG* promoters (Jovanovic *et al.*, 1996, Green and Darwin, 2004). PspA inhibits PspF by binding to it in the cytoplasm (Dworkin *et al.*, 2000, Elderkin *et al.*, 2002, Yamaguchi *et al.*, 2010). PspB and PspC are integral cytoplasmic membrane proteins hypothesized to detect an inducing stimulus and then sequester PspA (Adams *et al.*, 2003, Yamaguchi *et al.*, 2010). This frees PspF to activate its target promoters. This leads to a large increase in Psp protein concentrations, particularly PspA, which has been proposed to counter stress by maintaining the proton motive force (PMF; e.g. Kleerebezem *et al.*, 1996, Kobayashi *et al.*, 2007).

Many conditions that induce the Psp response also induce other systems, including the σ^E (RpoE) and Cpx extracytoplasmic stress responses. In contrast, secretin production only induces *psp* gene expression (Lloyd *et al.*, 2004, Seo *et al.*, 2007). This remarkably specific secretin-Psp relationship is further emphasized by the fact that in *Y. enterocolitica* only *psp* null mutants are specifically sensitive to secretin production (Seo *et al.*, 2007). Furthermore, a random screen for overexpression inducers of the *Y. enterocolitica* Psp system found various secretins and just three other (non-secretin) cytoplasmic membrane proteins of unknown function (Maxson and Darwin, 2004). Interestingly, although all of these proteins induced the Psp response, and not the σ^E and Cpx responses, only the secretins were toxic to a *psp* null strain. To date there has been no explanation for this.

Another unresolved issue is the Psp protein(s) that prevent secretin toxicity. PspA is considered to be the master effector protein involved in countering Psp-inducing stress. This is based on several observations, including the wide conservation of PspA homologues (Huvet *et al.*, 2011), the high level of PspA when the system is induced (Brissette *et al.*, 1990), that deletion of *pspA* decreased PMF during production of improperly translocated PhoE (Kleerebezem *et al.*, 1996), and that PspA prevented proton leakage from damaged membrane vesicles *in vitro* (Kobayashi *et al.*, 2007). Confounding this, deletion of *pspA* does not cause sensitivity to secretin production in *Y. enterocolitica* (Darwin and Miller, 2001, Maxson and Darwin, 2006). This result suggests that PspA is not involved in countering secretin-induced stress. However, an important caveat to that conclusion is that deletion of *pspA* causes massive overexpression of the remaining Psp regulon. This might mask any physiological consequence of losing PspA, especially as it is known that

overexpression of *pspB* and/or *pspC* promotes secretin-stress tolerance in a strain lacking all other *psp* genes (Maxson and Darwin, 2006).

Here, we have addressed some of these outstanding issues. What potentially lethal physiological defects correlate with secretin production in a *psp* null strain? Why do secretins kill a *psp* null strain whereas non-secretin inducers do not? Which Psp protein(s) promote secretin-stress tolerance, and was the surprising suggestion that PspA is not involved correct?

Results

A correlation between secretin production and the reduced growth yield in a *psp* null strain

Secretins inhibit the growth of *Y. enterocolitica*, *E. coli* and *Salmonella enterica* serovar Typhimurium strains with defective Psp systems (e.g. Seo *et al.*, 2007, Seo *et al.*, 2009). However, it has not been determined whether the magnitude of this growth defect correlates with the level of secretin production. We began by investigating this in *Y. enterocolitica*.

First, we used the YsaC secretin of the Ysa-Ysp T3SS produced from an arabinose-inducible expression plasmid. Growth of *psp*⁺ and Δ *psp* strains strain containing an empty vector plasmid, and of the *psp*⁺ strain with the plasmid encoding YsaC-His₆, was similar regardless of arabinose concentration (Fig. 1A). However, the growth yield of the Δ *psp* strain with YsaC-His₆ decreased as arabinose concentration increased. Immunoblot analysis confirmed more YsaC-His₆ production as arabinose concentration increased (Fig. 1B; note that all YsaC-His₆ multimers dissociated after heating prior to SDS-PAGE, data not shown). Experiments with the YscC secretin of the Ysc-Yop T3SS, produced from an IPTG-inducible (*tacp*) plasmid, led to similar conclusions (Fig. 1 panels C and D, which shows monomers and boiling-resistant multimers characteristic of YscC, e.g. Koster *et al.*, 1997). These data provide a correlation between secretin level and the reduced growth yield of a Δ *psp* strain.

YscC has a known pilot protein, YscW (Burghout *et al.*, 2004). Without YscW, fewer YscC multimers form, but the multimers that do form predominantly mislocalize to the cytoplasmic membrane (Koster *et al.*, 1997, Burghout *et al.*, 2004). In contrast, when YscW is present the more abundant YscC multimers localize mostly into the outer membrane (Burghout *et al.*, 2004). We extended the YscC titration experiments by including a *tacp-yscCyscW* co-expression plasmid. YscW increased the abundance of YscC multimers but alleviated the reduced growth yield of the Δ *psp* strain (Fig. 1 panels C and D). This suggests that secretin mislocalization is the toxic event, which is consistent with work in *E. coli* (Guilvout *et al.*, 2006).

Secretins decrease the membrane potential of a *psp* null strain whereas non-secretin Psp inducers do not

Next, we began our investigation into why secretins reduce the growth of a *psp* null strain, and why non-secretin Psp inducers do not (Maxson and Darwin, 2004). The Psp system has been proposed to maintain proton motive force (PMF; e.g. Kleerebezem *et al.*, 1996, Kobayashi *et al.*, 2007). The gold standard to monitor the membrane potential component of the PMF in *E. coli* K-12 uses uptake of the lipophilic cation tetraphenylphosphonium, which is proportional to the membrane potential (TPP⁺; Eisenbach, 1982). However, TPP⁺ was not taken up by *Y. enterocolitica* unless CCCP was added, and then uptake did not increase over time (data not shown). This is evidence for an energy dependent efflux pump that prevents TPP⁺ from accumulating in the cell (e.g. De Rossi *et al.*, 1998). Therefore, we used *E. coli* as a surrogate host. First, we tested whether the *Y. enterocolitica* Psp inducers behaved

similarly in *E. coli*. Production of the YsaC or YscC secretins, or the non-secretin YE0566 and AmpE proteins, induced $\Phi(pspA-lacZ)$ operon fusion expression in *E. coli* (Fig. 2A). However, only the secretins reduced the growth of a Δpsp strain (Fig. 2B). This is exactly how these proteins behave in *Y. enterocolitica* (Maxson and Darwin, 2004). Interestingly, YE0566 reduced the growth of the psp^+ strain but not the Δpsp strain (Fig. 2B). The reason for this is not yet known.

Next we measured TPP⁺ uptake. psp^+ and Δpsp strains containing an empty *tacp* expression plasmid accumulated [³H]-TPP⁺ similarly, indicating an intact membrane potential (Fig 2C). Treatment with CCCP abolished [³H]-TPP⁺ uptake, supporting its dependence on the membrane potential. Production of the YsaC or YscC secretins did not affect [³H]-TPP⁺ uptake in the psp^+ strain, but almost completely abolished it in the Δpsp strain. In contrast, AmpE or YE0566 production did not abolish [³H]-TPP⁺ uptake. Therefore, reduced membrane potential offers a potential explanation of why secretins are toxic in a psp null strain but non-secretin inducers are not.

Secretin toxicity correlates with increased cytoplasmic membrane permeability to ONPG

In typical growth experiments like those in Figure 1 we have found that once secretin-induced growth inhibition is evident, psp null cells die rapidly (colonies cannot be recovered; data not shown). However, several studies indicate that bacteria (*E. coli*) can remain viable for some time in the absence of a PMF and, if glucose is present, even resume growth after an adaptive lag period (e.g. Kinoshita *et al.*, 1984, Ohyama *et al.*, 1992, Gage and Neidhardt, 1993). Consequently, we considered it unlikely that reduced PMF was the sole explanation for the rapid secretin-induced cell death of a *Y. enterocolitica psp* null strain. Therefore, we investigated the possibility that secretins might increase cytoplasmic membrane permeability to molecules much larger than protons. For this we adapted a method used to monitor cytoplasmic membrane permeabilization by antimicrobial peptides (e.g. Lehrer *et al.*, 1989, Barker *et al.*, 2000, Arcidiacono *et al.*, 2009). This takes advantage of a permeabilized cytoplasmic membrane allowing ortho-nitrophenyl- β -galactoside (ONPG) to enter the cytoplasm, where it can be hydrolyzed by cytoplasmic β -galactosidase. To adapt this to *Y. enterocolitica* we introduced a constitutively expressed *lacZ* gene, but the LacY permease was absent.

A Δpsp strain with constitutively expressed *lacZ* and the *tacp-yscC* expression plasmid was grown with various amounts of IPTG as in Figure 1. A corresponding psp^+ strain was grown with the highest concentration of IPTG (200 μ M). All were harvested at the 4 h time point, incubated in phosphate buffered saline containing ONPG, and the absorbance at 420 nm was measured over time. When YscC was uninduced in the Δpsp strain (no IPTG), or was induced at low level (10 μ M IPTG), there was no ONPG hydrolysis above the basal level (Fig. 3A). However, there was a small increase in ONPG hydrolysis in the sample that had been grown with 25 μ M IPTG, and a substantial increase following growth with 200 μ M IPTG. These results correlate perfectly with the effects of YscC on growth yield at the 4 h time point, with the no IPTG and 10 μ M IPTG Δpsp cultures having no growth defect, the 25 μ M culture having a minor defect and the 200 μ M culture having a substantial defect (Fig. 1C). Furthermore, when these assays were extended to strains co-producing the YscW pilot, ONPG hydrolysis was essentially abolished, which correlates with YscW alleviating the growth defect (Fig. 1C). Finally, cells that were grown overproducing the non-secretin Psp inducers AmpE and YE0566 did not exhibit ONPG hydrolysis above the basal level (Fig. 3A). This is consistent with these proteins failing to inhibit the growth of a psp null strain compared to a psp^+ strain (Maxson and Darwin, 2004).

To confirm induction of the Psp response by the levels of YscC, AmpE and YE0566 used in these experiments, a separate set of psp^+ strains with a $\Phi(pspA-lacZ)$ operon fusion was

used. The strains were grown with the same concentrations of IPTG that had been used for the ONPG hydrolysis assays (only 200 μ M IPTG was used for strains producing YscC +/- YscW). Consistent with published data (e.g. Darwin and Miller, 2001, Maxson and Darwin, 2004), YscC induced the *pspA* promoter weakly, YscC + YscW did not cause any significant induction, and AmpE and YE0566 caused strong induction (Fig. 3B). Therefore, even though AmpE and YE0566 were much stronger inducers of the Psp response than YscC, they did not cause cytoplasmic membrane permeability when the Psp system was defective.

ONPG might be hydrolyzed by β -galactosidase leaking from the cytoplasm (e.g. by cell lysis). Therefore, a mock ONPG hydrolysis assay was set up with Δ *psp* + YscC, 200 μ M IPTG cells, but without adding ONPG. At the end of the incubation period bacteria were removed by filtration so that any extracellular β -galactosidase would be retained in the filtrate. However, when ONPG was added to the filtrate it was not hydrolyzed (data not shown). Therefore, although YscC permeabilized the Δ *psp* cytoplasmic membrane to ONPG, the membrane did not become permeable to the much larger β -galactosidase enzyme.

These results suggest that secretins are toxic to a Δ *psp* strain, but the non-secretin Psp inducers are not, because only secretins cause lethal cytoplasmic membrane permeability to molecules at least as large as ONPG (~ 300 Da).

YscC secretin production in a Δ *psp* strain causes a phenotype that resembles plasmolysis

Secretin production causes major cytoplasmic membrane permeability in a *psp* null strain. This prompted us to examine *psp*⁺ and Δ *psp* cells +/- *tacp-yscC* by transmission electron microscopy. The cells were harvested after three hours grown as in Figure 1C, when the growth defect of the *psp* null strain was first evident, but the culture OD was still increasing. When YscC was produced in the Δ *psp* strain some cells appeared to have a physical separation between layers of their cell envelope, which most frequently occurred at the pole (Fig. 4). In two independent experiments this phenotype was observed in approximately 13% of the cells (out of ~ 200) in the first experiment and 3% (out of ~ 350) in the second. The relatively low frequency is probably due to the fact that cells were harvested before growth had completely arrested. Significantly, we did not observe this phenotype in any *psp*⁺ or Δ *psp* cells without YscC production, or in any *psp*⁺ cells overproducing YscC. Furthermore, it was also not observed in any strain overproducing the non-secretin inducers AmpE and YE0566 (data not shown). Therefore, it is a phenotype specific to the *psp* null strain during secretin production. These sites of cell envelope separation resemble plasmolytic bays that have been observed at the pole of *E. coli* cells undergoing mild plasmolysis in hypertonic conditions (reviewed by Koch, 1998). It is linked to cytoplasmic shrinkage caused by loss of water, and so is consistent with increased cytoplasmic membrane permeability. In fact, a correlation between cytoplasmic membrane permeability and plasmolytic bays has also been established recently in *E. coli* (Reimann and Wolfe, 2011). Therefore, secretin production in a *psp* null strain correlates with growth inhibition, decreased membrane potential, increased cytoplasmic membrane permeability to large molecules and a plasmolysis-like phenotype.

A secretin that cannot multimerize does not affect the growth of a *psp* null strain

Our experiments had suggested that secretin toxicity is the result of major cytoplasmic membrane permeability caused by secretin multimers. A recent insightful review article grouped secretins into five classes depending on their behavior in the absence of pilot or other accessory proteins (Koo *et al.*, 2012). Secretins in classes 1-3 still form multimers whereas those in classes 4-5 do not. All secretins we have tested to date are toxic to a *Y.*

enterocolitica *psp* null strain: YscC and YsaC from *Y. enterocolitica*, InvG from *S. enterica* serovar Typhimurium, XcpQ from *P. aeruginosa* and pIV from filamentous phage f1 (e.g. Seo *et al.*, 2007, Seo *et al.*, 2009 and this study). All of those secretins can multimerize in the absence of helper proteins (Seo *et al.*, 2009, Koo *et al.*, 2012 and literature cited within; data not shown for YsaC). Therefore, their toxicity to a *psp* null strain is consistent with the proposal that it is only secretin multimers that impact the Psp system (e.g. Guilvout *et al.*, 2006). However, if this is true, we reasoned that a secretin unable to multimerize should not be toxic to a *psp* null strain.

To test this hypothesis we used PilQ from the *P. aeruginosa* type IV pilus, which is a class 5 secretin (Koo *et al.*, 2012). Without its pilot protein, PilQ mislocalizes to the cytoplasmic membrane but does not form the heat/SDS-resistant multimers detected when its pilot protein is present (Koo *et al.*, 2008). Therefore, PilQ produced in isolation in a *Y. enterocolitica* *psp* null mutant should not be toxic. This was tested by introducing a *tacp-pilQhis₆* expression plasmid, or the empty vector control, into *Y. enterocolitica* *psp*⁺ and Δ *psp* strains. The strains were grown with concentrations of IPTG up to 1 mM and optical density was measured over time. Regardless of the IPTG concentration there was no evidence of PilQ-dependent toxicity in either strain (Fig. 5A and data not shown). Immunoblot analysis revealed robust PilQ production (Fig. 5B). However, all of the PilQ was in monomeric form, consistent with the situation when PilQ was produced in *P. aeruginosa* without its pilot protein (Koo *et al.*, 2008). We also failed to detect any evidence of PilQ multimers after prolonged exposure of the immunoblots and regardless of whether samples were heated or not prior to SDS-PAGE (data not shown). Finally, we found that PilQ production also failed to induce Φ (*pspA-lacZ*) expression in a *psp*⁺ *Y. enterocolitica* strain (Fig. 5C).

These results are notable because PilQ is the first secretin protein we have found to be unable to induce the Psp response and to be non-toxic to a *psp* null strain. It is also the first secretin we have tested that does not form any multimers under the experimental conditions we used. Therefore, all of this is consistent with the contention that secretins are toxic to a *psp* strain when in their multimeric forms.

A physiologically relevant system where the regulatory link between Psp proteins is uncoupled

Next, we established a system to unequivocally identify which Psp protein(s) prevent multimeric secretin toxicity when present at a normal level. Deletion of *pspA* does not cause secretin-sensitivity (Darwin and Miller, 2001, Maxson and Darwin, 2006), suggesting that the proposed master effector PspA is not involved in secretin-tolerance. However, loss of PspA causes non-physiological overexpression of the remaining Psp regulon. In fact, because PspA, -B and -C all regulate *pspA* operon expression, removing any of them changes the levels of those remaining. Any phenotype could be due to loss of regulatory function, loss of physiological function, or both. Therefore, to carefully examine the contribution of Psp components to secretin tolerance we have established a chromosomal expression system where the proteins are produced at physiological levels, but without regulatory links between them. For this, a region encompassing the *Y. enterocolitica* *pspF* gene and the *pspA* promoter was replaced by a fragment encoding *E. coli* LacI repressor and the *tac* promoter (Fig. 6A). As a result, *psp* gene expression can be controlled by IPTG. We reported a preliminary use of this system to investigate PspC regulatory mutants (Yamaguchi *et al.*, 2010) but here we characterized it in detail.

YsaC production reduced the growth yield of the *tacp-psp* strain when IPTG was omitted from the medium, but the inclusion of 10 μ M IPTG prevented this (Fig. 6B). This is consistent with IPTG-dependent induction of the *pspA* operon, which was confirmed by

immunoblot analysis of PspA, -B and -C protein levels (Fig. 6C). Importantly, the Psp protein levels induced by 10 μ M IPTG were comparable to the YsaC-induced levels in the wild type strain. Therefore, the proteins were produced at a physiological level. We also used our ONPG hydrolysis assay to investigate YsaC-induced cytoplasmic membrane permeability. YsaC caused severe cytoplasmic membrane permeability in a complete *psp* deletion strain, but not in a wild type *psp* locus strain, which was similar to the effect of the YsaC secretin (compare Figs. 3A and 6D). In the *tacp-psp* strain, YsaC caused cytoplasmic membrane permeability in cells grown without IPTG, but not in cells grown with 10 μ M IPTG (Fig. 6D). Finally, these experiments also revealed that in the absence of IPTG, the reduced growth yield and cytoplasmic membrane permeability of the *tacp-psp* strain were not as severe as in a complete *psp* deletion strain (Fig. 6). This can be explained by low but detectable Psp protein levels in the *tacp-psp* strain without IPTG (Fig. 6C), probably due to so called leaky expression from the *tac* promoter.

PspB and PspC are both necessary to prevent secretin-induced toxicity

The *tacp-psp* strain produced physiological levels of PspA, -B and -C that allowed tolerance to secretin-induced stress (Fig. 6). We could now use this system to delete members of the *pspA* operon without affecting the expression of those remaining. This approach would allow a definitive determination of which Psp proteins are needed to prevent secretin-induced toxicity when produced at physiological levels. Therefore, derivatives of the *tacp-psp* strain were constructed with Δ *pspA*, Δ *pspB*, Δ *pspC*, Δ *pspBC* or Δ (*pspD-ycjXF*) in frame deletions.

Immunoblot analysis was used to test the effect of each deletion on the levels of PspA, -B, and -C during growth with 10 μ M IPTG (Fig. 7). The Δ (*pspD-ycjXF*) deletion did not affect PspA, -B, or -C. With only one exception, the Δ *pspA*, Δ *pspB*, Δ *pspC*, Δ *pspBC* deletions abolished production of the protein(s) encoded by the deleted gene(s) but did not significantly affect any others (Fig. 7A). The exception was Δ *pspB*, which also abolished detection of PspC. This was expected, because PspB is required to protect PspC from FtsH-dependent degradation (Singh and Darwin, 2011).

Next we determined the effect of the *psp* deletions on growth during YsaC secretin production (Fig. 7B). As expected, the Δ (*pspD-ycjXF*) deletion had no effect. Importantly, the same was true for Δ *pspA*, which demonstrates that a physiological level of PspA is not required for secretin stress tolerance. Furthermore, it shows that the lack of secretin sensitivity when *pspA* is deleted in a wild type strain (Darwin and Miller, 2001, Maxson and Darwin, 2006) cannot be explained by compensatory overproduction of PspBC, because this does not occur in the *tacp-pspA* operon strain (Fig. 7A). In contrast, the Δ *pspB*, Δ *pspC* and Δ *pspBC* deletions all caused secretin-sensitivity (Fig. 7), and comparison of their phenotypes suggested that both PspB and PspC contribute to secretin-stress tolerance. First, Δ *pspC* caused secretin sensitivity, which demonstrates a role for PspC because this mutation only abolishes PspC protein production. Second, the Δ *pspB* mutation caused a larger reduction in growth yield than Δ *pspC*, which indicates a contribution of PspB towards secretin-tolerance. Finally, results from ONPG hydrolysis assays correlated perfectly with the effects on growth yield, with only deletion of *pspB* and/or *pspC* causing detectable permeability to ONPG (Fig. 7C).

Taken together these data provide unequivocal evidence that of the six proteins encoded by the *Y. enterocolitica pspA* operon, only PspB and PspC are required to prevent the lethal cytoplasmic membrane permeability caused by secretin production.

Discussion

A *Y. enterocolitica* *psp* null mutant is avirulent in a mouse model of infection (Darwin and Miller, 1999). This phenotype correlates with bacterial cell death caused by mislocalization of the YscC secretin from the Ysc-Yop T3SS (Darwin and Miller, 2001). Secretin-sensitivity also occurs in *E. coli* and *S. enterica* *psp* null mutants (Seo *et al.*, 2007, Seo *et al.*, 2009). However, there has been no explanation for this secretin-sensitivity phenotype that might account for the rapid cell death. Here, we report that secretins probably kill *psp* null cells by causing a defect in the cytoplasmic membrane permeability barrier that is more profound than previously demonstrated. Our data also show unequivocally that PspA is not required to prevent this, whereas PspBC are.

In considering how a mislocalized secretin kills a *psp* null cell, it has long been hypothesized that the Psp system functions to maintain the PMF (Model *et al.*, 1997, Darwin, 2005, Joly *et al.*, 2010). The failure of *Y. enterocolitica* to accumulate the lipophilic cation TPP⁺ prevented us from monitoring its membrane potential. However, secretin production significantly reduced uptake of TPP⁺ in *E. coli* *psp* null cells (Fig. 2C), which is consistent with a reduced membrane potential, and with other studies (e.g. Guilvout *et al.*, 2006). Even so, reduced PMF is unlikely to explain abrupt death of *psp* null cells. *E. coli* can survive for a prolonged period and even divide in some situations after the PMF has been destroyed (Kinoshita *et al.*, 1984, Ohyama *et al.*, 1992, Gage and Neidhardt, 1993). In fact, our own data revealed that secretin production was sufficient to abolish the ability of *E. coli* *psp* null cells to take up TPP⁺ at the 3 hour time point, suggesting a dissipated PMF (Fig. 2C). Nevertheless, these cells continued to divide long after 3 h (Fig. 2B).

Secretin production allowed externally added ONPG to be hydrolyzed by cytoplasmic β -galactosidase in *Y. enterocolitica* *psp* null cells (e.g. Fig. 3A). Therefore, a mislocalized secretin permeabilizes the cytoplasmic membrane to molecules much larger than protons and other small ions. Importantly, the rate at which cells hydrolyzed ONPG correlated perfectly with the magnitude of the growth yield reduction. First, overproduction of YsaC (Fig. 1A, 0.02% arabinose) reduced the growth yield of a *psp* null strain more than overproduction of YscC (Fig. 1C, 200 μ M IPTG) and also led to a faster rate of ONPG hydrolysis (compare Figs. 3A and 6D, note the different y-axis scales). Second, increasing the level of YscC production correlated with increasingly reduced growth yield and an increasing rate of ONPG-hydrolysis (Figs. 1C and 3A). Third, YsaC production reduced the growth yield of a *psp* null strain more than the *tacp-pspA* operon strain without IPTG, and the former strain also had an increased rate of ONPG-hydrolysis (Fig. 6). Finally, using the *tacp-pspA* operon strain, the relative effect of *pspB* and/or *pspC* deletions on growth yield correlated exactly with their relative rate of ONPG hydrolysis (Fig. 7). Therefore, we suggest that secretins kill *psp* null cells by permeabilizing the cytoplasmic membrane and allowing exchange of molecules at least as large as ONPG.

We reported that overproduction of secretins and some non-secretin cytoplasmic membrane proteins (e.g. AmpE and YE0566) induced *Y. enterocolitica* *pspA* operon expression (Maxson and Darwin, 2004). However, in a *psp* null strain, secretins were toxic whereas AmpE and YE0566 were not. This can now be explained because only secretins permeabilize the cytoplasmic membrane of a *psp* null strain (Fig. 3A). Still unexplained is what secretin, AmpE and YE0566 production have in common that generates a Psp-inducing signal. It has been hypothesized that the Psp system is induced by dissipated PMF (Model *et al.*, 1997, Darwin, 2005, Joly *et al.*, 2010). This is unlikely because the PMF does not dissipate unless the Psp system is defective (Kleerebezem *et al.*, 1996, Guilvout *et al.*, 2006, Jovanovic *et al.*, 2006, Engl *et al.*, 2009, Jovanovic *et al.*, 2009, Jovanovic *et al.*, 2010). We also found this to be true for secretins, which induced $\Phi(\textit{pspA-lacZ})$ expression in *psp*⁺ cells

without affecting TPP⁺ uptake (Fig. 2). Furthermore, AmpE or YE0566 production did not abolish TPP⁺ uptake in *psp*⁺ or *psp* null cells (Fig. 2). It is possible that all of these Psp inducers reduce the PMF in *psp*⁺ cells but the difference is below the detection limit. However, a recent study also showed that reduced PMF is not sufficient to induce *E. coli psp* gene expression (Engl *et al.*, 2011).

Perhaps the Psp system responds to increased cytoplasmic membrane permeability, rather than specifically to reduced PMF. However, secretins, AmpE and YE0566 all induced $\Phi(\textit{pspA-lacZ})$ expression in *psp*⁺ cells but did not increase cytoplasmic membrane permeability to ONPG (e.g. Figs. 3A and 6D). This suggests that a Psp-inducing signal is generated in the absence of increased cytoplasmic membrane permeability to ONPG. Nevertheless, it remains possible that some altered physical property of the cytoplasmic membrane is sufficient to generate the Psp-inducing stimulus.

Another important part of this work was the question of whether PspA is involved in mitigating secretin toxicity. Formally, we have shown that it is not *required* because a *pspA* in frame deletion mutant is not secretin sensitive (Darwin and Miller, 2001, Maxson and Darwin, 2006). However, this does not rule out its *involvement* because the remaining *psp* genes are massively overexpressed without PspA, which might have provided non-physiological compensation for its loss. We engineered *Y. enterocolitica* to produce Psp proteins at near-physiological levels but no longer regulated by the Psp proteins themselves. This allowed us to show unequivocally that only PspB and PspC play a role in secretin stress tolerance. The removal of PspA had no effect, even though other *psp* genes could not be overexpressed to compensate. We still think that PspA is involved in countering adverse effects associated with activation of the Psp response. In particular, the genetics of one study convincingly linked PspA to maintenance of the PMF during production of a translocation-defective PhoE protein (Kleerebezem *et al.*, 1996). Perhaps PspA counters mild membrane permeabilization that might lead to leakage of small ions and/or as yet undetected defects caused by many Psp inducers, including YE0566 and AmpE, but secretins cause much more severe damage that demands the function(s) of PspB and PspC to counteract.

An obvious possibility is that cytoplasmic membrane permeability is caused by the passage of molecules through the mislocalized multimeric secretin channel itself. However, it is also possible that secretin multimers disrupt the integrity of the phospholipid bilayer around them, allowing passage of molecules through the membrane rather than through the secretin channel. Regardless of how secretins permeabilize the cytoplasmic membrane, a focus of future studies will be to understand how PspB and PspC prevent it. We have not found any evidence to support a model that Psp proteins simply inhibit secretin mislocalization into the cytoplasmic membrane. Differential detergent solubilization experiments did not reveal any difference in the distribution of YscC between inner and outer membrane fractions in *psp*⁺ and $\Delta\textit{psp}$ cells (N.K. Horstman and A.J. Darwin, unpubl. data). However, the severe toxicity of secretins to the $\Delta\textit{psp}$ strain makes these fractionation experiments difficult and it is possible that changes in cell envelope properties could impact the fractionation procedure. Nevertheless, this is consistent with mislocalization of PulD into the *E. coli* cytoplasmic membrane in the presence of a functional Psp system (Guilvout *et al.*, 2006). Also, a *pspA* insertion mutation did not affect pullulanase secretion in *E. coli* with a plasmid encoding the *Klebsiella oxytoca* Pul type 2 secretion system, suggesting that PulD secretin function/localization was similar with or without an intact *pspA* operon (Possot *et al.*, 1992). Similarly, a *pspC* null mutation did not affect Yop export by the *Y. enterocolitica* Ysc-Yop T3SS, suggesting that YscC secretin function/localization is not affected by the Psp system (Darwin and Miller, 1999). Other possibilities include PspBC preventing leakage through the secretin channel or physically modifying membrane properties to prevent permeability.

Experimental procedures

Bacterial strains, plasmids, and routine growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown at 37 °C and *Y. enterocolitica* at 26°C or 37°C as noted. Strains were grown in Luria-Bertani (LB) broth or on LB agar plates. Antibiotics were used as described previously (Maxson and Darwin, 2004) and tetracycline was used at 15 µg ml⁻¹ for *E. coli* and 7.5 µg ml⁻¹ for *Y. enterocolitica*.

Strain constructions

The $\Delta(pspF-pspE)::kan$ mutation (Maxson and Darwin, 2006) was introduced into *E. coli* strain MC3 by phage P1 *vir* transduction.

The *pspF-pspA* region of the *Y. enterocolitica* chromosome was replaced with a *lacIq-tacp* fragment using the *sacB*⁺ allelic exchange plasmid and strategy described previously (Yamaguchi *et al.*, 2010). The same allelic exchange plasmid was used to replace the *pspA* promoter with the *tac* promoter in *psp*⁺ $\Delta pspB$, $\Delta pspC$, $\Delta pspBC$ and $\Delta(pspD-ycjF)$ strains. However, to do this in a $\Delta pspA$ strain, a new pSR47S-based allelic exchange plasmid was constructed as described (Yamaguchi *et al.*, 2010), except that the downstream fragment flanking the *pspA* promoter region was derived from a $\Delta pspA$ strain.

For the ONPG hydrolysis assays, a promoterless *E. coli lacZ* gene was amplified from plasmid pAJD905 (Maxson and Darwin, 2005) by PCR with primers that incorporated EagI and NotI sites. This fragment was cloned into *sacB*⁺ suicide plasmid pAJD898 (Maxson and Darwin, 2005) at the unique NotI site, which is flanked by fragments surrounding *araGFB* on the *Y. enterocolitica* chromosome. Then the *cat* promoter (*catp*) region was amplified from plasmid pACYC184 with primers that incorporated XbaI and BglII restriction sites and cloned into these sites upstream of *lacZ* so that *catp* controlled *lacZ* expression. Standard *sacB*⁺ allelic exchange procedures were used to introduce this $\Phi(catp-lacZ)$ operon fusion into the *ara* locus of the *Y. enterocolitica* chromosome. This was confirmed by colony PCR analysis, as were all of the above-described chromosomal alterations.

Plasmid constructions

For cytoplasmic membrane permeability assays with strains that did not contain an *araBp* expression plasmid, a plasmid-encoded $\Phi(catp-lacZ)$ operon fusion was used (combining this plasmid with an *araBp* plasmid in the same strain led to toxicity). To make this plasmid, promoterless *lacZ* was amplified from pAJD905 with a primer that incorporated a BamHI site downstream of *lacZ*. The fragment was digested with EcoRI and BamHI and ligated between the EcoRI and BclI sites of pACYC184, which placed *lacZ* downstream of the *cat* promoter. The *araBp-ysaC-his₆* (pAJD935) plasmid was constructed by first transferring the EcoRI-BamHI insert of pAJD801 (Maxson and Darwin, 2004) into plasmid pWSK129 (Wang and Kushner, 1991), then excising it from pWSK129 as a KpnI-XbaI fragment and cloning it into pBAD33. The *tacp-pilQ-his₆* plasmid (pAJD1806) was constructed by amplifying the promoterless *pilQ* (PA5040) gene from the chromosome of *P. aeruginosa* strain PAK (Strom and Lory, 1986) with an upstream primer that incorporated an EcoRI site and a downstream primer that incorporated a His₆-encoding region immediately before the stop codon and an XbaI site downstream of the stop codon. The EcoRI-XbaI fragment was ligated between the same sites of plasmid pVLT35.

Growth curves

Saturated *Y. enterocolitica* cultures that had been grown at 26°C were diluted into fresh LB broth in 18 mm diameter test tubes to an optical density (600 nm) of 0.1. Arabinose and/or

IPTG were included at concentrations indicated in the figures. Growth on a roller drum at 37°C was monitored hourly by removing a 0.1 ml aliquot and determining optical density (600 nm). Growth curves of *E. coli* strains were done similarly except that cultures were diluted to an initial optical density (600 nm) of 0.04. All growth curves were done on two or more occasions to ensure reproducibility, but the figures report data from a single experiment.

β-galactosidase assays

For *Y. enterocolitica*, saturated cultures that had been grown at 26°C were diluted into 5 ml of fresh medium in 18 mm diameter test tubes to an optical density (600 nm) of 0.04. For *E. coli*, saturated cultures grown at 37°C were diluted into 4 ml fresh media to an optical density (600nm) of 0.02. For all, after 2 hours of growth in a roller drum at 37°C, IPTG and/or arabinose was added to induce *tac* or *araB* promoter expression unless otherwise indicated in figure legends. Growth continued at 37°C for two more hours prior to harvest. β-Galactosidase activity was determined at room temperature as described previously (Maloy *et al.*, 1996) and reported in arbitrary Miller units (Miller, 1972). The mean of the data from three independently grown cultures, each of which was assayed in duplicate, is reported.

Polyclonal antisera and immunoblotting

The region encoding the C-terminus of YscC (amino acids 321-607) was cloned into pET-32a(+) (Novagen) to encode a TrxA-His₆-YscC-His₆ fusion protein (TrxA = thioredoxin). The plasmid was transferred into *E. coli* strain ER2566 (NEB) and the strain was grown to mid-exponential phase at 37°C in LB broth containing 1 mM IPTG. Total cell lysates were prepared and the His₆-tagged protein purified under denaturing conditions using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography as recommended by the manufacturer (Qiagen). Polyclonal rabbit antiserum was raised against the purified TrxA-His₆-YscC-His₆ fusion protein at Covance Research Products.

For immunoblots, cells were resuspended at equivalent optical densities in SDS sample buffer and heated at 90-100°C for 10 min. Proteins were separated by 10% polyacrylamide SDS-PAGE (for detection of secretin monomers only, or Psp proteins) or by 4-12% gradient polyacrylamide SDS-PAGE (Lonza; for simultaneous detection of secretin monomers and multimers) and transferred to nitrocellulose by semidry electroblotting. The nitrocellulose was stained with Ponceau S and then destained prior to immunoblotting. Enhanced chemiluminescent detection followed sequential incubation in diluted polyclonal antisera or monoclonal antibody, followed by a 1 in 5000 dilution of goat α-rabbit IgG or goat α-mouse IgG horseradish peroxidase conjugate (Bio-Rad). Dilutions of polyclonal antisera or monoclonal antibody were 1 in 5000 for His₆ antibody (GenScript), 1 in 20,000 for PspA antiserum (Yamaguchi *et al.*, 2010), 1 in 100,000 for PspB antiserum (Gueguen *et al.*, 2009), 1 in 20,000 for PspC antiserum (Maxson and Darwin, 2006) and 1 in 10,000 for YscC antiserum. For detection of secretin multimers, after SDS-PAGE the gels were soaked in 10% trichloroacetic acid and washed prior to semidry electroblotting as described previously (Burghout *et al.*, 2004).

[³H]-tetraphenylphosphonium uptake assay

E. coli strains were grown as for β-galactosidase assays. Bacterial cells equivalent to 0.6 ml at an optical density (600 nm) of 2 were collected by centrifugation at room temperature, resuspended in 0.75 ml 100 mM Tris (pH 7.6) 1mM EDTA, and incubated at 37°C for 10 min to permeabilize the outer membrane. Cells were collected by centrifugation and resuspended in 0.6 ml 0.1 M sodium phosphate buffer (pH 7.6) containing 1 mM KCl and 10 mM glucose. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) in ethanol was added to aliquots of the empty pVLT35 samples to a final concentration of 80 μM and all other

samples were treated with an equivalent volume of ethanol. After incubating at 37°C for 5 min, 1.25 μ l of a 1:10 dilution of 1mCi/ml [3 H]-tetraphenylphosphonium bromide (TPP; American Radiolabeled Chemicals) and an equivalent volume of non-radioactive TPP were added so the total TPP concentration was 2 mM. After a further 10 min at 37°C, samples were captured onto GF/F filters (Whatman) using a vacuum manifold (Millipore) and washed twice with sodium phosphate buffer. Filters were air-dried and [3 H] was detected by liquid scintillation (Beckman Coulter). The mean of the data from three independently grown cultures is reported.

Cytoplasmic membrane permeability assay

The method to detect cytoplasmic membrane permeability to ONPG was adapted from a published procedure (Arcidiacono *et al.*, 2009). Bacterial cells equivalent to 1 ml at an optical density (600 nm) of 0.5 were collected by centrifugation at room temperature and washed twice with 1 ml phosphate buffer saline (PBS; 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.1). Cells were resuspended in 1 ml PBS and diluted 1:10 in PBS containing 2 mM ONPG. Absorbance at 420 nm was measured every 2 minutes for 3 hours using the kinetics program of a Beckman Coulter DU® 730 spectrophotometer. The blank was PBS with 2 mM ONPG. To control for changes in β -galactosidase enzyme production, the total β -galactosidase of cells permeabilized with SDS and chloroform (Miller, 1972) was determined in all experiments, but no significant differences were detected (data not shown).

Transmission Electron Microscopy

Cultures were grown at 37°C with 200 μ M IPTG for 3 h and collected by centrifugation. Bacteria were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde, washed with cacodylate buffer (50 mM, pH 7.2), then fixed with 2% osmium tetroxide and washed with Kellenberger buffer. Pellets were embedded in 2% agar, cut and stained in the dark with 0.5% (w/v) uranyl acetate. Samples were dehydrated with alcohol, transferred to propylene oxide/Epon mixtures and finally embedded in EMbed 812 (Electron Microscopy Sciences, Hatfield, PA). Thin sections were cut, adsorbed on electron microscope grids, and stained with uranyl acetate and lead citrate. Stained grids were imaged in a Philips CM12 electron microscope (FEI; Eindhoven, the Netherlands) and photographed with a Gatan (4kx2.7k) digital camera (Gatan Inc., Pleasanton, CA). Sample fixation, preparation, and microscopy were performed by the Microscopy Core at the New York University Langone Medical Center (New York, NY).

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References

- Adams H, Teertstra W, Demmers J, Boesten R, Tommassen J. Interactions between phage-shock proteins in *Escherichia coli*. *J Bacteriol.* 2003; 185:1174–1180. [PubMed: 12562786]
- Arcidiacono S, Soares JW, Meehan AM, Marek P, Kirby R. Membrane permeability and antimicrobial kinetics of cecropin P1 against *Escherichia coli*. *J Pept Sci.* 2009; 15:398–403. [PubMed: 19308920]

- Barker HC, Kinsella N, Jaspe A, Friedrich T, O'Connor CD. Formate protects stationary-phase *Escherichia coli* and *Salmonella* cells from killing by a cationic antimicrobial peptide. *Mol Microbiol.* 2000; 35:1518–1529. [PubMed: 10760151]
- Bergler H, Abraham D, Aschauer H, Turnowsky F. Inhibition of lipid biosynthesis induces the expression of the *pspA* gene. *Microbiology.* 1994; 140(Pt 8):1937–1944. [PubMed: 7921245]
- Brissette JL, Russel M, Weiner L, Model P. Phage shock protein, a stress protein of *Escherichia coli*. *Proc Natl Acad Sci U S A.* 1990; 87:862–866. [PubMed: 2105503]
- Burghout P, Beckers F, de Wit E, van Boxtel R, Cornelis GR, Tommassen J, Koster M. Role of the pilot protein YscW in the biogenesis of the YscC secretin in *Yersinia enterocolitica*. *J Bacteriol.* 2004; 186:5366–5375. [PubMed: 15292137]
- Chang AC, Cohen SN. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J Bacteriol.* 1978; 134:1141–1156. [PubMed: 149110]
- Cornelis GR, Boland A, Boyd AP, Geuijen C, Iriarte M, Neyt C, et al. The virulence plasmid of *Yersinia*, an antihost genome. *Microbiol Mol Biol Rev.* 1998; 62:1315–1352. [PubMed: 9841674]
- Cornelis GR. The *Yersinia* Ysc-Yop “type III” weaponry. *Nat Rev Mol Cell Biol.* 2002; 3:742–752. [PubMed: 12360191]
- Darwin AJ, Miller VL. Identification of *Yersinia enterocolitica* genes affecting survival in an animal host using signature-tagged transposon mutagenesis. *Mol Microbiol.* 1999; 32:51–62. [PubMed: 10216859]
- Darwin AJ, Miller VL. The *psp* locus of *Yersinia enterocolitica* is required for virulence and for growth *in vitro* when the Ysc type III secretion system is produced. *Mol Microbiol.* 2001; 39:429–444. [PubMed: 11136463]
- Darwin AJ. The phage-shock-protein response. *Mol Microbiol.* 2005; 57:621–628. [PubMed: 16045608]
- de Lorenzo V, Eltis L, Kessler B, Timmis KN. Analysis of *Pseudomonas* gene products using *lacIq*/*Ptp-lac* plasmids and transposons that confer conditional phenotypes. *Gene.* 1993; 123:17–24. [PubMed: 8380783]
- De Rossi E, Branzoni M, Cantoni R, Milano A, Riccardi G, Ciferri O. *mmr*, a *Mycobacterium tuberculosis* gene conferring resistance to small cationic dyes and inhibitors. *J Bacteriol.* 1998; 180:6068–6071. [PubMed: 9811672]
- Dworkin J, Jovanovic G, Model P. The PspA protein of *Escherichia coli* is a negative regulator of sigma(54)-dependent transcription. *J Bacteriol.* 2000; 182:311–319. [PubMed: 10629175]
- Edwards RA, Keller LH, Schifferli DM. Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. *Gene.* 1998; 207:149–157. [PubMed: 9511756]
- Eisenbach M. Changes in membrane potential of *Escherichia coli* in response to temporal gradients of chemicals. *Biochemistry.* 1982; 21:6818–6825. [PubMed: 6760894]
- Elderkin S, Jones S, Schumacher J, Studholme D, Buck M. Mechanism of action of the *Escherichia coli* phage shock protein PspA in repression of the AAA family transcription factor PspF. *J Mol Biol.* 2002; 320:23–37. [PubMed: 12079332]
- Engl C, Jovanovic G, Lloyd LJ, Murray H, Spitaler M, Ying L, et al. *In vivo* localizations of membrane stress controllers PspA and PspG in *Escherichia coli*. *Mol Microbiol.* 2009; 73:382–396. [PubMed: 19555453]
- Engl C, Beek AT, Bekker M, de Mattos JT, Jovanovic G, Buck M. Dissipation of proton motive force is not sufficient to induce the phage shock protein response in *Escherichia coli*. *Curr Microbiol.* 2011; 62:1374–1385. [PubMed: 21259006]
- Foultier B, Troisfontaines P, Muller S, Opperdoes FR, Cornelis GR. Characterization of the *ysa* pathogenicity locus in the chromosome of *Yersinia enterocolitica* and phylogeny analysis of type III secretion systems. *J Mol Evol.* 2002; 55:37–51. [PubMed: 12165841]
- Gage DJ, Neidhardt FC. Adaptation of *Escherichia coli* to the uncoupler of oxidative phosphorylation 2,4-dinitrophenol. *J Bacteriol.* 1993; 175:7105–7108. [PubMed: 8226654]
- Green RC, Darwin AJ. PspG, a new member of the *Yersinia enterocolitica* phage shock protein regulon. *J Bacteriol.* 2004; 186:4910–4920. [PubMed: 15262928]

- Gueguen E, Savitzky DC, Darwin AJ. Analysis of the *Yersinia enterocolitica* PspBC proteins defines functional domains, essential amino acids and new roles within the phage-shock-protein response. *Mol Microbiol.* 2009; 74:619–633. [PubMed: 19775245]
- Guilvout I, Chami M, Engel A, Pugsley AP, Bayan N. Bacterial outer membrane secretin PulD assembles and inserts into the inner membrane in the absence of its pilotin. *EMBO J.* 2006; 25:5241–5249. [PubMed: 17082772]
- Guzman LM, Belin D, Carson MJ, Beckwith J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J Bacteriol.* 1995; 177:4121–4130. [PubMed: 7608087]
- Haller JC, Carlson S, Pederson KJ, Pierson DE. A chromosomally encoded type III secretion pathway in *Yersinia enterocolitica* is important in virulence. *Mol Microbiol.* 2000; 36:1436–1446. [PubMed: 10931293]
- Huvet M, Toni T, Sheng X, Thorne T, Jovanovic G, Engl C, et al. The evolution of the phage shock protein response system: interplay between protein function, genomic organization, and system function. *Mol Biol Evol.* 2011; 28:1141–1155. [PubMed: 21059793]
- Joly N, Engl C, Jovanovic G, Huvet M, Toni T, Sheng X, et al. Managing membrane stress: the phage shock protein (Psp) response, from molecular mechanisms to physiology. *FEMS Microbiol Rev.* 2010; 34:797–827. [PubMed: 20636484]
- Jones SE, Lloyd LJ, Tan KK, Buck M. Secretion defects that activate the phage shock response of *Escherichia coli*. *J Bacteriol.* 2003; 185:6707–6711. [PubMed: 14594846]
- Jovanovic G, Weiner L, Model P. Identification, nucleotide sequence, and characterization of PspF, the transcriptional activator of the *Escherichia coli* stress-induced *psp* operon. *J Bacteriol.* 1996; 178:1936–1945. [PubMed: 8606168]
- Jovanovic G, Lloyd LJ, Stumpf MP, Mayhew AJ, Buck M. Induction and function of the phage shock protein extracytoplasmic stress response in *Escherichia coli*. *J Biol Chem.* 2006; 281:21147–21161. [PubMed: 16709570]
- Jovanovic G, Engl C, Buck M. Physical, functional and conditional interactions between ArcAB and phage shock proteins upon secretin-induced stress in *Escherichia coli*. *Mol Microbiol.* 2009; 74:16–28. [PubMed: 19682256]
- Jovanovic G, Engl C, Mayhew AJ, Burrows PC, Buck M. Properties of the phage-shock-protein (Psp) regulatory complex that govern signal transduction and induction of the Psp response in *Escherichia coli*. *Microbiology.* 2010; 156:2920–2932. [PubMed: 20595257]
- Kinder SA, Badger JL, Bryant GO, Pepe JC, Miller VL. Cloning of the YenI restriction endonuclease and methyltransferase from *Yersinia enterocolitica* serotype O8 and construction of a transformable R^M⁺ mutant. *Gene.* 1993; 136:271–275. [PubMed: 8294016]
- Kinoshita N, Unemoto T, Kobayashi H. Proton motive force is not obligatory for growth of *Escherichia coli*. *J Bacteriol.* 1984; 160:1074–1077. [PubMed: 6389506]
- Kleerebezem M, Tommassen J. Expression of the *pspA* gene stimulates efficient protein export in *Escherichia coli*. *Mol Microbiol.* 1993; 7:947–956. [PubMed: 8387148]
- Kleerebezem M, Crielgaard W, Tommassen J. Involvement of stress protein PspA (phage shock protein A) of *Escherichia coli* in maintenance of the protonmotive force under stress conditions. *EMBO J.* 1996; 15:162–171. [PubMed: 8598199]
- Kobayashi R, Suzuki T, Yoshida M. *Escherichia coli* phage-shock protein A (PspA) binds to membrane phospholipids and repairs proton leakage of the damaged membranes. *Mol Microbiol.* 2007; 66:100–109. [PubMed: 17725563]
- Koch AL. The biophysics of the gram-negative periplasmic space. *Crit Rev Microbiol.* 1998; 24:23–59. [PubMed: 9561823]
- Koo J, Tammam S, Ku SY, Sampaleanu LM, Burrows LL, Howell PL. PilF is an outer membrane lipoprotein required for multimerization and localization of the *Pseudomonas aeruginosa* Type IV pilus secretin. *J Bacteriol.* 2008; 190:6961–6969. [PubMed: 18776008]
- Koo J, Burrows LL, Howell PL. Decoding the roles of pilotins and accessory proteins in secretin escort services. *FEMS Microbiol Lett.* 2012; 328:1–12. [PubMed: 22098485]
- Korotkov KV, Gonen T, Hol WG. Secretins: dynamic channels for protein transport across membranes. *Trends Biochem Sci.* 2011; 36:433–443. [PubMed: 21565514]

- Koster M, Bitter W, de Cock H, Allaoui A, Cornelis GR, Tommassen J. The outer membrane component, YscC, of the Yop secretion machinery of *Yersinia enterocolitica* forms a ring-shaped multimeric complex. *Mol Microbiol.* 1997; 26:789–797. [PubMed: 9427408]
- Lehrer RI, Barton A, Daher KA, Harwig SS, Ganz T, Selsted ME. Interaction of human defensins with *Escherichia coli*. Mechanism of bactericidal activity. *J Clin Invest.* 1989; 84:553–561. [PubMed: 2668334]
- Lloyd LJ, Jones SE, Jovanovic G, Gyaneshwar P, Rolfe MD, Thompson A, et al. Identification of a new member of the phage shock protein response in *Escherichia coli*, the phage shock protein G (PspG). *J Biol Chem.* 2004; 279:55707–55714. [PubMed: 15485810]
- Maloy, SR.; Stewart, VJ.; Taylor, RK. Genetic analysis of pathogenic bacteria: a laboratory manual. Cold Spring Harbor Laboratory Press; Plainview, N.Y.: 1996.
- Matsumoto H, Young GM. Proteomic and functional analysis of the suite of Ysp proteins exported by the Ysa type III secretion system of *Yersinia enterocolitica* Biovar 1B. *Mol Microbiol.* 2006; 59:689–706. [PubMed: 16390460]
- Maxson ME, Darwin AJ. Identification of inducers of the *Yersinia enterocolitica* phage shock protein system and comparison to the regulation of the RpoE and Cpx extracytoplasmic stress responses. *J Bacteriol.* 2004; 186:4199–4208. [PubMed: 15205422]
- Maxson ME, Darwin AJ. Improved system for construction and analysis of single-copy β -galactosidase operon fusions in *Yersinia enterocolitica*. *Appl Environ Microbiol.* 2005; 71:5614–5618. [PubMed: 16151161]
- Maxson ME, Darwin AJ. PspB and PspC of *Yersinia enterocolitica* are dual function proteins: regulators and effectors of the phage-shock-protein response. *Mol Microbiol.* 2006; 59:1610–1623. [PubMed: 16468999]
- Merriam JJ, Mathur R, Maxfield-Boumil R, Isberg RR. Analysis of the *Legionella pneumophila fliI* gene: intracellular growth of a defined mutant defective for flagellum biosynthesis. *Infect Immun.* 1997; 65:2497–2501. [PubMed: 9169800]
- Miller, JH. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, N.Y.: 1972.
- Model P, Jovanovic G, Dworkin J. The *Escherichia coli* phage-shock-protein (*psp*) operon. *Mol Microbiol.* 1997; 24:255–261. [PubMed: 9159513]
- Ohshima T, Mugikura S, Nishikawa M, Igarashi K, Kobayashi H. Osmotic adaptation of *Escherichia coli* with a negligible proton motive force in the presence of carbonyl cyanide *m*-chlorophenylhydrazine. *J Bacteriol.* 1992; 174:2922–2928. [PubMed: 1314804]
- Possot O, d'Enfert C, Reyss I, Pugsley AP. Pullulanase secretion in *Escherichia coli* K-12 requires a cytoplasmic protein and a putative polytopic cytoplasmic membrane protein. *Mol Microbiol.* 1992; 6:95–105. [PubMed: 1738317]
- Reimann SA, Wolfe AJ. Constitutive expression of the maltoporin LamB in the absence of OmpR damages the cell envelope. *J Bacteriol.* 2011; 193:842–853. [PubMed: 21131484]
- Revell PA, Miller VL. *Yersinia* virulence: more than a plasmid. *FEMS Microbiol Lett.* 2001; 205:159–164. [PubMed: 11750796]
- Seo J, Savitzky DC, Ford E, Darwin AJ. Global analysis of tolerance to secretin-induced stress in *Yersinia enterocolitica* suggests that the phage-shock-protein system may be a remarkably self-contained stress response. *Mol Microbiol.* 2007; 65:714–727. [PubMed: 17608794]
- Seo J, Brencic A, Darwin AJ. Analysis of secretin-induced stress in *Pseudomonas aeruginosa* suggests prevention rather than response and identifies a novel protein involved in secretin function. *J Bacteriol.* 2009; 191:898–908. [PubMed: 19028883]
- Shao F. Biochemical functions of *Yersinia* type III effectors. *Curr Opin Microbiol.* 2008; 11:21–29. [PubMed: 18299249]
- Singh S, Darwin AJ. FtsH-dependent degradation of phage shock protein C in *Yersinia enterocolitica* and *Escherichia coli*. *J Bacteriol.* 2011; 193:6436–6442. [PubMed: 21965563]
- Strom MS, Lory S. Cloning and expression of the pilin gene of *Pseudomonas aeruginosa* PAK in *Escherichia coli*. *J Bacteriol.* 1986; 165:367–372. [PubMed: 2867989]
- Wang P, Kuhn A, Dalbey RE. Global change of gene expression and cell physiology in YidC-depleted *Escherichia coli*. *J Bacteriol.* 2010; 192:2193–2209. [PubMed: 20061485]

- Wang RF, Kushner SR. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene*. 1991; 100:195–199. [PubMed: 2055470]
- Weiner L, Model P. Role of an *Escherichia coli* stress-response operon in stationary-phase survival. *Proc Natl Acad Sci U S A*. 1994; 91:2191–2195. [PubMed: 8134371]
- Yamaguchi S, Gueguen E, Horstman NK, Darwin AJ. Membrane association of PspA depends on activation of the phage-shock-protein response in *Yersinia enterocolitica*. *Mol Microbiol*. 2010; 78:429–443. [PubMed: 20979344]
- Yamaguchi S, Darwin AJ. Recent findings about the *Yersinia enterocolitica* phage shock protein response. *J Microbiol*. 2012; 50:1–7. [PubMed: 22367931]

Fig. 1.

Correlation between secretin production and reduced growth yield of a *psp* null strain

A. YsaC-dependent growth inhibition depends on the amount of YsaC produced. *psp*⁺ (AJD3) and Δ *psp* (AJD1171) strains containing pBAD33 (– YsaC) or pAJD935 (+ YsaC) were grown with the indicated arabinose concentrations. Optical density was measured every hour.

B. Anti-His₆ (for YsaC-His₆) immunoblot analysis of cells grown as in panel A, harvested at the 3 h time point. Ponceau S stain of the YsaC region of the nitrocellulose membrane is shown as a loading control.

C. YscC-dependent growth inhibition depends on the amount of YscC produced but is alleviated by the pilot protein YscW. *psp*⁺ (AJD3) and Δ *psp* (AJD1171) strains containing pVLT35 (– YscC), pAJD126 (+ YscC), or pAJD136 (+ YscC YscW) were grown with the indicated amounts of IPTG. Optical density was measured every hour.

D. Anti-YscC immunoblot analysis of cells grown as in panel C, harvested at the 3 h time point. Ponceau S stain of the YscC monomer region of the nitrocellulose membrane is shown as a loading control. Multimers and monomers were detected on the same nitrocellulose membrane but the intervening region, which was blank, has been excised.

Fig. 2.

Evidence that secretins decrease the membrane potential in an *E. coli psp* null strain but non-secretin Psp-inducers do not. *E. coli psp* $^+$ (MC3) and $\Delta(pspF-pspE)$ (AJDE2419) strains contained pVLT35-based vectors encoding *Y. enterocolitica* Psp-inducers. For all panels IPTG was added to cultures at 200 μ M for empty pVLT35 vector (–) and YscC, none for YsaC, 25 μ M for AmpE, and 5 μ M for YE0566.

A. $\Phi(pspA-lacZ)$ operon fusion expression in the psp^+ strain. Cultures were grown as described in Experimental procedures. Error bars represent one positive standard deviation. B. Growth of the psp^+ and $\Delta(pspF-pspE)$ stains. Optical density was determined hourly. C. $[^3H]$ -TPP $^+$ accumulation by whole cells grown as in panel A. Error bars represent one positive standard deviation. CPM = counts per minute.

Fig. 3.

The YscC secretin causes cytoplasmic membrane permeability to ONPG in a *Y. enterocolitica* *psp* null strain.

A. ONPG hydrolysis by whole cells of *psp*⁺ (AJD3) and Δ *psp* (AJD1171) strains containing Φ (*catp-lacZ*) plasmid pAJD2049 as well as *tacp* expression plasmids pAJD126 (YscC), pAJD136 (YscC YscW), pAJD633 (AmpE), or pAJD634 (YE0566). Strains had been grown with the indicated concentrations of IPTG for the YscC and YscC YscW panels, with 25 μ M IPTG for the AmpE panel, or with 10 μ M IPTG for the YE0566 panel. ONPG hydrolysis was monitored by increased absorbance at 420 nm. Readings were normalized so absorbance at 2 min was zero for all samples. Total β -galactosidase activities of cells permeabilized with SDS and chloroform were not significantly different, indicating no differences in β -galactosidase enzyme content (data not shown).

B. $\Phi(pspAp-lacZ)$ operon fusion expression in psp^+ strain AJD977 containing either pVLT35 (-), pAJD126 (YscC), pAJD136 (YscCW), pAJD633 (AmpE), or pAJD634 (YE0566). Cultures were grown as described in Experimental procedures with 200 μ M IPTG (-, YscC and YscCW), 25 μ M IPTG (AmpE) or 10 μ M IPTG (YE0566). Error bars represent one positive standard deviation.

Fig. 4. Transmission electron microscopy of *psp*⁺ (AJD3) and Δ *psp* (AJD1171) cells containing pVLT35 (– YscC) or pAJD126 (+ YscC). Samples were grown for 3 hr with 200 μ M IPTG. Arrows point to areas of apparent cell envelope layer separation that resembles a plasmolysis phenotype. Bars in the lower left hand corner of each panel represent 500 nm.

Fig. 5.

A secretin that does not multimerize has no impact on the Psp system.

A. PilQ production is not toxic in *Y. enterocolitica* *psp*⁺ or *psp* null strains. *psp*⁺ (AJD3) and Δ *psp* (AJD1171) strains containing pVLT35 (– PilQ) or pAJD1806 (+ PilQ) were grown in medium containing 1 mM IPTG. Optical density was measured hourly. B. Anti-His₆ (for PilQ-His₆) immunoblot analysis of cells grown as in panel A and harvested at the 4 h time point. Ponceau S stain of the PilQ monomer region of the nitrocellulose membrane is shown as a loading control. The region corresponding to the top of the original 4-12% polyacrylamide gradient gel is indicated (any secretin multimers would be slightly below the top). C. PilQ does not induce the Psp response. $\Phi(pspA-lacZ)$ operon fusion expression in *psp*⁺ strain AJD977 containing pVLT35 (– PilQ) or pAJD1806 (+ PilQ) grown with 1mM IPTG. Error bars represent one positive standard deviation

Fig. 6.

Characterization of a strain with a *tac* promoter-controlled *pspA* operon.

A. Diagrams of the wild type *pspA* locus (*pspAp-ppspA*) and the derivative with the *pspA* operon expressed from the *tac* promoter (*tacp-ppspA*). In the *tacp-ppspA* strain, the region encompassing *pspF* and the *pspA* promoter was replaced by the *E. coli lacI^q* gene and the *tac* promoter.

B. Growth of *pspAp-ppspA* and *tacp-ppspA* operon strains. A derivative of the *pspAp-ppspA* strain with all *psp* genes deleted was included as a secretin-sensitivity control (Δ *psp*; strain AJD1171). Strains contained pBAD33 ($-YsaC$) or pAJD935 ($+YsaC$) and were grown in the presence of 0.02% arabinose. 10 μ M IPTG was included in all cultures except to one set of the *tacp-ppspA* strains as indicated ($-IPTG$). Optical density was measured hourly.

C. Anti-PspA, -PspB and -PspC immunoblot analysis of samples taken at the 4 h time point from the cultures in panel B. Ponceau S stain of the PspA-PspC region of the nitrocellulose membrane is shown as a loading control.

D. ONPG hydrolysis by derivatives of the *pspAp-ppspA* and *tacp-ppspA* operon strains with a Φ (*catp-lacZ*) operon fusion on the chromosome. Strains contained either pBAD33 ($-YsaC$) or pAJD935 ($+YsaC$). Arabinose was added to all cultures at 0.02% and 10 μ M IPTG was also included where indicated. ONPG hydrolysis was monitored by increased absorbance at 420 nm. Readings were normalized so absorbance at 2 min was zero for all samples. Total β -galactosidase activities of cells permeabilized with SDS and chloroform were not significantly different, indicating no differences in β -galactosidase enzyme content (data not shown).

Fig. 7.

Only PspB and PspC are necessary to prevent secretin-induced toxicity and cytoplasmic membrane permeability to ONPG.

A. Anti-PspA, -PspB, and -PspC immunoblot analysis of *tacp-psiA* strain derivatives with the indicated gene deletions. Samples were taken at the 4 h time point from the + YsaC cultures in panel B. Ponceau S staining of the PspC region of the nitrocellulose membrane is shown as a loading control.

B. Growth of *tacp-psiA* operon strain derivatives with the indicated gene deletions. Strains contained either pBAD33 (- YsaC) or pAJD935 (+ YsaC). All culture media contained 0.02% arabinose. 10 μ M IPTG was also included in the medium as indicated above each graph. Optical density was measured hourly.

C. ONPG hydrolysis by *tacp-psiA* operon strain derivatives with a $\Phi(catp-lacZ)$ operon fusion on the chromosome. Strains contained pBAD33 (- YsaC) or pAJD935 (+ YsaC). All were grown with 0.02% arabinose and 10 μ M IPTG. ONPG hydrolysis was monitored by increased absorbance at 420 nm. Readings were normalized so absorbance at 2 min was zero for all samples. Total β -galactosidase activities of cells permeabilized with SDS and chloroform were not significantly different, indicating no differences in β -galactosidase enzyme content (data not shown).

Table 1

Strains and plasmids

Name	Genotype/Features	Reference or Source
<i>E. coli</i> K-12 strain		
MC3	F ⁻ <i>araD1396</i> (<i>argF-lac</i>) <i>U169 relA1 rpsL150 flbB5301 depC1 ptsF25 rbsR</i> λ [(<i>pspAp-lacZY</i>)]	Bergler et al., 1994
AJDE2419	MC3 Δ (<i>pspF-E</i>):: <i>karF</i>	This study
<i>Y. enterocolitica</i> strains		
AJD3 ^a	Δ <i>yenR</i> (R ⁻ M ⁺) Nal ^r	Laboratory collection
AJD977	Δ <i>yenR</i> (R ⁻ M ⁺) Δ <i>araGFB</i> ::[(<i>pspAp-lacZY</i>)]	Maxson and Darwin, 2005
AJD1171	Δ <i>yenR</i> (R ⁻ M ⁺) Δ (<i>pspF-ycjF</i>) Δ <i>pspG</i>	Maxson and Darwin, 2006
AJD3298	Δ <i>yenR</i> (R ⁻ M ⁺) Δ <i>pspF</i> Δ <i>pspAp</i> ::[<i>lacP-tacp</i>]	Yamaguchi et al., 2010
AJD3463	Δ <i>yenR</i> (R ⁻ M ⁺) Δ <i>pspF</i> Δ <i>pspAp</i> ::[<i>lacP-tacp</i>] Δ <i>pspB</i>	This study
AJD3465	Δ <i>yenR</i> (R ⁻ M ⁺) Δ <i>pspF</i> Δ <i>pspAp</i> ::[<i>lacI^q-tacp</i>] Δ <i>pspC</i>	This study
AJD3469	Δ <i>yenR</i> (R ⁻ M ⁺) Δ <i>pspF</i> Δ <i>pspAp</i> ::[<i>lacP-tacp</i>] Δ <i>pspBC</i>	This study
AJD3494	Δ <i>yenR</i> (R ⁻ M ⁺) Δ <i>pspF</i> Δ <i>pspAp</i> ::[<i>lacI^q-tacp</i>] Δ (<i>pspD-ycjF</i>)	This study
AJD3497	Δ <i>yenR</i> (R ⁻ M ⁺) Δ <i>pspF</i> Δ <i>pspAp</i> ::[<i>lacI^q-tacp</i>] Δ <i>pspA</i>	This study
AJD4250	Δ <i>yenR</i> (R ⁻ M ⁺) Δ <i>araGFB</i> ::[(<i>catp-lacZ</i>)]	This study
AJD4251	Δ <i>yenR</i> (R ⁻ M ⁺) Δ (<i>pspF-ycjF</i>) Δ <i>pspG</i> Δ <i>araGFB</i> ::[(<i>catp-lacZ</i>)]	This study
AJD4505	Δ <i>yenR</i> (R ⁻ M ⁺) Δ <i>pspF</i> Δ <i>pspAp</i> ::[<i>lacI^q-tacp</i>] Δ <i>araGFB</i> ::[(<i>catp-lacZ</i>)]	This study
AJD4507	Δ <i>yenR</i> (R ⁻ M ⁺) Δ <i>pspF</i> Δ <i>pspAp</i> ::[<i>lacI^q-tacp</i>] Δ <i>araGFB</i> ::[(<i>catp-lacZ</i>)] Δ <i>pspB</i>	This study
AJD4508	Δ <i>yenR</i> (R ⁻ M ⁺) Δ <i>pspF</i> Δ <i>pspAp</i> ::[<i>lacI^q-tacp</i>] Δ <i>araGFB</i> ::[(<i>catp-lacZ</i>)] Δ <i>pspC</i>	This study
AJD4510	Δ <i>yenR</i> (R ⁻ M ⁺) Δ <i>pspF</i> Δ <i>pspAp</i> ::[<i>lacI^q-tacp</i>] Δ <i>araGFB</i> ::[(<i>catp-lacZ</i>)] Δ <i>pspBC</i>	This study
AJD4511	Δ <i>yenR</i> (R ⁻ M ⁺) Δ <i>pspF</i> Δ <i>pspAp</i> ::[<i>lacI^q-tacp</i>] Δ <i>araGFB</i> ::[(<i>catp-lacZ</i>)] δ (<i>pspD-ycjF</i>)	This study
AJD4512	Δ <i>yenR</i> (R ⁻ M ⁺) Δ <i>pspF</i> Δ <i>pspAp</i> ::[<i>lacI^q-tacp</i>] Δ <i>araGFB</i> ::[(<i>catp-lacZ</i>)] Δ <i>pspA</i>	This study
Plasmids		
pACYC184	Cm ^r Tet ^r , p15A <i>ori</i>	Chang and Cohen, 1978
pBAD33	Cm ^r , p15A <i>ori</i> , <i>araBp</i> expression vector	Guzman et al., 1995
pRE112	Cm ^r , R6K <i>ori</i> , <i>mob</i> ⁺ (RP4), <i>sacB</i> ⁺	Edwards et al., 1998
pSR47S	Km ^r , R6K <i>ori</i> , <i>mob</i> ⁺ (RP4), <i>sacB</i> ⁺	Merriam et al., 1997
pVLT35	Sm ^r , Sp ^r , RSF1010 <i>ori</i> , <i>tacp</i> expression	vector de Lorenzo et al., 1993
pAJD126	<i>tacp-yscC</i> in pVLT35	Darwin and Miller, 2001
pAJD136	<i>tacp-yscCyscW</i> in pVLT35	Darwin and Miller, 2001
pAJD555	<i>tacp-ysaC</i> in pVLT35	Maxson and Darwin, 2004
pAJD633	<i>tacp-ampE</i> in pVLT35	Maxson and Darwin, 2004
pAJD634	<i>tacp-YE0566</i> in pVLT35	Maxson and Darwin, 2004
pAJD935	<i>araBp-ysaC-his₆</i> in pBAD33	This study
pAJD1806	<i>tacp-pilQ-his₆</i> in pVLT35	This study
pAJD2049	Φ (<i>catp-lacZ</i>) in pACYC184	This study

^a AJD3 is a virulence plasmid cured derivative of strain JB580v (Kinder *et al.*, 1993). All other *Y. enterocolitica* strains listed are derivatives of AJD3.