The flagellar-specific transcription factor, $\sigma^{28}$, is the Type III secretion chaperone for the flagellar-specific anti-$\sigma^{28}$ factor FlgM

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The $\sigma^{28}$ protein is a member of the bacterial $\sigma^{70}$-family of transcription factors that directs RNA polymerase to flagellar late (class 3) promoters. The $\sigma^{28}$ protein is regulated in response to flagellar assembly by the anti-$\sigma^{28}$ factor FlgM. FlgM inhibits $\sigma^{28}$-dependent transcription of genes whose products are needed late in assembly until the flagellar basal motor structure, the hook-basal body (HBB), is constructed. A second function for the $\sigma^{28}$ transcription factor has been discovered: $\sigma^{28}$ facilitates the secretion of FlgM through the HBB, acting as the FlgM Type III secretion chaperone. Transcription-specific mutants in $\sigma^{28}$ were isolated that remained competent for FlgM-facilitated secretion separating the transcription and secretion-facilitation activities of $\sigma^{28}$. Conversely, we also describe the isolation of mutants in $\sigma^{28}$ that are specific for FlgM-facilitated secretion. The data demonstrate that $\sigma^{28}$ is the Type III secretion chaperone for its own anti-sigma factor FlgM. Thus, a novel role for a $\sigma^{70}$-family transcription factor is described.

[Keywords: Flagella; gene regulation; Type III secretion]

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The bacterial flagellum is a self-assembling macromolecular machine anchored in the bacterial membrane that allows bacteria to move through liquid environments or crawl along surfaces (Macnab 1992). Flagellar assembly and function is a complex process, which in Salmonella typhimurium involves over 60 genes (Frye et al. 2006). The construction of individual flagella requires an ordered assembly pathway (Macnab 1992). The assembly process involves the secretion of individual subunits through the hollow core of the growing flagellum where they assemble at the tip of the structure. In addition, multi-flagellated bacteria can have individual flagella in the same cell at different stages of assembly (Bardy et al. 2003). Consequently, the assembly process is tightly regulated at the levels of substrate selection by the associated secretion apparatus and coupled gene regulation. This paper reports an unexpected discovery related to the current understanding of how a cell regulates flagellar gene expression in response to intermedi-
subunits at the right moment to the growing flagellar structures (Aldridge and Hughes 2002).

In *S. typhimurium*, the flagellar genes are organized into a transcriptional hierarchy composed of three promoter classes [class 1, 2, and 3] (Kutsukake et al. 1990). The class 1 promoters drive the expression of the flagellar master operon, which encodes the transcription factors FlhD and FlhC. A FlhDC$_2$ heterodimer directs $\sigma^{70}$, RNA polymerase to initiate transcription from class 2 promoters [Liu and Matsumura 1994]. Genes transcribed from class 2 promoters encode the structural subunits of the hook-basal body (HBB) and a number of regulatory factors that include the flagellar-specific transcription factor $\sigma^{28}$ and its inhibitor, the anti-$\sigma^{28}$ factor FlgM. Class 3 promoters require $\sigma^{28}$-RNA polymerase for transcription [Ohnishi et al. 1992]. Flagellar class 3 operons encode genes whose products are required late in flagellar assembly, including the flagellin filament subunit and genes of the chemosensory system (Chilcott and Hughes 2000).

A major checkpoint in flagellar assembly is the completion of the hook-basal body (HBB) structure. Associated with HBB completion are two crucial regulatory steps that couple flagellar gene expression to flagellar assembly. First, upon HBB completion, the flagellar specific T3S apparatus undergoes a substrate specificity switch from hook-rod secretion substrate specificity to late secretion substrate specificity, including flagellin filament subunits [Minamino et al. 1999; Muramoto et al. 1999]. The anti-$\sigma^{28}$ factor FlgM is also a late secretion substrate, and FlgM secretion is the signal that the HBB is complete [Gillen and Hughes 1991a]. Prior to HBB completion, $\sigma^{28}$ activity is inhibited by direct interaction with FlgM. The physical binding of $\sigma^{28}$ to FlgM prevents $\sigma^{28}$ from interacting with DNA or core RNA polymerase [Daughdrill et al. 1997; Chadsey et al. 1998; Sorenson et al. 2004]. Secretion of FlgM from the cell by completed HBB structures [Hughes et al. 1993] releases $\sigma^{28}$ to transcribe the late flagellar genes, which are needed only after HBB completion. Thus, FlgM secretion is coupled to HBB completion, which releases $\sigma^{28}$ to transcribe the class 3 flagellin subunit genes. However when completed HBB structures are present onto which flagellin must polymerize outside the cell [Karlinsey et al. 2000b].

The relative cellular levels of FlgM and $\sigma^{28}$ are tightly controlled. Both the *flgM* gene and the $\sigma^{28}$ structural gene, *fliA*, are transcribed from class 2 and class 3 flagellar promoters. Upon HBB completion FlgM is secreted and both *flgM* and *fliA* genes are transcribed from their class 3 promoters until a steady state is reached between FlgM and $\sigma^{28}$ that balances expression and FlgM-inhibition of $\sigma^{28}$-dependent class 3 transcription. Upon HBB completion, FlgM secretion will result in a reduction of the intracellular concentration of FlgM. However $\sigma^{28}$-dependent *flgM* transcription has the ability to immediately compensate for this drop by increasing class 3 *flgM* gene expression.

The flagellar-associated T3S apparatus is not restricted to flagellar assembly. Virulence-associated T3S systems facilitate the secretion of virulence factors either into the surrounding environment or directly into host cells [Stebbins and Galan 2003]. The secretion of all T3S substrates is influenced by at least one of three factors. All T3S substrates possess an N-terminal secretion signal that is strictly required for recognition and secretion by any given T3S apparatus [Namba 2001]. Two other factors, Type III secretion-facilitators, referred to as T3S-chaperone proteins, and mRNA signals play a greater role in regulating the timing and location of secretion rather than secretion per se [Karlinsey et al. 2000a; Lee and Galan 2004].

T3S-chaperones are defined as a family of proteins that bind specific T3S substrates and are required for their efficient secretion [Parsot et al. 2003]. Unfortunately, the nomenclature leads to confusion between the T3S-chaperone protein family and with the families of molecular chaperones such as the chaperoin GroEL, whose major role is to assist the folding of proteins [Ellis 2005; Swain and Gierasch 2005]. In contrast, the T3S-chaperones facilitate the secretion of their bound substrates by guiding them to the associated T3S apparatus and even hold these substrates in an unfolded state prior to secretion. Recent evidence suggests that the T3S-chaperones play a crucial role in T3S-pathway specificity in cells possessing both flagellar and virulence-associated T3S systems. Removal of a specific T3S-chaperone from the cell by mutation allows the cognate secretion substrate to be secreted by either flagellar or virulence T3S systems [Lee and Galan 2004; Lilic et al. 2006]. In *S. typhimurium* there are three T3S-chaperones, FlgN, FliT, and FliS, which are only required for efficient secretion of their cognate substrates [Fraser et al. 1999; Auvray et al. 2001; Aldridge et al. 2003]. To date, the only flagellar late secretion substrate without a defined T3S-chaperone is the anti-$\sigma^{28}$ factor FlgM.

In this paper we demonstrate that $\sigma^{28}$ acts as the FlgM T3S-chaperone. Using a genetic screen based on the current knowledge of the $\sigma^{28}$:FlgM interaction, two classes of $\sigma^{28}$ mutant proteins were isolated. One class is defective as a transcription factor but functional as a facilitator of FlgM secretion. The second class is defective in FlgM secretion but still functional as a transcriptional factor. The major class of FlgM secretion-defective $\sigma^{28}$ mutant proteins is altered in the ability to interact with FlgM. The implications these findings have for general substrate secretion by T3S systems are discussed.

**Results**

*Known flagellar T3S-chaperones do not affect FlgM secretion*  

The flagellar system of *Salmonella* includes six late secretion substrates, which are secreted only after HBB completion. These are the hook-filament junction proteins FlgK and FlgL, the filament cap FlhD, the flagellins FlIC and FljB, and the anti-$\sigma^{28}$ factor FlgM [Aldridge and Hughes 2002]. Of these six proteins only two lack a de-
S. typhimurium directly facilitates FlgM secretion or another
increased itself or a fliA levels. The data for fliS structural gene, that is referred to FliC OUT led to a significant reduction in FlgM = TH5737; = TH5937; Hin flgN IN is defined as the amount was null mutant strains was compared are defined as fliC fliS fliT for transcription facilitates IN fliT was the level of protein. A in Fig. 2). For clarity, it may be a candidate T3S-chaperone for FlgM secretion is dependent upon the IN OUT mutants compared with the wild-type strain LT2 (TH437): \( \Delta \text{flgN} = \text{TH5937}; \Delta \text{fliS} = \text{TH5737}; \Delta \text{fliT} = \text{TH5831}; \Delta \text{flis} \Delta \text{fliT} = \text{TH5935}; \Delta \text{flis} \Delta \text{fliT} \Delta \text{flgN} = \text{TH5999}. \) All data, including error bars, are shown relative to LT2 FlgM IN levels. The data shown are an average of three independent repeats of the secretion assays.

Figure 1. Known flagellar T3S-chaperones do not facilitate FlgM secretion. A graph showing the relative amount of intracellular FlgM [FlgM\textsubscript{IN}—unshaded bars] compared with extracellular FlgM levels [FlgM\textsubscript{OUT}—black bars]. Secretion assays were performed as described in Materials and Methods. FlgM\textsubscript{IN} is defined as the level of FlgM present in whole cell lysates of mid-log phase cultures, while FlgM\textsubscript{OUT} is defined as the amount of FlgM detected in the supernatant of the same culture. The secretion assays were performed in a wild-type background (HBB ‘fliC’) using null mutants of all three known T3S-chaperones compared with the wild-type strain LT2 (TH437): \( \Delta \text{flgN} = \text{TH5937}; \Delta \text{fliS} = \text{TH5737}; \Delta \text{fliT} = \text{TH5831}; \Delta \text{flis} \Delta \text{fliT} = \text{TH5935}; \Delta \text{flis} \Delta \text{fliT} \Delta \text{flgN} = \text{TH5999}. \) All data, including error bars, are shown relative to LT2 FlgM\textsubscript{IN} levels. The data shown are an average of three independent repeats of the secretion assays.

FlgM secretion is dependent upon \( \sigma^{28} \)

The fact that \( \sigma^{28} \) interacts with FlgM and that known T3S-chaperones do not promote FlgM secretion suggests that \( \sigma^{28} \) itself may be a candidate T3S-chaperone for FlgM. FlgM secretion was tested in the absence of \( \sigma^{28} \) using a strain deleted for the \( \sigma^{28} \) structural gene, fliA (designated as \( \Delta \sigma^{28} \) in Fig. 2). For clarity, \( \sigma^{28} \) is referred to by its protein name (\( \sigma^{28} \)) rather than by its gene name (\( \text{fliA} \)). In contrast to null mutations in flgN, fliT, and fliS, the absence of \( \sigma^{28} \) led to a significant reduction in FlgM secretion (Fig. 2). Compared with wild type, levels of FlgM outside the cell [FlgM\textsubscript{OUT}] in the absence of \( \sigma^{28} \) (\( \Delta \sigma^{28} \)) were down 117-fold, while FlgM\textsubscript{IN} increased threefold. These data suggest that either \( \sigma^{28} \) itself or a protein dependent on \( \sigma^{28} \) for transcription facilitates FlgM secretion.

To distinguish between the two possibilities that either \( \sigma^{28} \) directly facilitates FlgM secretion or another protein dependent on \( \sigma^{28} \) for its production, \( \sigma^{28} \) derivatives that were unable to bind FlgM, but still able to bind RNA polymerase and direct flagellar class 3 promoter transcription, were tested for the ability to facilitate FlgM secretion. Previously, single amino acid substitutions in \( \sigma^{28} \) were isolated that bypass the inhibitory effect of FlgM (Chilcott and Hughes 1998; Chadsey and Hughes 2001). A bypass mutant works in the presence of an inhibitor or activator to give the same phenotype as loss of that inhibitor or activator. A mutation in \( \sigma^{28} \) that allows \( \sigma^{28} \)-dependent flagellar class 3 transcription in the presence of inhibitory concentrations of FlgM (such as in HBB mutants unable to secrete FlgM) is a FlgM-bypass mutation. Such mutations in \( \sigma^{28} \) are defined as \( \sigma^{28} \)- (FlgM-bypass) mutant proteins (Chadsey and

Figure 2. FlgM secretion is dependent upon the \( \sigma^{28} \) protein. A graph showing the FlgM secretion profiles for different \( \sigma^{28} \) mutant alleles compared with \( \sigma^{28} \). All secretion assays were performed in a strain that had a HBB ‘FliC·Hin’ phenotype (\( \sigma^{28} \)) where ‘FliC expression is “ON”’ (Bonifield and Hughes 2003). The data shown are an average of three independent repeats and are represented as relative to FlgM\textsubscript{IN} levels for \( \sigma^{28} \). Strains used are highlighted in Table 3 with a full description of each genotype.

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Hughes 2001). Two classes of $\sigma^{28+}$ proteins were obtained. One class is defective in FlgM binding, while the second class of $\sigma^{28+}$ mutant proteins was more stable to proteolysis. Both $\sigma^{28+}$ mutant types result in an excess of free $\sigma^{28}$ in the cell and accompanying $\sigma^{28}$-dependent transcription under FlgM inhibitory conditions. If $\sigma^{28}$ directly facilitates FlgM secretion, then a $\sigma^{28+}$ protein defective in FlgM binding [such as the $\sigma^{28+}$[V213E] mutant protein] would be defective in FlgM secretion, while the more stable $\sigma^{28+}$ protein that binds FlgM similar to wild-type $\sigma^{28+}$ [such as $\sigma^{28+}$[H14D]] would facilitate FlgM secretion. If another protein dependent on $\sigma^{28}$ for its production facilitates FlgM secretion, then FlgM secretion should occur at high levels in strains with either $\sigma^{28+}$ mutant type [FlgM-binding defective $\sigma^{28}$ or more stable $\sigma^{28}$ protein] expressed. The $\sigma^{28+}$[V213E] mutant protein is 70-fold reduced in its ability to bind FlgM, while the $\sigma^{28+}$[H14D] mutant binds FlgM with wild-type efficiency but is a more stable $\sigma^{28}$ protein (Chadsey and Hughes 2001). The secretion of FlgM in the presence of $\sigma^{28+}$[V213E] and $\sigma^{28+}$[H14D] was compared with secretion in the presence of wild-type $\sigma^{28}$ [Fig. 2]. For $\sigma^{28+}$[H14D], no significant change in the secretion profile of FlgM was detected apart from a fourfold increase in FlgM$_{\text{OUT}}$ presumably due to increased class 3 $flgM$ gene transcription by $\sigma^{28+}$[H14D]-RNA polymerase (Chadsey and Hughes 2001).

In contrast to FlgM secretion in the presence of $\sigma^{28+}$[H14D], a change in FlgM secretion was observed for $\sigma^{28+}$[V213E], which is defective in binding FlgM. No significant change to FlgM$_{\text{OUT}}$ was observed for $\sigma^{28+}$[V213E], whereas FlgM$_{\text{IN}}$ increased 19-fold presumably resulting from increased class 3 $flgM$ gene transcription by $\sigma^{28+}$[V213E]-RNA polymerase (Chadsey and Hughes 2001). These data suggest that, even though FlgM is secreted in the presence of $\sigma^{28+}$[V213E], secretion is significantly reduced and some of the secretion defect is compensated by a large increase of FlgM$_{\text{IN}}$. This is consistent with previous findings that the lack of the FlgN T3S-chaperone can be overcome by the overexpression of its cognate secretion substrates [FlgK and FlgL] (Aldridge et al. 2003). Thus FlgM secretion is dependent on its ability to bind to $\sigma^{28}$ and not on $\sigma^{28}$-dependent transcription of an unknown FlgM T3S-chaperone gene.

**FlgM and $\sigma^{28}$ stability are interdependent**

Many T3S substrates are relatively unstable prior to secretion in the absence of their cognate T3S-chaperone (Bennett and Hughes 2000; Aldridge et al. 2003). To investigate whether protein stability of FlgM and $\sigma^{28}$ has a role in FlgM secretion, the stabilities of both proteins were tested in wild-type, $\Delta flgM$, and $\Delta \sigma^{28}$ strains [Fig. 3; see Supplemental Material for protein half-life calculations]. $\sigma^{28}$ was slightly more stable in the presence of FlgM; its half-life increased from 24 min when FlgM was absent to 30 min. Significantly, after 60 min ~5% of $\sigma^{28}$ is detected in the $\Delta flgM$ strain whereas ~40% of the detectable $\sigma^{28}$ protein is present in the $flgM^+$ strain. In contrast, FlgM was more stable in the absence of $\sigma^{28}$ increasing from 18 min in the wild-type strain to 32 min in the $\Delta \sigma^{28}$ strain. However, these strains are competent for FlgM secretion [HBB+ backgrounds], and therefore FlgM turnover also includes FlgM secretion for $\sigma^{28}$ strains as only whole cell lysates were assayed. Recently FlgM was demonstrated to be a stable protein (Aldridge et al. 2006). This suggests that the interaction of FlgM with $\sigma^{28}$ facilitates its secretion and not its degradation. Thus, for FlgM, the changes observed are a reflection of FlgM accumulation in cells when $\sigma^{28}$ is absent, consistent with our hypothesis that $\sigma^{28}$ facilitates its secretion.

**Separation of $\sigma^{28}$-dependent FlgM secretion from $\sigma^{28}$ transcription**

We wanted to determine if the transcription activity of $\sigma^{28}$ could be genetically separated from its FlgM secretion-facilitator activity. The first step was the isolation of mutants in $\sigma^{28}$ specific to its flagellar class 3 transcription activity, defined as $\sigma^{28-}$. To obtain $\sigma^{28-}$ mutants, a deletion of the $\sigma^{28}$ structural gene, fliA ($\Delta fliA5805::tetRA$), was replaced with PCR mutagenized fliA-containing DNA fragments using the $\lambda$-RED recombination system (see Materials and Methods; Karlinsky and Hughes 2006). The $\Delta fliA5805::tetRA$ allele is a deletion of the entire fliA gene replaced with a tetracycline-resistance element, tetRA, of transposon Tn10. A positive selection for loss of tetracycline resistance allows for the direct selection of recombinants in which the $\Delta fliA5805::tetRA$
allele was replaced with the mutagenized DNA that includes fliA [Maloy and Nunn 1981]. Tetracycline-sensitive (Tc<sup>+</sup>) recombinants were screened, in the absence of flgM, for those defective in transcription of the σ<sup>28</sup>-dependent [flagellar class 3] motA gene using a lac operon transcriptional fusion vector in motA [motA5461::MudJ]. From 697 Tc<sup>+</sup> transformants, two Lac<sup>−</sup> isolates that had wild-type levels of σ<sup>28</sup> protein were isolated. Subsequent DNA sequence analysis revealed that both were double mutants: σ<sup>28</sup>−[R91C L207P] and σ<sup>28</sup>−[Y190C S209L]. The σ<sup>28</sup>−[R91C L207P] mutant had an amino acid substitution located in each of the −10 and −35 promoter recognition regions of σ<sup>28</sup> while σ<sup>28</sup>−[Y190C S209L] had two amino acid substitutions located in the −35 promoter recognition region (Fig. 4). Interestingly no single amino acid changes were isolated, suggesting that complete loss of transcriptional activity required more than one change in σ<sup>28</sup>. Furthermore, because the screen demanded that the transcription-defective σ<sup>28</sup> mutants had a wild-type protein stability, it is likely that all transcription-defective mutants resulting from defects in interactions with RNA polymerase were unstable since none was isolated.

The transcription-defective σ<sup>28</sup> proteins were found to be competent at facilitating FlgM secretion. FlgM secretion assays using σ<sup>28</sup>−[R91C L207P] when compared with wild type and Δσ<sup>28</sup> showed that, even though FlgM<sub>INS</sub> was significantly reduced [presumably due to loss of flgM gene transcription from its σ<sup>28</sup>-dependent class 3 promoter], σ<sup>28</sup>−[R91C L207P] did facilitate FlgM secretion (Fig. 2). Thus, we were able to separate the transcription and FlgM T3S-chaperone activities of σ<sup>28</sup> by mutation. Therefore, the transcriptional function of σ<sup>28</sup> is not essential for σ<sup>28</sup> to facilitate FlgM secretion.

Genetic screen rationale for the isolation of FlgM secretion-defective mutants in σ<sup>28</sup>  

The ability to isolate transcription-specific mutants in σ<sup>28</sup> demonstrated that its two functions could be separated. This suggested that a region of σ<sup>28</sup> might be specific to the facilitation of FlgM secretion [T3S-chaperone activity]. It is known that a twofold increase in the intracellular FlgM concentration results in a 100-fold reduction of σ<sup>28</sup>-dependent class 3 transcription due to the stoichiometric nature of the interaction between σ<sup>28</sup> and FlgM [Karlinsey et al. 2000a]. The above data predict that when σ<sup>28</sup> is unable to facilitate FlgM secretion, then FlgM would accumulate in the cell [Figs. 2, 3] and result in reduced σ<sup>28</sup>-dependent class 3 promoter activity. Unfortunately, this phenotype [decreased class 3 transcription] is the same as σ<sup>28</sup> mutants that are defective in transcription. Therefore, we needed to develop a genetic screen that we could use to separate transcription mutants from FlgM-secretion mutants.

Mutants in σ<sup>28</sup> defective in FlgM secretion [defined here as σ<sup>28</sup>−] are expected to show reduced class 3 transcription only in the presence of FlgM while transcription-specific mutants should exhibit reduced class 3 transcription in the presence and absence of FlgM. This required a method that allowed for the isolation of σ<sup>28</sup> mutants exhibiting lower class 3 promoter activity in the presence of wild-type FlgM<sub>INS</sub> levels followed by a screen for the restoration of class 3 transcription in the absence of FlgM. For this screen, a strain background was constructed where flgM gene expression was independent of σ<sup>28</sup> transcription. The flgM<sup>−</sup> coding region was placed under the control of the arabinose promoter P<sub>araBAD</sub> at the araBAD locus [see Materials and Methods; Supplemental Material]. This allowed for the induction of flgM<sup>−</sup> expression independent of flagellar assembly by the addition of arabinose to the growth medium. In this construct, expression of flgM<sup>−</sup> from the arabinose promoter resulted in too much intracellular FlgM. Compared with wild-type expression of flgM<sup>+</sup> from its normal chromosomal location, expression of P<sub>araBAD</sub>−flgM<sup>−</sup> resulted in reduced class 3 promoter expression by 40% (Table 1). Unfortunately, this was already the phenotype expected for FlgM-secretion σ<sup>28</sup> mutants.

### Table 1. Expression of FlgM from the araBAD locus leads to decreased σ<sup>28</sup> activity

| Relevant genotype<sup>a</sup> | Class 3 promoter activity (motA5461::MudJ)<sup>b</sup> |  |
|-------------------------------|---------------------------------|---|
|                               | No FlgM<sup>c</sup> | With FlgM<sup>c</sup> |
| flgM<sup>+</sup>               | NA                        | 115 ± 10 |
| P<sub>araBAD</sub>−flgM<sup>+</sup> | 273 ± 32    | 70 ± 5    |
| P<sub>araBAD</sub>−flgM<sup>−</sup> | 270 ± 27    | 99 ± 15   |

<sup>a</sup>Strains used included flgM<sup>−</sup>: motA5461::MudJ [TH9393]; P<sub>araBAD</sub>−flgM<sup>−</sup>: ΔflgM5628::FRT motA5461::MudJ araBAD923::flgM-FK [TH7250]; P<sub>araBAD</sub>−flgM<sup>−</sup>: ΔflgM5628::FRT motA5461::MudJ araBAD923::flgM-FK ParaB934 [TH7394].

<sup>b</sup>Class 3 promoter activity is represented by β-galactosidase activity of the motA-lac operon fusion created by the motA5461::MudJ insertion.

<sup>c</sup>For P<sub>araBAD</sub>−flgM<sup>−</sup> and P<sub>araBAD</sub>−flgM<sup>−</sup>; No FlgM = no arabinose added to the growth media and With FlgM = arabinose added.
In order to express the flgM* gene from the P_{araBAD} promoter to levels comparable with what is expressed from its native promoters, the P_{araBAD} promoter was mutagenized and screened for FlgM anti-σ^{28} activity using the motA5461::Mu{\textregistered} as a reporter of class 3 promoter activity [see Materials and Methods]. Four P_{araBAD} promoter mutants were obtained that exhibited a Lac-phenotype from the motA5461::Mu{\textregistered} reporter similar to what we observed in a wild-type flgM strain. These were further characterized by DNA sequence analysis [see Supplemental Material]. One mutant, P_{araBAD}934-flgM*, resulted from a TA to CT double change between the –35 and –10 boxes and exhibited class 3 transcription activity that was comparable with wild type and was subsequently used in all further experiments.

Isolating σ^{28} mutants defective in FlgM-secretion

Using the P_{araBAD}934-flgM* expression construct, σ^{28}-dependent class 3 transcription in the presence of FlgM [with arabinose inducer present] again by recombinational replacement of the ΔflaA5805::tetRA allele with PCR-mutagenized flIA-containing DNA [Karlinsey and Hughes 2006]. These mutants were then screened for normal σ^{28}-dependent class 3 transcription in the absence of FlgM [no arabinose inducer present] [see Materials and Methods]. From a total of 3050 Tc^{28} recombinants, 96 (3.1%) putative σ^{28} FlgM secretion-defective (σ^{28}*) mutants were isolated. DNA sequence analysis identified 24 mutants that resulted from single amino acid substitutions, eight had two amino acid changes, and seven resulted from greater than two amino acid substitutions. The rest possessed either truncation mutations or mutations outside the coding sequence including promoter region mutations. The amino acid changes did not cluster to a single region of σ^{28} but were located throughout the protein (Table 2). Twenty-one mutants were assayed for β-galactosidase activity [Table 2]. The majority of the putative σ^{28}* mutants had reduced class 3 promoter activity in the presence of FlgM. However, many exhibited a reciprocal reduction in class 3 promoter activity in the absence of FlgM [false-positive mutants] and were discarded. Five mutants were found to have the predicted phenotype, i.e., wild-type activity in the absence of FlgM and reduced activity in the presence of FlgM. One of these mutations, V33E, had been previously identified as a σ^{28}* mutant that was defective in binding to FlgM [Chadsey and Hughes 2001]. The σ^{28}*-proteins allow class 3 transcription in a HBB strain that does not secrete FlgM.

The four other mutants were assayed for σ^{28}* activity by measuring class 3 promoter activity using a different class 3 reporter gene construct [flIC5050::Mu{\textregistered}] in a HBB flgM* strain. β-Galactosidase assays showed that all four mutants resulted in a three- to fourfold increase in class 3 transcription in the HBB background, which was comparable to that seen for σ^{28}* [V33E] [Fig. 5]. In comparison, the σ^{28}*[V213E] mutant, which is 70-fold reduced in its ability to bind FlgM, and σ^{28}*[H14D] mutant, which exhibited increased stability, possessed a 148-fold or 36-fold increase in class 3 promoter activity, respectively. The fourth mutant [G77W L135S] showed a much stronger σ^{28}* phenotype (12.5-fold increase in class 3 transcription in the HBB background) and was not characterized further. This suggests that the three new mutants could be classed as σ^{28}* mutants that had defects in their ability to bind FlgM. This assumption is based on the fact that all four mutants, V33E, S226R, E193V, and [E209G, V221A], are still responsive to FlgM inhibition [cf. HBB with HBB* in Fig. 5] but still exhibit some σ^{28}* activity, whereas σ^{28}*[V213E] was completely insensitive to FlgM inhibition.

The σ^{28}* mutants isolated in a σ^{28}+ screen are defective for FlgM secretion

In the above screen for FlgM secretion-defective mutants in σ^{28} (σ^{28}+), the mutants isolated appeared to have a slight defect in binding to FlgM (weak σ^{28}+). However,
levels did not result from mutants were compared with transcriptional activity. FlgM se-
mutants inactive for tran-
scriptional fusion protein, are
and HBB (white bars) or HBB strain suggested a defect in Putative strain. Even though all mutants isolated possessed strains. In contrast, significant increases in gene expression. As all four transcriptional activity for HBB strains exhibited lower transcriptional activity for HBB‘ strains compared with HBB‘ α28*, they all showed a weak α28* phenotype in the HBB‘ strains. In contrast, significant increases in fliC-lacZ transcription for both HBB‘ and HBB‘ strains were observed for the α28*[H14D] and α28*[V213E] mutants. A complete description of the genotypes of all HBB‘ strains used in this analysis is to be found in Table 3. HBB‘ strains were con-
structed by transduction of the flgG574::Tn10 allele.

all exhibited a significant reduction in class 3 transcription in a strain competent for FlgM secretion [HBB‘], consistent with a α28*- phenotype [Table 2; Fig. 5]. In contrast, α28* [V213E] and α28*[H14D] mutants exhibited a significant increase in class 3 promoter activity in the HBB‘ strain. Even though all mutants isolated possessed weak α28* activity, the strong inhibition of class 3 promoter activity for the HBB‘ strain suggested a defect in FlgM secretion. Thus, there appears to be an overlap be-
tween the α28* [FlgM-binding] and α28* (FlgM-secretion) activities. Furthermore, the class 3 promoter activity in the absence of FlgM for the four mutants was compar-
ible with wild type [Table 2], indicating that the lower class 3 transcription in the presence of FlgM did not re-
sult from reduced α28* transcriptional activity. FlgM secretion assays were therefore performed for the four mu-
tants possessing overlapping α28* and α28* phenotypes [V33E, S226R, E193V, and [E209G, V221A]] [Fig. 2]. Compared with α28*, all four mutants exhibited a strong reduction in FlgM secretion [FlgMOUT]. Significantly, intracellular FlgM levels [FlgMINS] were increased com-
pared with wild type [V33E: 1.2-fold; S226R: 2.7-fold; E193V: 1.3-fold; and [E209G, V221A]: 1.4-fold], indicating that the reduced FlgMOUT levels did not result from reduced FlgM gene expression. As all four α28* mutants isolated during this study possessed a minor α28* phen-
totype, the data are consistent with an overlap in the FlgM binding and FlgM secretion-facilitator activities of α28*.

Discussion

Efficient flagellar assembly requires tight control of protein subunit production, secretion, and self-assembly. Bacteria that possess multiple flagella per cell might also require mechanisms to allow the different flagella within a single cell to develop independently. A major checkpoint for flagellar assembly in all bacteria is the completion of the intermediate structure known as the hook-basal body (HBB). In S. typhimurium, HBB completion coincides with FlgM secretion and the initiation of α28*-dependent flagellar class 3 transcription of genes that encode late flagellar subunits and the chemosensory machinry. Prior to secretion, FlgM binds and inhibits the activity of α28*. The interaction between FlgM and α28* allows the cell to couple flagellar gene expression to the assembly process.

Prior to HBB completion, the flagellar T3S system is specific for hook and rod-type secretion substrates. Upon HBB completion, the specificity of the T3S apparatus is switched to late secretionstrate recognition. Five out of the six late secretion substrates have been shown to require a T3S-chaperone for efficient secretion coupled to assembly. The only flagellar late secretion substrate without a defined chaperone was FlgM. Evidence from the T3S field suggested that all substrates secreted after the substrate-specificity switch required a T3S-chaper-
one for efficient secretion. Based on this evidence, the identity of the T3S-chaperone for FlgM secretion was investigated. Surprisingly, α28* itself facilitates FlgM se-
cretion defining a novel role for a σ70-family transcription factor as a T3S-chaperone. The following results demonstrate that FlgM secretion is facilitated by α28*, but is independent of α28* transcriptional activity; (1) A strain deleted for the α28* gene (complete null mutant) is defec-
tive for FlgM secretion; (2) a α28* mutant that is defec-
tive in binding FlgM α28* [V213E] is defective in FlgM secretion; (3) a α28* mutant with increased stability that shows normal binding to FlgM α28*[H14D]] is proficient in FlgM secretion; and (4) α28* mutants inactive for tran-
scription activity, but still produce the α28* protein, are proficient for FlgM secretion. Thus α28* is defined as the FlgM T3S-chaperone based on the strong evidence ob-
tained from the analysis presented here.

The conclusions drawn from these data have come from experiments performed using a system in which extra care has been taken not to deviate from a physi-
ologically relevant condition. Only the genetic screen employed α28*-independent flgM expression. However, all subsequent analysis of FlgM protein levels, α28* activity, and FlgM-secretion for α28* mutants with the re-
quired phenotype were performed using a wild-type background with α28*-dependent flgM expression.

The conclusions drawn here are also in agreement with the physical characteristics that define a T3S-chap-
erone. The majority of T3S-chaperones identified to date have a low molecular size and a low pI, usually in the

Figure 5. Putative α28* mutants exhibit weak α28* activity. β-Galactosidase activity of a fliC-lacZ transcriptional fusion (fliC5050::MuΔ] in various flagellar backgrounds. The activities of α28* mutants were compared with α28*, flgM*, α28*[H14D] [increased-stability α28* with wild-type FlgM-binding], and α28*[V213E] [severe FlgM binding defective α28*] in strains with either a HBB‘ [white bars] or HBB‘ [black bars] phenotype. Surprisingly, even though α28*[V33E], α28*[G77W L135S], α28*[E193V], α28*[S226R], and α28*[E209G, V221A] all exhibited lower α28* transcriptional activity for HBB‘ strains compared with HBB‘ α28*, the interaction between FlgM and α28* allows the cell to couple flagellar gene expression to the assembly process.
range of pI = 3.9–5.3 (Parsot et al. 2003; $\sigma^{28}$ has a theoretical pI of 5.12 and a molecular weight of 28 kDa. The accepted flagellar T3S-chaperones FlgN, FliT, and FlIS all have a molecular weight of 14 kDa and pIs that also fall in the range defined by Parsot et al. (2003). One difference is that the known flagellar T3S-chaperones act as dimers whereas $\sigma^{28}$ acts as a monomer.

Recent studies of a growing number of T3S-chaperones associated with both flagellar and virulence T3S systems have identified a subset of T3S-chaperones to possess multiple functions: facilitation of T3S substrate recognition, secretion, and regulation of gene expression. Some examples include SicA (SPI-1) (Darwin and Miller 2001), FlgN [Flagella] (Karlinsey et al. 2000a), and FliT [Flagella] (Kutsukake et al. 1999) from S. typhimurium, Spa15 and IpGC from Shigella flexneri (Parsot et al. 2005), and SycD and SycH from Yersinia spp (Feldman and Cornelis 2003). Apart from FlgN and FliT whose regulatory mechanisms are still poorly understood the majority of T3S-chaperones to be studied regulate gene expression via secondary interactions with transcriptional activators. By comparison, $\sigma^{28}$ being itself a transcriptional activator, secretion of its partner FlgM has the immediate effect of freeing $\sigma^{28}$ to interact with core RNA polymerase and activate $\sigma^{28}$-dependent flagellar gene transcription. With a growing number of T3S-chaperones possessing a gene regulatory function, the question arises to whether all or the majority of the protein family are bi-functional.

The bi-functionality of T3S-chaperones allows for gene regulation to be coupled to secretion of the bound substrate. Thus, the dual nature of T3S-chaperones provides a molecular device that coordinates gene regulation in a checkpoint manner, whether the checkpoint is in structural assembly or in pathogenesis. The main objective of the interaction between $\sigma^{28}$ and FlgM is to couple flagellar gene expression to flagellar assembly. Therefore, it is not surprising that the $\sigma^{28}$:FlgM complex has evolved into a T3S-chaperone:substrate partnership in order to “sense” HBB completion.

How does the flagellar secretion apparatus dissociate FlgM from $\sigma^{28}$?

The fact that the $\sigma^{28}$-mutant isolates in this study as defective in FlgM secretion possess at least some $\sigma^{28}$-activity (FlgM-binding defective) indicates that, even though $\sigma^{28}$-mutants still secreted FlgM in a $\sigma^{28}$-dependent manner, the two functions of $\sigma^{28}$ have overlap within the $\sigma^{28}$ protein. This is not surprising since $\sigma^{28}$ must bind FlgM in order to facilitate its secretion, and conversely FlgM secretion must be concomitant with its release from $\sigma^{28}$. Given the remarkable strength of the $\sigma^{28}$:FlgM interaction (Kd = 0.2 nM) (Chadsey and Hughes 2001), the dissociation of the $\sigma^{28}$:FlgM complex by the flagellar T3S system must occur by a mechanism that is able to unravel this tight complex. FlgM is a very small protein of 97 amino acids. A cocrystal of the Aquifex aeolicus $\sigma^{28}$:FlgM complex demonstrates that FlgM essentially wraps around $\sigma^{28}$ (Sorenson et al. 2004).

While FlgM binds $\sigma^{28}$ at multiple sites throughout its three structural regions [conserved regions 2, 3, and 4] mutants of $\sigma^{28}$ most defective in binding FlgM are all in region 4 (Fig. 4; Chadsey and Hughes 2001). Recently, it was shown that the T3S-chaperone, FlgN, associates with FliI in the presence of its substrate, FlgK (Thomas et al. 2004). The exact nature of the interaction with FliI has not yet been determined. In the case of the $\sigma^{28}$:FlgM complex, we propose that the binding of $\sigma^{28}$ to FlgM exposes the N-terminal secretion domain of FlgM and facilitates interaction of the $\sigma^{28}$:FlgM complex to the flagellar T3S apparatus. If the flagellar T3S apparatus is competent for FlgM secretion (HBB $^+$), the action of FlgM secretion would effectively peel FlgM from $\sigma^{28}$, resulting in secreted FlgM and $\sigma^{28}$ that is free to interact with RNA polymerase and transcribe flagellar class 3 promoters. Our model is consistent with the disassociation of SptP from the SicP:SptP complex by the ATPase InvC of the SPI-1 virulence-associated T3S in Salmonella (Akeda and Galan 2005). From our understanding of the $\sigma^{28}$/FlgM interaction and our genetic data we conclude that the structural conformation of the T3S-chaperone:substrate complex is crucial for efficient substrate recognition by the ATPase. With the $\sigma^{28}$ mutants used during this study we are now in a position to determine if and how the $\sigma^{28}$:FlgM complex interacts with the T3S apparatus.

The evolution of the multifunctional T3S-chaperone and would T3S-facilitator be a better name?

It appears that T3S-chaperones can play multiple roles as general facilitators of secretion either through protein stabilization or targeted delivery (or both) and as regulators of gene expression, but in a manner such that the regulatory function coincides with the timing of secretion for a given substrate. Such a mechanism would determine which T3S-chaperones evolve to bind a given substrate and for T3S-chaperones with regulatory functions. The cognate secretion substrate would be selected according to when it is secreted in the assembly process or in the virulence pathway so that the regulatory function can be appropriately timed with the secretion and assembly of a specific protein.

Finally we would like to highlight once more the confusion that can occur with the current nomenclature of proteins deemed to have “chaperone” functions. T3S-chaperones can be confused with other molecular chaperones most of which are involved in folding or degrading proteins that never leave the cell. Even though proteins that guide protein folding, like GroEL, are occasionally now referred to as chaperonins (Ellis 2005; Swain and Gierasch 2005), the term “chaperone” is being used, even if correctly, too often. One solution would be to rename the T3S-chaperones as T3S-facilitators. Data is available suggesting that this family of accessory proteins is in fact facilitating the secretion of cognate substrates. Furthermore, many, if not all, T3S-facilitator substrates can be secreted via a T3S secretion system in the absence of their T3S-facilitator (Aldridge et al. 2003;
Lee and Galan 2004; Lilic et al. 2006). However, if current terminology is to be kept, then we stress that whenever mentioning chaperones associated with T3S systems they should be referred to as “T3S-chaperones” always prefixed with T3S.

### Materials and methods

**Bacterial strains, plasmids, and growth conditions**

Bacterial strains and plasmids used in this study are shown in [Table 3. Cultivation of bacteria and the concentration of antibiotics used throughout this study have been previously described (Bonfield and Hughes 2003). The generalized transducing phage of *S. typhimurium* P22 HT105/1 int-201 was used in all transductional crosses (Davis et al. 1980). Media used during this study included Luria Bertin (LB) broth, MacConkey-lactose agar (Difco), and Triphenyltetrazolium chloride (TTC) lactose agar (Gillen and Hughes 1991a,b). Tetracycline sensitivity (Tc<sup>+</sup>) selection was performed as described (Maloy 1990). All experiments involving the growth of strains containing the plasmid pKD46 were performed at 30°C. Loss of pKD46 from given strains was achieved simply by growth at 37°C after electroporation of PCR products. All FlgM secretion assays and β-galactosidase assays were performed on cultures grown in LB at 37°C with shaking to an *OD<sub>600</sub> = 0.6-0.8.

**Recombinant DNA techniques**

Standard molecular DNA manipulation techniques have been previously described (Aldridge and Jenal 1999). The plasmid pJOY was constructed as follows. The FRT-kan-FRT (FKF) cassette from pKD4 was amplified using primers BamHPI (5'-CGCGGTACCGTGACGAGCTGCTTC-3') and HindIII (5'-GGGAACTTTATATGATATATCCCTCC TTAG-3') digested with BamHI and HindIII and cloned into pUC19 creating plasmid pPA122. The flgM coding sequence was amplified with primers FlgA5Fkp (5'-CGCGGTACCC AGCCTTACGATGACCG-3') and FlgMspkp (5'-CCG TACCTATTTTACTCTGTAAGTAGC-3') and subcloned into pPA122 upstream of the FKF cassette by restriction digestion with KpnI, resulting in pJOY.

### Obtaining arabinose-controlled FlgM expression at the araBAD locus

To achieve arabinose-inducible expression of *flgM*, the primer ARAFLG (5'-ACTGTTTCTTCATACCTGTTTTTCTGAGT GAAGTACAGGATGAGCATGCTACCTC-3') was used with AraGTR (5'-TTATGACTCATCCTCGCATATTT TACCCAGCGAATCGACCATTGTAACCGCAAGC-3') to amplify the *flgM*-FKF insert of pJOY. The primers possessed 5' 40-nucleotide overhangs complementary to the start codon of *araB* [ARAFLG] and codons 174-162 of *araD* for AraGTR.

### Table 3. Strains and plasmids used

| Strain number | Genotype<sup>a</sup> | Reference or source |
|---------------|----------------------|---------------------|
| TH437         | LT2 wild-type (WT)<sup>b</sup> | John Roth          |
| TH5737        | ΔfliS720::Frt-Kan-Frt (ΔfliS)<sup>b</sup> |                  |
| TH5831        | ΔfliT758::Frt-Cm-Frt (ΔfliT)<sup>b</sup> |                  |
| TH5935        | ΔfliST774::Frt-Kan-Frt (ΔfliST)<sup>b</sup> |                  |
| TH5937        | flgM5220::MudCm (ΔflgN)<sup>b</sup> |                  |
| TH5999        | flgM5220::MudCm ΔfliST774::Frt-Kan-Frt (ΔflgN ΔfliS ΔfliT)<sup>b</sup> |                  |
| TH7032        | ΔflgM5628::FRT motA5461::MudJ ΔaraBAD923::UTR-flgM-FKF flgI5886 |                  |
| TH7250        | ΔflgM5628::FRT motA5461::MudJ ΔaraBAD923::flgM-FKF |                  |
| TH7253        | ΔflgM5628::FRT motA5461::MudJ ΔaraBAD923::flgM-FKF ΔaraBAD923::tetRA |                  |
| TH7470        | ΔflgM5628::FRT motA5461::MudJ ΔaraBAD923::flgM-FKF ΔflgM5805::tetRA |                  |
| TPA368        | Δhin-5717::FRT [Δc5050::MudJ] [Δ28<sup>c</sup>]<sup>c,d,e</sup> |                  |
| TPA370        | Δhin-5717::FRT [Δc5050::MudJ] flaA5225 [Δ28<sup>+</sup>][H14]<sup>de</sup> |                  |
| TPA375        | Δhin-5717::FRT [Δc5050::MudJ] flaA6555 [Δ28<sup>+</sup>]<sup>V33E</sup> |                  |
| TPA376        | Δhin-5717::FRT [Δc5050::MudJ] flaA5467::FRT [Δ28<sup>+</sup>]<sup>c,d,e</sup> |                  |
| TPA378        | Δhin-5717::FRT [Δc5050::MudJ] flgM5628::FRT ΔflgM<sup>d,e</sup> |                  |
| TPA469        | Δhin-5717::FRT [Δc5050::MudJ] flaA7009 [Δ28<sup>+</sup>]<sup>[S226R]<sup>c,d</sup></sup> |                  |
| TPA490        | Δhin-5717::FRT [Δc5050::MudJ] flaA7010 [Δ28<sup>+</sup>]<sup>[E209G V221A]<sup>c</sup></sup> |                  |
| TPA598        | Δhin-5717::FRT [Δc5050::MudJ] ΔflgM5805::tetra |                  |

**Plasmids**

- pKD46: Plasmid containing the FRT-Kan-FRT cassette
- pNT-ts derivative containing the λ-Red recombinase under the control of the P<sub>aroBAD</sub> promoter
- pCP20: Temperature-sensitive plasmid containing the FLP recombinase
- pUC19: Ap<sup>b</sup> cloning vector
- pPA122: FRT-Kan-FRT cassette from pKD4 cloned in pUC19
- pJOY: flgM<sup>c</sup> in pPA122

<sup>a</sup>For simplicity only descriptions of the strains have been used in the text to help the reader understand our conclusions and results.

<sup>b</sup>Strains used in Figure 1.

<sup>c</sup>Strains used in Figure 2.

<sup>d</sup>Strains used in Figure 3.

<sup>e</sup>“HBB” strains used in Figure 5, “HBB” strains were constructed by transduction of *flgG574::Tn10.*
The design of the primers meant that ARAFLGM produced a transcriptional fusion of flgM, resulting in a transcript with the araBAD 5'-untranslated region. The ARAFLGM-AraGTR PCR product was electrophoretically into strain TH437 (pKD46), grown at 30°C in Luria Broth with 0.2% arabinose as previously described (Aldridge et al. 2003). ParaBAD-flgM* (strain TH6599 [AaraBAD923::[flgM-FK6])] was isolated by screening kanamy-cin-resistant transformants for a Ara* phenotype and confirmed by PCR. The ParaBAD-flgM* construct was moved by cotransduction using leu-1151 ::Tn10 selecting for Leu* Ara* Tc* transductants into a flgM motA5461 ::MuJ (TH6662) background. The leu-1151 ::Tn10 allele was replaced with a leu* allele from wild-type strain LT2 by P22-mediated transduction. Confirmation that ParaBAD-flgM* had been retained was possible by plating transductants on MacConkey Lactose plates ±0.2% arabinose and screening for arabinose-independent inhibition of class 3 promoter activity using the motA5461 ::MuJ reporter construct. The ParaBAD-flgM* construct showed reduced class 3 promoter activity compared with TH3933 in the presence of arabinose (Table 1).

To obtain PParaBAD-flgM* expression levels comparable with PParaD-flgM* expression levels, recombinational replacement PCR mutagenesis was used (Karlinsey and Hughes 2006). First, the araC-araBAD intergenic region in TH7250 was replaced with a PCR product amplified using ParaBtetR (5'-GTGTC TACTCCATCCGAAGAAAACGTTAGGAAACAGATATTAAAGCCCCACTTCCCA-3') and ParaBtetA (5'-GTGTCATATCGACAGGAGCAAGTCTCTCCGCAACTAAGCACCTGATCTCTCG-3') and a chromosomal Tn10dTc insert as a template. Second, ΔPParaBAD-927::tetR: tetRA in TH7253 was then replaced with PCR products amplified using 1, 2, 5, or 5 Units Taq DNA polymerase (Promega) with primers FlgM-34R (5'-CGGTTTCAAGGTGAGG-3') and AraC-5R (5'-CCATGTTATCCTTACCCCA-3') using the PCR clean up kits from either Qiagen or Sigma before somomal DNA preparation of LT2. PCR products were purified and used as donors in transduction of TPA598. This allowed the ΔflgG-L2157 strains with α28* dependent flgM expression rather than flgM* expression from ParaBAD Tc* transductants were replica-printed onto Tc-sensitive and Tc-sensitive + kanamycin plates to iso late Tc* transductants that retained the flhA-linked Hic5050::MuJ reporter insert. The HBB* mutation flgG-L2157::Tn10 was then introduced by transduction by selecting for Tn10-encoded Tc*. Transductants were purified and the activity of Hic5050::MuJ was determined by β-galactosidase assays. Alternatively, the deletion ΔflgG-L2157 was introduced by cotransduction of a linked pyrC691::Tn10 marker. Whether the alleles assayed possessed α28* activity was determined by replica-picking 100 Tc* transductants onto MacConkey-lactose + Tetracycline and TTC-lactose + Tetracycline plates. α28* mutants were defined as α28* mutants in this assay if on Lac indicator plates no Lac* transductants were observed, α28* activity was confirmed for positive isolates by β-galactosidase assays.

Analysis of FlgM and α28* by immunodetection

All samples used for secretion assays in this study were taken at mid-log phase corresponding to an OD600 = 0.6–0.8. For immunodetection of FlgM and α28*, tricine SDS polyacrylamide gels were used instead of standard glycine SDS gels. All immunode ntions were performed as described by Aldridge et al. (2003). Protein stability assays and quantification of immunoblots were performed essentially as described by Aldridge et al. (2003). Secretion assays for FlgM have been previously described (Karlinsey et al. 2000b). Briefly, strains were inoculated into 3 mL of LB broth and incubated with continuous shaking until the OD600 was ~0.6–0.8. Two-thirds of the culture was centrifuged and the cell pellet was resuspended in SDS-sample buffer (Karlinsey et al. 2000b); this whole-cell lysate sample was defined as FlgMint. A 1.8 mL aliquot of the supernatant was centrifuged a second time, filtered through a 0.2-μm PES filter, and the proteins were extracted by filtration over a nitrocellulose filter (BA85). The filter was soaked in SDS sample buffer for 15 min at 65°C to recover the proteins present, and the sample was defined as FlgMout. Prior to loading onto SDS-tricine gels, all samples were normalized to the OD of the culture and volume used in preparation (Karlinsey et al. 2000b). Detection of chemiluminescence produced by the ECL plus western detection kit (GE Healthcare) was performed using either a Storm 840 phospho-imager (GE Healthcare) or a Fuji LAS3000 image analyzer. Statistical analysis and calculation of the half-lives of FlgM and α28* were performed using Microsoft Excel (see Supplemental Material).

β-Galactosidase reporter gene assays

All β-galactosidase assays were performed on mid-log phase cultures. Strains were inoculated into 3 mL of LB broth and incu bated with continuous shaking until the OD600 was ~0.6–0.8.
Cells were recovered by centrifugation for 10 min at 4000 rpm and resuspended in 3 mL saline buffer. β-Galactosidase assays have been described previously by Aldridge et al. (2003).

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