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Analysis of Factors That Affect FlgM-Dependent Type III Secretion for Protein Purification with *Salmonella enterica* Serovar Typhimurium

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The FlgM protein is secreted in response to flagellar hook-basal body secretion and can be used as a secretion signal to direct selected protein secretion via the flagellar type III secretion (T3S) system [H. M. Singer, M. Erhardt, A. M. Steiner, M. M. Zhang, D. Yoshikami, G. Bulaj, B. M. Olivera, and K. T. Hughes, mBio 3(3):e00115-12, 2012, http://dx.doi.org/10.1128/mBio.00115-12]. Conditions known to affect flagellar gene expression, FlgM stability, and flagellar T3S were tested either alone or in combination to determine their effects on levels of secreted FlgM. These conditions included mutations that affect activity of the flagellar $FlhD_4C_2$ master regulatory protein complex or the FlgM T3S chaperone σ^{28}, the removal of *Salmonella p*athogenicity *i*sland 1 (Spi1), the removal of flagellar late secretion substrates that could compete with FlgM for secretion, and changes in the ionic strength of the growth medium. Conditions that enhanced FlgM secretion were combined in order to maximize levels of secreted FlgM. An optimized FlgM secretion strain was used to secrete and isolate otherwise difficult-to-produce proteins and peptides fused to the C terminus of FlgM. These include cysteine-rich, hydrophobic peptides (conotoxins δ-SVIE and MrVIA), nodule-specific, cysteine-rich antimicrobial peptides (NCR), and a malaria surface antigen domain of apical membrane antigen AMA-1.

Many bacteria utilize flagella to move in a directed manner, either away from stressful environments or toward nutrients, O_2, light, and other positive stimuli (1). The bacterial flagellum is a complex cellular machine that requires more than 30 gene products for its construction. For *Salmonella enterica*, there are currently more than 60 genes involved in the biogenesis and function of its flagella (2). These genes are organized into a transcriptional hierarchy of 3 promoter classes. At the top of the flagellar transcriptional hierarchy is the *flhDC* operon, encoding the master regulator proteins FlhD and FlhC, which form a heteromultimeric, transcriptional activation complex (3). The $FlhD_4C_2$ complex directs σ^{70} RNA polymerase to transcribe from class 2 flagellar promoters. Class 2 flagellar genes encode proteins required for the structure and assembly of a rotary motor called the hook-basal body (HBB), a key structural intermediate in flagellum assembly (4). The HBB includes the flagellar type III secretion (T3S) system, which exports flagellar proteins from the cytoplasm through the growing structure during assembly (5). In addition to HBB gene expression, flagellar class 2 transcription produces σ^{28} (FliA) and FlgM. These are regulatory proteins that couple transcription of the flagellar class 3 promoters to completion of the HBB. The σ^{28} protein is a flagellum-specific transcription factor that directs RNA polymerase to transcribe from the flagellar class 3 promoters (6). Class 3 genes include the structural genes of the flagellar filament and genes of the chemosensory signal transduction system, which controls the direction of flagellar rotation according to changing concentrations of extracellular ligands (7). Prior to HBB completion, FlgM binds σ^{28} and prevents flagellar class 3 promoter transcription (8). Upon HBB completion, a change in flagellar T3S substrate specificity results in FlgM secretion and initiation of σ^{28}-dependent transcription from flagellar class 3 promoters (9, 10). The secretion signal requirements for T3S substrates remains poorly defined, but all substrates utilize an N-terminal peptide secretion signal that is disordered in structure and, unlike the case with type II secretion, is not cleaved during the secretion process (11). Substrate secretion is often facilitated by T3S chaperone-assisted delivery to the secretion apparatus (12). The FlgM protein is 97 amino acids in length, and its secretion is dependent on an N-terminal secretion signal. FlgM secretion is greatly enhanced by its secretion chaperone, σ^{28}, which binds to the C-terminal half of FlgM (13–15).

The FlgM type III secretion system can be used to express and purify recombinant proteins (16). Here we have identified factors that improve yields of secreted FlgM. These factors include those controlling flagellar gene expression, ionic conditions, cell growth phase, and removal of cellular proteases or secretion competitors. This characterization allowed us to construct strains that produce high yields of secreted protein for the purposes of protein purification via flagellar T3S.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study are derivatives of LT2, a wild-type strain of *Salmonella enterica* serovar Typhimurium, and are listed in Table 1. Strain construction utilized either P22-mediated generalized transduction or λ Red-mediated targeted mutagenesis (17–19). For strains with the *flhDC* operon expressed from its native promoter, flagellar gene expression was induced by 100-fold dilution from overnight stationary cultures into fresh LB medium (10 g Bacto tryptone, 5 g Bacto yeast extract, and 5 g NaCl [per liter]). For strains with the *flhDC* operon expressed from the *tetA* promoter of transposon Tn*10* (ΔP_{flhDC}*8089*::*tetR*-P*tetA*), flagellar gene expression was induced by addition of the tetracycline analog, anhydrotetracycline (1 μg/ml). For all

Received 13 February 2014 Accepted 30 March 2014
Published ahead of print 4 April 2014
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doi:10.1128/JB.01572-14

TABLE 1 Strains used in this work

Strain[a]	Genotype[b]
TH17831	Δ*araBAD1124*::*flgM*-6His-*TEV*-δ-*SVIE*
TH18353	Δ*araBAD1124*::*flgM*-6H-*TEV*-δ-*SVIE* Δ*clpX*::*tetRA*
TH18500	Δ*araBAD1156*::*flgM*$^+$
TH18527	Δ*araBAD1156*::*flgM*$^+$ Δ*clpX*::*tetRA*
TH18528	Δ*araBAD1156*::*flgM*$^+$ Δ*flhDC*::FKF
TH18549	Δ*araBAD1156*::*flgM*$^+$ Δ*flhDC*::FKF Δ*clpX*::*tetRA*
TH18624	Δ*araBAD1156*::*flgM*$^+$ Δ*fliA5805*::*tetRA*
TH18636	Δ*araBAD1156*::*flgM*$^+$ *fliA8088*(H14D, R91C, L207P)
TH18647	Δ*araBAD1124*::*flgM*-6His-*TEV*-δ-*SVIE* ΔP_{flhDC}*8089*::(*tetR*-P_{tetA})
TH18649	Δ*araBAD1156*::*flgM*$^+$ ΔP_{flhDC}*8089*::(*tetR*-P_{tetA})
TH18704	Δ*araBAD1156*::*flgM*$^+$ Δ*invH-sprB*::FKF(ΔSpi1)
TH18729	Δ*araBAD1156*::*flgM*$^+$ Δ*fliC7715*::*tetRA*
TH18730	Δ*araBAD1156*::*flgM*$^+$ Δ*ompT*::Km
TH18731	Δ*araBAD1156*::*flgM*$^+$ Δ*clpA74*::FKF
TH18732	Δ*araBAD1156*::*flgM*$^+$ Δ*sseA-ssaU*::FKF(ΔSpi2)
TH18733	Δ*araBAD1156*::*flgM*$^+$ Δ*clpP*::mini-Tn*5*
TH18737	Δ*araBAD1156*::*flgM*$^+$ Δ*fliA5999*(R91C, L207P)
TH18739	Δ*araBAD1156*::*flgM*$^+$ *fliA5225*(H14D)
TH18752	Δ*araBAD1156*::*flgM*$^+$ Δ*ydiV251*::*tetRA*
TH18769	Δ*araBAD1124*::*flgM*-6His-*TEV*-δ-*SVIE* Δ*fliT*::Km
TH18778	Δ*araBAD1156*::*flgM*$^+$ *fliA5228*(V33E)
TH18780	Δ*araBAD1156*::*flgM*$^+$ *fliA5223*(T138I)
TH18782	Δ*araBAD1156*::*flgM*$^+$ *fliA5224*(E203D)
TH18787	Δ*araBAD1156*::*flgM*$^+$ Δ*flgM5628*::FKF
TH18788	Δ*araBAD1156*::*flgM*$^+$ Δ*fliT*::FKF
TH18790	Δ*araBAD1156*::*flgM*$^+$ Δ*flgK7665*
TH18793	Δ*araBAD1156*::*flgM*$^+$ Δ*flgL7666*
TH18796	Δ*araBAD1156*::*flgM*$^+$ Δ*fliD5630*::FRT
TH18798	Δ*araBAD1156*::*flgM*$^+$ Δ*degP*::*tetRA*
TH18822	Δ*araBAD1156*::*flgM*$^+$ Δ*csrA101*::Cm
TH18823	Δ*araBAD1156*::*flgM*$^+$ Δ*dksA*::FKF
TH18824	Δ*araBAD1156*::*flgM*$^+$ Δ*rcsB*::*tetRA*
TH18830	Δ*araBAD1124*::*flgM*-6H-*TEV*-δ-*SVIE* Δ*rcsB*::*tetRA*
TH18850	Δ*araBAD1156*::*flgM*$^+$ Δ*lrhA*
TH18880	Δ*araBAD1124*::*flgM*-6H-*TEV*-δ-*SVIE* P_{flhDC}*7793*
TH18881	Δ*araBAD1156*::*flgM*$^+$ P_{flhDC}*7793*
TH18897	Δ*araBAD1156*::*flgM*$^+$ *fliA5240*(L199R)
TH18973	Δ*araBAD1156*::*flgM*$^+$ Δ*ecnR5*::FCF
TH19086	Δ*araBAD1156*::*flgM*$^+$ Δ*flgN5626*::FKF
TH19087	Δ*araBAD1156*::*flgM*$^+$ Δ*fliST5775*::FCF
TH19099	Δ*araBAD1156*::*flgM*$^+$ Δ*hin-fljA7731*::*tetRA*
TH19104	Δ*araBAD1156*::*flgM*$^+$ Δ*flgN5626*::FKF Δ*fliST5775*::FCF
TH19106	Δ*araBAD1156*::*flgM*$^+$ Δ*fliS5728*::FRT
TH19113	Δ*araBAD1156*::*flgM*$^+$ Δ*hin-fljA7731*::*tetRA* Δ*fliC7861*::FCF
TH19116	Δ*araBAD1164*::*flgM*-6H-ETK-δ-*SVIE*
TH19118	Δ*araBAD1164*::*flgM*-6H-ETK-δ-*SVIE* P_{flhDC}*7793*
TH19120	Δ*araBAD1164*::*flgM*-6H-*ETK*-δ-*SVIE* Δ*lrh*A Δ*ecnR4*::FRT Δ*fliB-T7771* P_{flhD}*7793* Δ*ydiV252* Δ*hin-fljA7752* Δ*flgMN7753* Δ*flgKL7770*
TH19145	Δ*araBAD1164*::*flgM*-6H-*ETK*-δ-*SVIE* P_{flhDC}7793 Δ*flgM5628*:: FKF Δ*clpX80*::*tetRA*
TH19149	Δ*araBAD1156*::*flgM*$^+$ Δ*flgKL5636*::FKF
TH19320	Δ*araBAD1156*::*flgM*$^+$ *fliA5226*(H14N)
TH19323	Δ*araBAD1156*::*flgM*$^+$ Δ*clpA74*::FKF Δ*flhDC8040*::*tetRA*
TH19324	Δ*araBAD1156*::*flgM*$^+$ Δ*clpP*::mini-Tn*5* Δ*flhDC8040*::*tetRA*
TH19325	Δ*araBAD1156*::*flgM*$^+$ Δ*degP*::*tetRA* Δ*flhDC*::FKF
TH19326	Δ*araBAD1156*::*flgM*$^+$ Δ*hin-fljA7731*::*tetRA* Δ*fliC7861*::FCF Δ*flgKL5739*::FKF
TH19481	Δ*araBAD1156*::*flgM*$^+$ Δ*fliS8156* (replaced *fliST* with *fliS*)
TH19673	Δ*araBAD1124*::*flgM*-6His-*TEV*-δ-*SVIE* Δ*fliT*::Km Δ*rcsB*::*tetRA*
TH19675	Δ*araBAD1124*::*flgM*-6H-*TEV*-δ-*SVIE* P_{flhDC}*7793* Δ*rcsB*::*tetRA*
TH20042	Δ*araBAD1156*::*flgM*$^+$ Δ*hin-5717*::FRT
TH20043	Δ*araBAD1156*::*flgM*$^+$ Δ*hin-5717*::FRT **Δ***fliC7715*::*tetRA*
TH20044	Δ*araBAD1124*::*flgM*-6H-*TEV*-δ-*SVIE* P_{flhDC}*7793* Δ*rcsB*::*tetRA* Δ*flgM5628*::FKF

TABLE 1 (Continued)

Strain[a]	Genotype[b]
TH20047	Δ*araBAD1156*::*flgM*$^+$ *fljB*^{enx}
TH20048	Δ*araBAD1124*::*flgM*-6His-*TEV*-δ-*SVIE fljB*^{enx} *vh2*
TH20049	Δ*araBAD1156*::*flgM*$^+$ *fljB*^{enx} *vh2* Δ*fliC7715*::*tetRA*
TH20050	Δ*araBAD1124*::*flgM*-6His-*TEV*-δ-*SVIE fljB*^{enx} *vh2* **Δ***fliC7715*::*tetRA*
TH20053	Δ*araBAD1156*::*flgM fljB*^{enx} *vh2* P_{flhDC}*7768*::*tetRA*
TH20055	Δ*araBAD1156*::*flgM fliA8176* (−18 to +1G replaced by ataaAGGAGGtaaataA)
TH20056	Δ*araBAD1124*::*flgM*-6His-*TEV*-δ-*SVIE fliA8176*
TH20057	Δ*araBAD1156*::*flgM*$^+$ Δ*hin-5718*::FRT
TH20058	Δ*araBAD1156*::*flgM*$^+$ *fljB*^{enx} *vh2 flhD8089*::(*tetR*-P_{tetA})
TH20059	Δ*araBAD1156*::*flgM*$^+$ Δ*hin-5718*::FRT **Δ***fliC7715*::*tetRA*
TH20060	Δ*araBAD1156*::*flgM*$^+$ Δ*fliC7861*::FCF *fljB*^{enx} *vh2 flhD8089*::(*tetR*-P_{tetA})
TH20061	Δ*araBAD1156*::*flgM*$^+$ *fliA8177*(H14N, R91C, L207P)
TH20062	Δ*araBAD1156*::*flgM*$^+$ *fliA8178*(ATG H14N)
TH20063	Δ*araBAD1156*::*flgM*$^+$ *fljB*^{enx} *vh2 flhD8089*::(*tetR*-P_{tetA}) Δ*clpX*::Tn*10d*Cm
TH20064	Δ*araBAD1156*::*flgM*$^+$ *fljB*^{enx} *vh2* Δ*clpX*::Tn*10d*Cm
TH20065	Δ*araBAD1156*::*flgM*$^+$ *fljB*^{enx} *vh2* Δ*ydiV251*::*tetRA*
TH20066	Δ*araBAD1156*::*flgM*$^+$ *fljB*^{enx} *vh2* Δ*fliA5805*::*tetRA*
TH20067	Δ*araBAD1156*::*flgM*$^+$ *fljB*^{enx} *vh2* Δ*fliB-T7727*::*tetRA*
TH20068	Δ*araBAD1156*::*flgM*$^+$ *fljB*^{enx} *vh2* Δ*fliC7715*::*tetRA* Δ*clpX*::Tn*10d*Cm
TH20069	Δ*araBAD1156*::*flgM*$^+$ *fliA8179*(GTG:ATG)
TH20071	Δ*araBAD1156*::*flgM*$^+$ *fljB*^{enx} *vh2 fliA8176* Δ*fliC7861*::FCF
TH20072	Δ*araBAD1156*::*flgM*$^+$ *fljB*^{enx} *vh2 flhD8089*::(*tetR*-P_{tetA}) Δ*fliA5805*::*tetRA*
TH20073	Δ*araBAD1156*::*flgM*$^+$ *fljB*^{enx} *vh2 fliA8176* (−18 to +1G replaced by ataaAGGAGGtaaataA) Δ*fliC7861*::FCF Δ*clpX72*::FKF
TH20074	Δ*araBAD1156*::*flgM*$^+$ Δ*flgMN7753* Δ*flgKL7770* Δ*trg-7774* Δ*ycgR7775* Δ*fliB-T7771* Δ*hin-fljA7752 fliA8176*(−18 to +1G replaced by ataaAGGAGGtaaataA)
TH20075	Δ*araBAD1124*::*flgM*-6His-*TEV*-δ-*SVIE fljB*^{enx} *vh2* Δ*fliB-T7727*::*tetRA*
TH20077	Δ*araBAD1124*::*flgM*-6His-*TEV*-δ-*SVIE fljB*^{enx} *vh2* Δ*clpX80*::*tetRA*
TH20078	Δ*araBAD1156*::*flgM*$^+$ *fliA8176* Δ*flgM5794*::FCF
TH20079	Δ*araBAD1156*::*flgM*$^+$ *fljB*^{enx} *vh2 flhD8089*::(*tetR*-P_{tetA}) Δ*fliA5805*::*tetRA motA5461*::*Mud*J
TH20080	Δ*araBAD1156*::*flgM*$^+$ *fljB*^{enx} *vh2 flhD8089*::(*tetR*-P_{tetA}) *motA5461*::MudJ *fliA8176* Δ*fliC7861*::FCF
TH20081	Δ*araBAD1156*::*flgM*$^+$ *fliA5226*(H14N) Δ*fliC7861*::FCF
TH20082	Δ*araBAD1156*::*flgM*$^+$ *fljB*^{enx} *vh2 flhD8089*::(*tetR*-P_{tetA}) *motA5461*::MudJ *fliA5226*(H14N) Δ*fliC7861*::FCF
TH20083	Δ*araBAD1124*::*flgM*-6His-TEV-δ-SVIE *fljB*^{enx} *vh2 flhD8089*::(*tetR*-P_{tetA}) Δ*clpX*::Tn*10d*Cm
TH20221	Δ*araBAD1190*::*flgM*-6His-ETK-NCR247 Δ*flgMN7753* Δ*flgKL7770* Δ*fliB-T7771* Δ*hin-fljA7752* P_{flhD}*7793* *fliA5226*(H14N) Δ*lrhA* Δ*ydiV252* Δ*ecnR4*::FRT
TH20222	Δ*araBAD1191*::*flgM*-6His-ETK-NCR057 Δ*flgMN7753* Δ*flgKL7770* Δ*fliB-T7771* Δ*hin-fljA7752* P*flhD7793* *fliA5226*(H14N) Δ*lrhA* Δ*ydiV252* Δ*ecnR4*::FRT
TH20223	Δ*araBAD1192*::*flgM*-6His-ETK-NCR224 Δ*flgMN7753* Δ*flgKL7770* Δ*fliB-T7771* Δ*hin-fljA7752* P_{flhD}*7793* *fliA5226*(H14N) Δ*lrhA* Δ*ydiV252* Δ*ecnR4*::FRT
TH20492	Δ*araBAD1163*::*flgM*-6His-ETK-MrVIA Δ*flgMN7753* Δ*flgKL7770* Δ*fliB-T7771* Δ*hin-fljA7752* P_{flhD}*7793* *fliA5226*(H14N) Δ*lrhA* Δ*ydiV252* Δ*ecnR4*::FRT
TH20685	Δ*araBAD1195*::*flgM*-6His-ETK-AMA1 Δ*flgMN7753* Δ*flgKL7770* Δ*fliB-T7771* Δ*hin-fljA7752* P*flhD7793* *fliA5226*(H14N) Δ*lrhA* Δ*ydiV252* Δ*ecnR4*::FRT

[a] All strains were constructed during the course of this study.
[b] FRT, FLP recombination target. Amino acid changes encoded by *fliA* alleles are shown in parentheses.

strains used in this study, the arabinose utilization operon, *araBAD*, was replaced with the *flgM*$^+$ gene or *flgM* gene fusion *flgM*-6His-*TEV*-δ-*SVIE* or *flgM*-6His-*ETK*-δ-*SVIE* (Δ*araBAD*::*flgM*$^+$, Δ*araBAD*::*flgM*-6His-*TEV*-δ-*SVIE*, or Δ*araBAD*::*flgM*-6His-*ETK*-δ-*SVIEI*, respectively). This allowed for the induction of FlgM, FlgM-6His-TEV-δ-SVIE, and FlgM-6His-ETK-δ-SVIE production from the P_{araBAD} promoter by the addition of L-arabinose to the growth medium. Arabinose was added to a 0.2% final concentration 2 h after the induction of the *flhDC* operon. After another 5 h, the cultures were centrifuged at 10,000 × *g* for 30 min to pellet the cells. The supernatant was filtered with a 0.2-μm low-protein-binding filter (Acrodisk syringe filter; Pall Life Sciences) to remove remaining cells. Secreted proteins in the filtered supernatant were precipitated by addition of TCA (trichloroacetic acid) to a 10% final concentration. The cell pellet was resuspended in cold PBS (phosphate-buffered saline) containing 5 mM PMSF (phenylmethylsulfonyl fluoride), followed by sonication to lyse the cell suspension. The cell lysate was either analyzed directly for whole-cell protein or separated into soluble and insoluble fractions for analysis by 30 min of centrifugation (15,000 × *g*) at 4°C. To test the effect of different concentrations of NaCl and KCl on FlgM secretion, LB medium was prepared without NaCl and either NaCl or KCl was added to the desired concentrations.

SDS-PAGE and Western blotting assays. SDS-polyacrylamide gel electrophoresis (14%) was carried out using standard procedures using a Bio-Rad system. Levels of secreted, soluble, insoluble, or whole-cell proteins were analyzed by Western blotting. Expressed DnaK, FlgM, and σ^{28} levels in the whole-cell lysates and culture supernatants were determined. For analysis of strains containing Δ*araBAD*::*flgM*$^+$, equivalents of 50 and 100 optical density (OD) units were loaded for the cellular and supernatant fractions, respectively. In order to analyze strains for FlgM-6His-TEV-δ-SVIE and FlgM-6His-ETK-δ-SVIE secretion, 50 and 300 OD units were loaded for the cellular and supernatant fractions, respectively. For FlgM-6His-ETK-AMA1, FlgM-6His-ETK-NCR peptides, and FlgM-6His-ETK-MrVIA, 10 μl of the supernatant was loaded without the concentration step and detected using anti-His tag antibodies (mouse; Abcam). For NCR and AMA1 constructs, SDS-PAGE followed by Coomassie staining of 20-fold-concentrated supernatant (TCA concentration) was performed.

Anti-DnaK (mouse; Abcam), anti-His tag antibodies (mouse; Abcam), anti-σ^{28}, and anti-FlgM antibodies (rabbit; Hughes lab) were used for detection of Western blots. DnaK was used as a protein standard control for loading concentration and for the presence of protein in the supernatant due to cell lysis. To visualize antigen-antibody complexes, secondary anti-rabbit-IRDye690 and anti-mouse-IRDye800 antibodies (Li-Cor) were used. Densiometric measurements of FlgM, σ^{28}, and DnaK bands were performed using the Li-Cor Odyssey infrared imaging system software. All assays were performed in triplicate.

Motility assay. Motility assays utilized soft agar tryptone plates (per liter: 10 g Bacto tryptone, 5 g NaCl, and 3 g Bacto agar). A bacterial colony was picked by toothpick and poked through the soft agar, followed by incubation at 37°C for about 5 h. If necessary, either arabinose (0.2%) or anhydrotetracycline (1 μg/ml) was added for P_{araBAD} or *flhDC* operon induction, respectively.

RESULTS

FlgM produced from P_{araBAD}::*flgM* is secreted. FlgM is secreted through a completed HBB into the external spent growth medium. It has been shown that fusion of foreign proteins to the C terminus of FlgM can be used for protein purification purposes without the need to lyse cells prior to purification (16). In order to develop the flagellar T3S system for protein purification using FlgM as a secretion signal, we decided to characterize known aspects of FlgM production and secretion to maximize protein production using this system. About 80% of steady-state *flgM* transcription is from its class 3 promoter. Since FlgM is an anti-σ^{28} factor, its production via the class 3 promoter is under autoinhibition. For the studies presented here, we removed *flgM* gene transcription from FlgM autoinhibition by using a construct with the *flgM* gene transcribed from the arabinose-inducible chromosomal *araBAD* promoter (P_{araBAD}). This was accomplished by a targeted deletion of the chromosomal *araBAD* operon followed by insertion of the *flgM*$^+$ gene in its place (13). This resulted in the arabinose-dependent induction of FlgM production in strains where the arabinose inducer is not degraded due to deletion of the *araBAD* structural genes. FlgM transcribed from P_{araBAD} was produced in the presence of arabinose and secreted at levels higher than those of FlgM produced and secreted from its native promoters (Fig. 1). A *fliF* deletion strain, which does not form flagellar structures, was unable to secrete P_{araBAD}-induced FlgM (Fig. 1). These results indicate that FlgM intrinsic-peptide secretion signals are sufficient for high-level FlgM secretion and the flagellar-dependent T3S system is required for FlgM secretion.

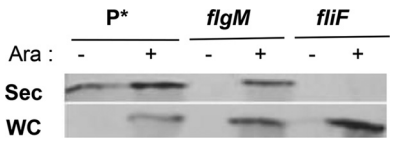

FIG 1 Increased levels of secreted FlgM under P_{araBAD} overexpression conditions. Western blot analysis of supernatant fractions of strains overexpressing FlgM from the chromosomal P_{araBAD} promoter. Overnight cultures were diluted 100-fold in LB medium and incubated at 37°C for 2 h, followed by addition of arabinose to 0.2% to induce excess FlgM expression. After 5 h of further incubation at 37°C, cells were separated from the supernatant by centrifugation and TCA was added to the cell supernatant to precipitate secreted proteins (see Materials and Methods). For the secreted proteins, 100 OD units sample was loaded; for the whole-cell protein, 50 OD units sample was loaded. Anti-FlgM antibody was used to determine levels of secreted and whole-cell FlgM (Sec, secreted FlgM; WC, whole-cell FlgM; Ara, 0.2% arabinose). P*, parent strain, TH18500 (Δ*araBAD1156*::*flgM*$^+$).

Mutations affecting $FlhD_4C_2$ activity also affect FlgM secretion. The *flhDC* operon is at the top of the flagellar transcriptional hierarchy. The regulation of *flhDC* is complex, and there are six known transcription initiation sites within the *flhDC* promoter region (20). It was previously reported that changes in the −10 sequences for the P1 and P4 transcription initiation sites within the *flhDC* promoter region to the canonical TATAAT sequence (the P_{flhDC}*7793* allele) resulted in the doubling of the number of HBB structures per cell and increased production and secretion of the flagellar hook protein (21). Other mutations resulting in increased hook protein secretion and presumably increased HBB structures per cell resulted in reduced expression or removal of known inhibitors of *flhDC* operon transcriptional or posttranscriptional control. We decided to test the effects of *flhDC* regulatory mutations on the secretion of FlgM transcribed from P_{araBAD}. The known transcriptional and posttranscriptional inhibitors of *flhDC* expression included in this study were EcnR, RscB, LrhA, FliT, DskA, and YdiV. EcnR is responsible for FlhDC-mediated autorepression (22). The $FlhD_4C_2$ complex directs transcription of *ecnR*, which in turn results in repression of *flhDC* transcription in concert with the RcsB protein. RcsB, which regulates capsular polysaccharide synthesis and a number of genes in response to membrane and cell wall damage, is a known repressor of *flhDC* transcription (23, 24). LrhA is also a known regulator of *flhDC* that has been shown to bind within the *flhDC* promoter region to inhibit *flhDC* operon transcription (25). FliT is transcribed from both class 2 and class 3 flagellar promoters (26). FliT binds to the

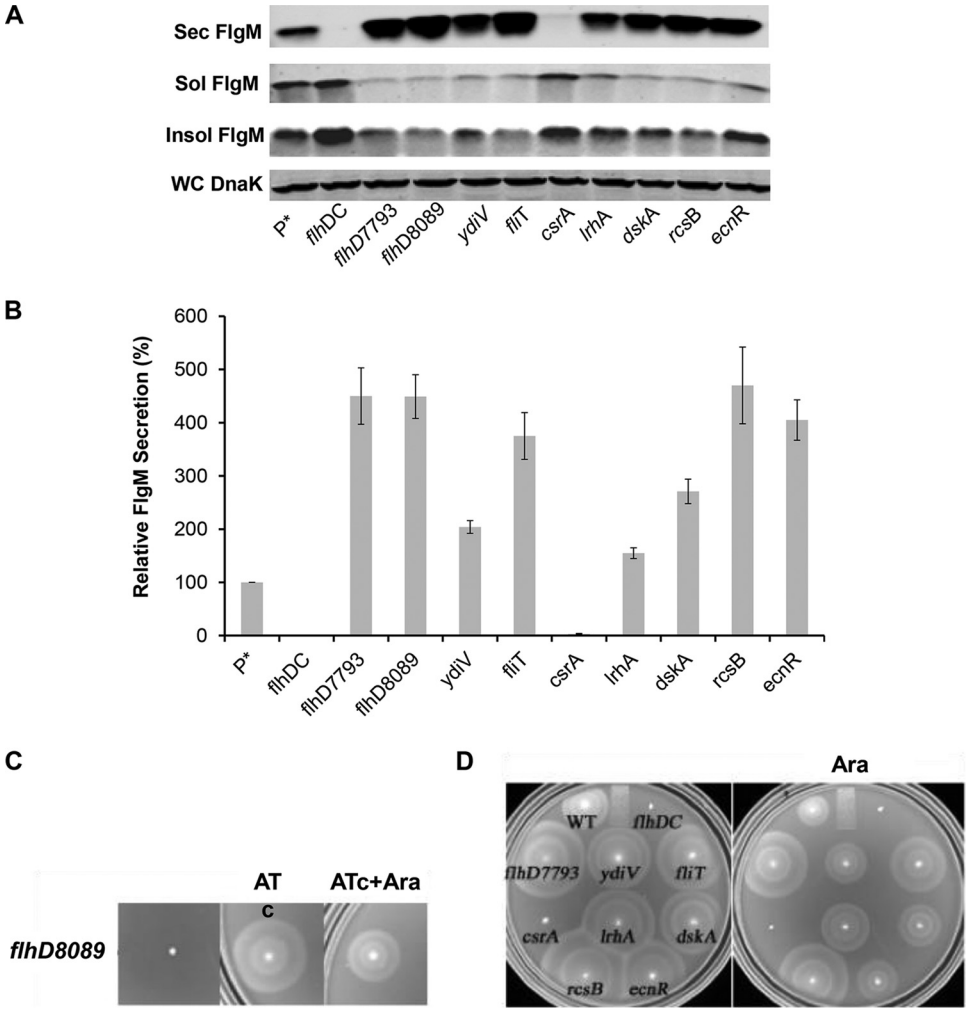

FIG 2 Effect of *flhDC* operon expression on levels of secreted FlgM and cell motility. (A) Secreted levels of FlgM in strains affected in *flhDC* operon expression were determined by quantitative Western blot analysis using anti-FlgM antibody to detect FlgM in the supernatant of the spent growth medium. (Sec FlgM, secreted FlgM; Sol FlgM, cellular soluble FlgM; Insol FlgM, cellular insoluble FlgM; WC DnaK, whole cellular DnaK). P*, parent strain, TH18500 (Δ*araBAD1156*::*flgM*$^+$). (B) Relative FlgM secreted levels. (C) Swim phenotype on soft agar plates of strain TH18649 (Δ*PflhDC8089*::*tetR*-P*tetA* Δ*araBAD1156*::*flgM*$^+$) with no inducer added, 1 μg/ml anyhdrotetracycline (ATc) added, and both ATc and 0.2% arabinose (Ara) added. (D) Motility assay for various mutant backgrounds depicted with Δ*araBAD1156*::*flgM*$^+$ in the absence and presence of added 0.2% arabinose. WT, wild type.

$FlhD_4C_2$ complex and prevents activation of flagellar class 2 transcription (27, 28). FliT is also the secretion chaperone for the flagellar filament capping protein FliD (29). It is thought that secretion of FliD after HBB completion couples inhibition of further class 2 transcription by $FlhD_4C_2$ to HBB completion. The DskA protein acts with ppGpp to inhibit *flhDC*'s transcription (30). DskA can also stimulate *rpoS* translation, which may inhibit *flhDC* transcription through an RpoS-mediated mechanism (31). YdiV is a posttranscriptional regulator that targets $FlhD_4C_2$ to the ClpXP protease for degradation in response to changes in nutrient growth conditions (32, 33). As a control, we included CsrA, which is a positive regulator of *flhDC* mRNA stability (34). The P_{flhDC}*8089* allele was constructed by replacing the *flhDC* promoter control region with a *tetR*-P_{tetA} cassette from transposon Tn*10*. This resulted in the placement of the *flhDC* operon under the control of the *tetA* promoter, which can be induced by the addition of the tetracycline analog anhydrotetracycline. We also tested the *flhDC* promoter changes described previously that increase hook production and secretion (the P_{flhDC}*7793* allele) (21). The effects of the *flhDC* regulatory mutations on levels of secreted FlgM expressed from P_{araBAD} are shown in Fig. 2.

As expected, with deletion of the *flhDC* structural operon or deletion of the *csrA* gene, which is required for *flhDC* mRNA stability, there was no detectable FlgM in the secreted fraction, and FlgM accumulated in the cytoplasm. Mutations defective in known transcriptional and posttranscriptional inhibitors of *flhDC* expression resulted in increased levels of secreted FlgM, as did the presence of the P_{flhDC}*7793* allele. Secreted levels of FlgM in P_{flhDC}*7793*, P_{flhDC}*8089*(*tetR*-P_{tetA}-*flhDC*), *fliT*, *rcsB*, and *ecnR* mutant strains were 4.5-, 4.5-, 3.8-, 4.7-, and 4-fold higher than that for the wild type, respectively. Secreted levels of FlgM were less in *ydiV*, *lrhA*, and *dskA* mutant strains at 2-, 1.5-, and 2.7-fold compared to that for the wild type, respectively (Fig. 2B). For each strain tested, the accumulated cellular levels of FlgM were inversely proportional to secreted FlgM levels. Significantly, replacement of the *flhDC* promoter region with the *tetA* promoter

and regulatory region from transposon Tn*10* allowed for the production of secreted FlgM at levels meeting or exceeding FlgM secreted levels observed in loss-of-function mutants for the negative regulators of *flhDC* expression that were tested. Induction of *flhDC* in the P_{flhDC}*8089*(*tetR*-P_{tetA}-*flhDC*) strain also conferred motility on swim plates (Fig. 2C).

In strains missing negative regulators of *flhDC*, the swimming phenotypes on soft agar plates indicated increased motility compared to that of wild-type LT2 (Fig. 2D). We were surprised to see substantial levels of motility in the same strains with *flgM* overexpressed from P_{araBAD} (Fig. 2D). FlgM inhibits σ^{28} at a stoichiometry of 1:1. It was expected that induction of FlgM from P_{araBAD} would prevent all σ^{28}-dependent flagellar class 3 promoter transcription, especially in the wild-type LT2 background. This observation led to the conclusion that overexpressed cellular FlgM aggregated into an inactive form. Thus, the cellular component of FlgM was analyzed from both soluble and insoluble fractions from the cell pellet in the Western blot analysis of secreted and cellular FlgM levels. Although FlgM is a soluble protein, most cellular FlgM produced under overexpression conditions was insoluble (Fig. 2A), suggesting that excess cellular FlgM went into inclusion bodies, and the observed motility under FlgM-inducing conditions suggested that the insoluble form of FlgM was not active.

Effect of the FlgM T3S chaperone, σ^{28}, on FlgM secretion. As mentioned above, completion of the HBB coincides with a flagellar T3S substrate specificity switch from rod-hook-type secretion substrates to late or filament-type secretion substrates. The late secretion substrates include hook-filament junction proteins (FlgK and FlgL), filament cap protein (FliD), the alternately expressed filament proteins (FliC or FljB), and the anti-σ^{28} factor FlgM. Efficient secretion of each late secretion substrate requires the aid of a cognate T3S chaperone. These include FlgN (for FlgK and FlgL), FliT (for FliD), FliS (for FliC and FljB), and σ^{28} (for FlgM) (13, 29, 35). T3S chaperones fall into three classes: (i) those that bind and protect substrates from proteolysis in the system prior to secretion, (ii) those that facilitate substrate secretion, and (iii) those that both stabilize and facilitate secretion. It has been shown that interactions of FlgN, FliS, and FliT flagellar chaperones with the C-terminal cytoplasmic domain of FlhA are required for efficient export of their cognate substrates and that these interactions contribute to coordinating assembly of the flagellar filament (36–38). The σ^{28} protein falls into the category of T3S chaperones that both stabilize and facilitate secretion; it protects FlgM from proteolysis in the cytoplasm and facilitates the secretion of FlgM, presumably by helping to localize FlgM to the base of the flagellum. A mutant of σ^{28} with two amino acid substitutions that render it defective in recognition of the −10 and −35 promoter sequences (R91C and L207P) retains its T3S chaperone activity for FlgM secretion (13). This indicated that the T3S chaperone function of σ^{28} was a separate process from its transcription activity.

We tested the effects of FlgM bypass mutations in σ^{28} regions 2, 3, and 4 on secretion of FlgM expressed from P_{araBAD} (Fig. 3A and B). The region 2.1 mutations included H14D and H14N, which result in increased protein stability, and the V33E allele, whose mutant is defective in binding FlgM. The other FlgM bypass mutations tested included the T138I allele from region 3.1 and L199R from region 4.1 and E203D at the initiation point of region 4.2.

As expected from previous studies, the strain with the *fliA* null allele showed a strong reduction in FlgM secretion, to a level that was 5% of that of the *fliA*$^+$ strain. The V33E and L199R alleles resulted in FlgM secretion levels that were 12% and 59% of that of the wild type, respectively. Also expected, strains with the H14D and H14N alleles exhibited secreted FlgM levels that were 2.1- and 1.5-fold that seen for the wild type. Unexpectedly, the T138I and E203D alleles, which are defective in binding FlgM, resulted in increased secreted FlgM levels, 1.2- and 1.4-fold that of the wild type, respectively. The promoter binding-defective *fliA* double mutant (R91C L207P), when also tested and compared to the wild type, showed a 39% level of secreted FlgM. We combined the promoter binding-defective R91C L207P substitutions with the H14D increased-stability substitution and observed secreted FlgM levels in between those observed with either the R91C L207P double mutant or the H14D single mutant.

For wild-type FliA and the H14N, H14D, R91C L207P, and H14D R91C L207P mutants, the FliA level in the cell was consistent with the secreted FlgM level (Fig. 3A). We speculated that the FlgM secretion level was related to the FliA concentration within the cell. Therefore, we introduced other mutations, which would potentially increase the intracellular FliA concentration, to see whether they could affect FlgM secretion. These mutations included an *fliA* start codon change from GTG to ATG that was combined either with an H14N stabilization substitution or with a change of the *fliA* ribosome binding sequence (RBS) to a canonical sequence (CRBS). All of these changes resulted in increased FliA intracellular levels and secreted FlgM levels from 2- to 4-fold that of the wild type (Fig. 3A and B). Deletion of the chromosomal *flgM* gene in the *fliA* CRBS ATG double mutant background resulted in a reduction of both of intracellular FliA and secreted FlgM. We conclude that the excess σ^{28} produced in the *fliA* CRBS ATG double mutant background requires chromosomal *flgM* expression in addition to P_{araBAD}-expressed *flgM* to contribute to σ^{28} stability, which would also improve FlgM secretion. This is consistent with the results showing that FlgM not only acts as an anti-sigma factor but also protects FliA from being degraded (39).

Motility assays of the *fliA* mutants under FlgM-induced conditions are showed in Fig. 3C. As expected, the *fliA* null allele and mutants containing the R91C L207P alleles, which are unable to transcribe flagellar class 3 promoters and therefore unable to produce flagellin, are nonmotile under any condition. The H14N, H14D, and ATG substitution mutants showed increased swarm sizes on motility plates compared to that of the wild type, consistent with previous results (40). V33E, T138I, L199R, and E203D mutants are reported to have flagellar transcriptional activities in the presence of FlgM that are 8.0-, 31-, 45-, and 7-fold higher than that of the wild type, respectively, and their motility phenotypes on swim plates under FlgM-inducing conditions correlate with these levels. The H14N ATG and CRBS ATG double mutants showed decreased motility on swim plates, although they have higher cellular levels of σ^{28} than the wild-type strain.

Effect of flagellar late T3S substrate competitors on FlgM secretion. The initial report of FlgM secretion upon HBB completion presented qualitative data suggesting that secreted FlgM levels were higher in strains missing filament protein, a potential secretion competitor for FlgM (9). We tested for the effects of removing potential late secretion substrate competitors on secreted FlgM levels, anticipating that their removal would improve the yield of secreted FlgM. The number of late substrate subunits in the assembled flagellum is about 11 each for the hook-filament

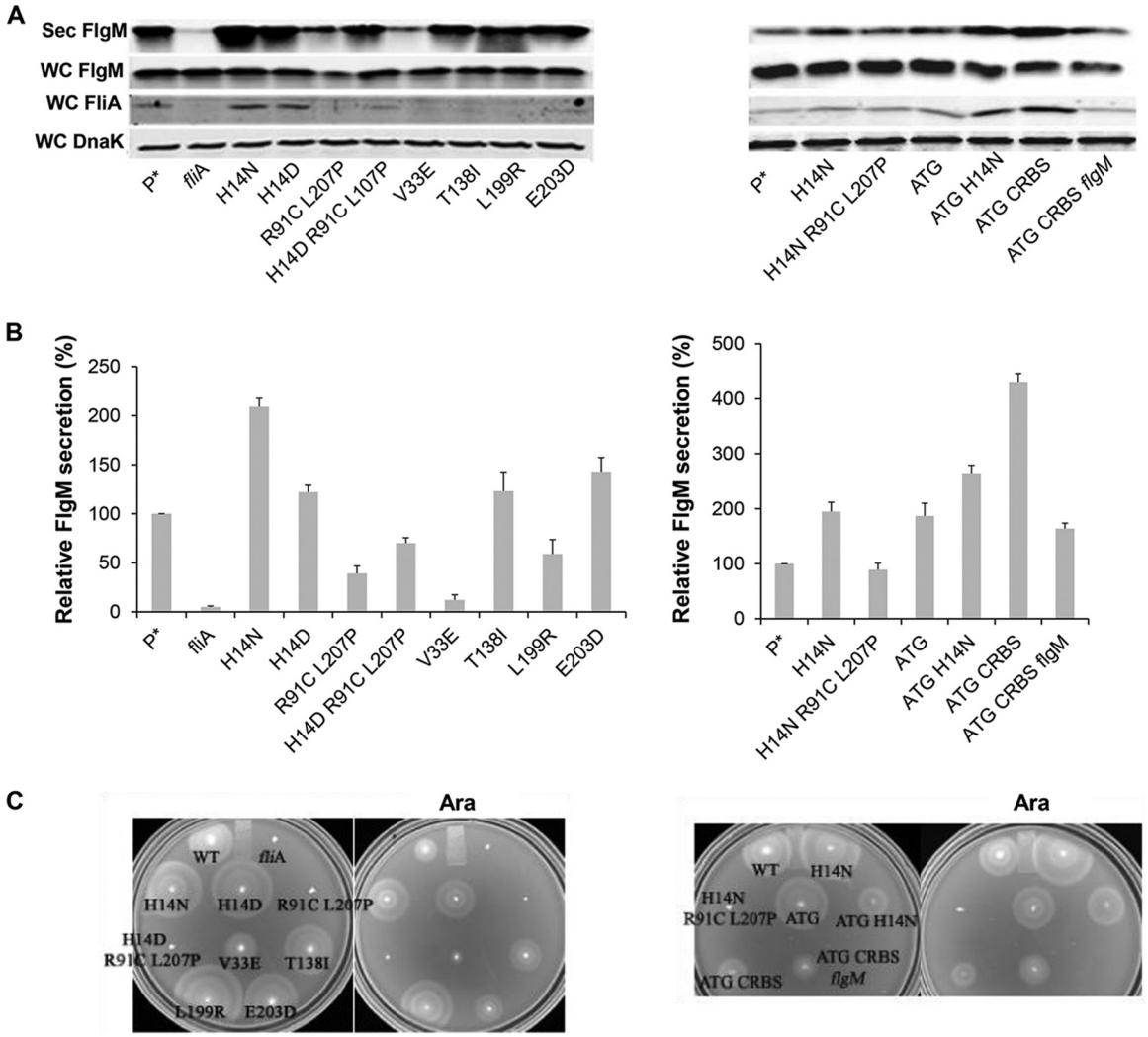

FIG 3 Effect of *fliA* (σ^{28}) alleles on levels of secreted FlgM and cell motility. (A) Levels of secreted FlgM were determined for strains carrying different alleles of the σ^{28} structural gene *fliA* by quantitative Western blot analysis using anti-FlgM antibody to detect FlgM in the supernatant of the spent growth medium (Sec FlgM, secreted FlgM; WC FlgM, whole cellular FlgM; WC FliA, whole cellular FliA; WC DnaK, whole cellular DnaK). P*, parent strain, TH18500 (Δ*araBAD1156*::*flgM*$^+$). (B) Relative levels of secreted FlgM. (C) Motility assay with various mutant backgrounds, depicted with the Δ*araBAD1156*::*flgM*$^+$ strain (WT), in the absence and presence of added 0.2% arabinose.

junction proteins FlgK and FlgL, 5 for the filament capping protein FliD, and depending on filament length, up to 20,000 for FliC or FljB. The results, presented in Fig. 4A and B, show that removal of FlgK, FlgL, or FliD has little or no effect on secreted FlgM levels, while removal of the filament substrates results in increased levels of secreted FlgM, although only up to 1.9-fold higher than the wild-type level. This result suggests that late T3S substrate levels are not a significant limiting factor in the secretion of FlgM through the flagella.

We also tested the effects of flagellar phase variation on FlgM secretion (Fig. 4A and B). *Salmonella enterica* alternately expresses one of two flagellin subunit genes, *fliC* or *fljB*. Strains carrying the *hin-5717* allele are locked in the FliC^{ON} FljB^{OFF} flagellin expression mode, while strains carrying the *hin-5718* allele are FliC^{OFF} FljB^{ON} (41). *fljB*^{enx} *vh2* is a historical relic that is also FliC^{ON} FljB^{OFF} and resulted from replacement of the *hin-fljBA* region from *S. enterica* strain LT2 with the same region from *Salmonella enterica* serovar Abortus-equi locked in the FliC^{ON} FljB^{OFF} mode (42). The FlgM secretion level in a strain carrying the *hin-5717* allele was similar to that of the wild-type strain. Deletion of the *fliC* gene in the *hin-5717* background resulted in a 1.9-fold increase in secreted FlgM compared to that for the wild type. The presence of the *hin-5718* allele alone increased secreted FlgM levels by 1.9-fold, and the additional removal of the *fliC* gene in this background further increased the secreted FlgM level 2.7-fold that of the wild type even though FliC flagellin is not produced in the *hin-5718* background. However, *fliC* mRNA is produced but not translated in the *hin-5718* background (41), suggesting that *fliC* mRNA has a negative effect on FlgM protein secretion. Secreted levels of FlgM in the *fljB*^{enx} *vh2* background were 2.9-fold higher than those in the Δ*hin-5717* strain, which is also locked for *fliC*^{ON} expression, and further removal of the *fliC* gene in this background increased secreted FlgM levels about 5-fold of those of the Δ*hin-5717* strain. We also observed a 2-fold increase in secreted

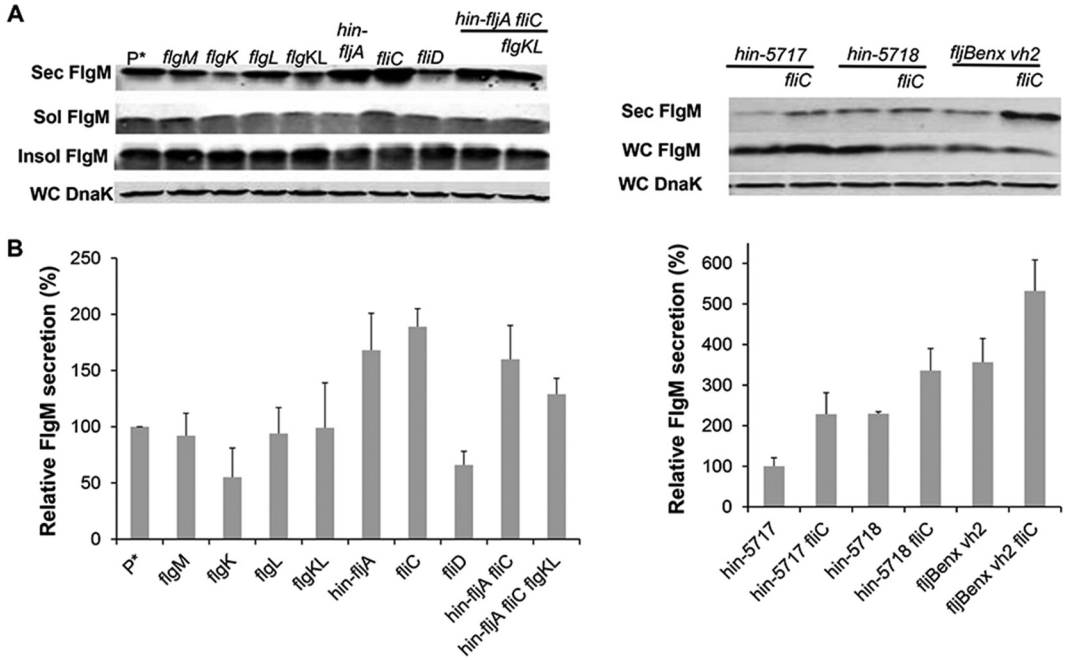

FIG 4 Effect of flagellar late substrate deletion, growth phase, and *fljB*^{enx} *vh2* mutations on levels of secreted FlgM. (A) Secreted levels of FlgM were determined for strains carrying different flagellum late substrate gene (*flgK*, *flgL*, *fliC*, *fliD*, and *fljB*) deletion mutants, flagellum-specific phase (Δ*hin-5717* and Δ*hin-5718*) mutants, and *fljB*^{enx} *vh2* mutants by quantitative Western blot analysis using anti-FlgM antibody to detected FlgM in the supernatant of the spent growth medium. Sec FlgM, secreted FlgM; Sol FlgM, cellular soluble FlgM; Insol FlgM, cellular insoluble FlgM; WC DnaK, whole cellular DnaK. P*, parent strain, TH18500 (Δ*araBAD1156*::*flgM*$^{+}$). (B) Relative secreted FlgM levels.

FlgM in the locked *fljB*^{ON} background, which could be accounted for by differences in *fliC* and *fljB* promoter strengths. The reason for the difference between the two locked *fliC*^{ON} strain backgrounds (*fljB*^{enx} *vh2* and Δ*hin-5717*) remains to be determined.

The effect of the flagellar late T3S chaperones on FlgM secretion. Next, we tested the effects of removing the late secretion chaperones FlgN, FliS, and FliT on secreted levels of FlgM expressed from P_{araBAD}. We reasoned that they might compete with σ^{28} for delivery of FlgM to the flagellar secretion system for export. Also, T3S chaperones are associated with regulatory functions in the absence of their cognate secretion substrates. FlgN, the T3S chaperone for FlgK and FlgL, is reported to inhibit *flgM* mRNA translation (43), σ^{28} is a transcription factor for flagellar class 3 promoters (6), and FliT acts as an anti-$FlhD_4C_2$ factor (28). Only FliS is not reported to have a regulatory function in the absence of its cognate secretion substrates, FliC and FljB. However, FliS has recently been shown to bind FlgM with a 1 FliS:1 FlgM stoichiometry, suggesting it does have some regulatory role that has yet to be elucidated (44, 45).

Removal of FlgN had little effect on FlgM secretion (Fig. 5A and B). This was expected, since FlgK and FlgL did not have a significant effect on secreted FlgM levels either (Fig. 4A and B). Removal of FliT, which is also analyzed in Fig. 1, resulted in a 4-fold increase in secreted FlgM levels. We do not have alleles of *fliT* that separate its anti-$FlhD_4C_2$ activity from its chaperone activity. Thus, the increase in secreted FlgM levels is likely due to enhanced $FlhD_4C_2$ activity in the absence of FliT. We did observe a 3-fold increase in secreted FlgM levels in the absence of FliS. However, *fliS* is transcribed in an operon upstream of the *fliT* gene. Thus, any polar effect of the *fliS* alleles on *fliT* would result in increased secreted levels of FlgM due to decreased *fliT* gene expression. Since we do not have a null allele of *fliS* resulting from missense mutation, we could not determine an effect of FliS removal on FlgM secretion that was independent of the effects of *fliS* deletion on FliT expression.

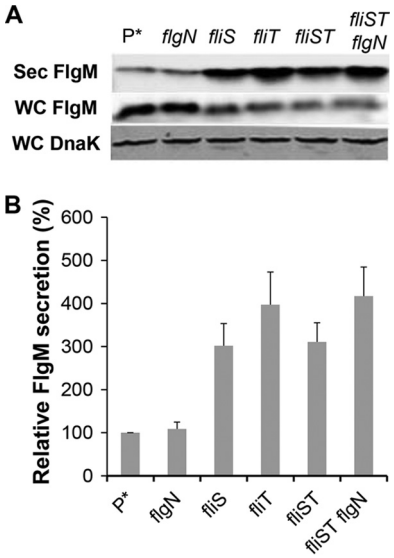

FIG 5 Effects of late flagellum T3S chaperone deletion mutations on levels of secreted FlgM. (A) Secreted levels of FlgM were determined for strains carrying different deletion mutant alleles of the flagellar T3S chaperone genes *flgN*, *fliS*, and *fliT* by quantitative Western blot analysis using anti-FlgM antibody to detect FlgM in the supernatant of the spent growth medium. Sec FlgM, secreted FlgM; WC FlgM, whole cellular FlgM; WC DnaK, whole cellular DnaK. P*, parent strain, TH18500 (Δ*araBAD1156*::*flgM*$^{+}$). (B) Relative levels of secreted FlgM.

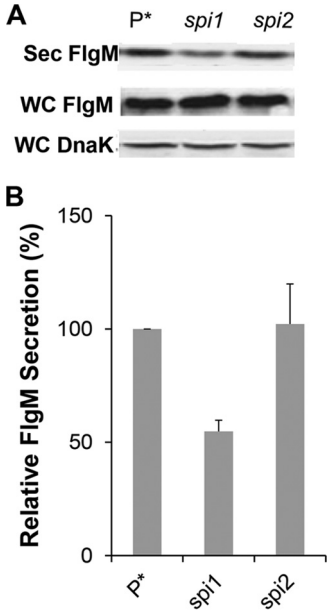

FIG 6 Effects of Spi-1 and Spi-2 deletions on levels of secreted FlgM. (A) Secreted levels of FlgM were determined for strains carrying deletion mutant alleles of the either the Spi1 or Spi2 gene by quantitative Western blot analysis using anti-FlgM antibody to detect FlgM in the supernatant of the spent growth medium. Sec FlgM, secreted FlgM; WC FlgM, whole cellular FlgM; WC DnaK, whole cellular DnaK. P*, parent strain, TH18500 (Δ*araBAD1156*::*flgM*$^+$). (B) Relative levels of secreted FlgM.

Deletion of *Salmonella* pathogenicity island 1 (Spi1) results in increased levels of secreted FlgM. The *flhDC* operon is the master operon of both the flagellar regulon and the genes of Spi1 (46, 47). The Spi1 regulon encodes genes needed for the structure and assembly of the Spi1 injectisome T3S system. The *fliZ* gene is transcribed in the *fliAZ* operon, and FliZ activates the Spi1 transcriptional activator HilD, which in turn activates Spi1 transcription under high salt (200 mM NaCl) microaerobic growth conditions (48). One HilD-activated gene product, RtsB, acts to repress *flhDC* transcription, providing a feedback loop for the entire flagellar-Spi1 regulon (49). Recent work has demonstrated that HilD activates *flhDC* transcription late in the cell's growth phase under non-Spi1 induction conditions (50, 51). We tested the effects of deletions of both the Spi1 and Spi2 *Salmonella* virulence systems on the secreted levels of overexpressed FlgM (from P_{araBAD}). The loss of Spi1 resulted in decreased FlgM secretion levels, to 55% of that of the wild type under non-Spi1-inducing conditions (aerobic, 86 mM NaCl), whereas loss of Spi2 had no significant effect on secreted levels of FlgM (Fig. 6A and B).

Effect of protease removal on FlgM secretion levels. A common technique used to improve protein yield from the cytoplasm is removing cellular proteases. In addition, proteases present in the outer membrane, such as OmpT, can decrease protein yield by degradation after cell lysis (52, 53). The ClpA and ClpX proteins interact with different substrates for delivery to the ClpP serine protease for degradation (54). DegP is a periplasmic protease that exhibits broad substrate specificity. DegP is exclusively directed against unfolded, mislocalized, hybrid and recombinant proteins that are improperly folded from overexpression in the periplasm (55, 56).

The results of protease removal on FlgM secretion levels are shown in Fig. 7. Removal of OmpT resulted in a slight increase in the yield of secreted FlgM, while loss of DegP had little effect. Removal of ClpA, ClpX, and ClpP increased the FlgM secretion yield by 1.7-, 5.4-, and 6.1-fold, respectively, compared to that of the wild type (Fig. 7A and B). This is consistent with the observation that $FlhD_4C_2$ is a substrate for YdiV-directed degradation by the ClpXP protease system (32, 33). As a control for cell lysis, deletion of *flhDC* in the protease mutant backgrounds showed no detectable FlgM in the secreted fraction. In this background, the cellular level of FlgM remained unaffected by loss of DegP mutation, while null alleles of *clpA*, *clpX*, and *clpP* mutation in the *flhDC* background increased intracellular FlgM accumulation by 1.3-, 1.5-, and 2.1-fold, respectively (Fig. 7B). Our results suggest that ClpA, ClpX, and ClpP are involved in FlgM degradation independent of FlhDC; the increased FlgM secretion in the *clpA* null strain was due to an increased cellular level of FlgM alone. The effects of the ClpXP protease in the presence of *flhD*$^+$*C*$^+$ were likely due to both FlgM stability and $FlhD_4C_2$ stability. This is consistent with increased motility observed in the *clpP* and *clpX* mutant strains (Fig. 7C).

Effect of ionic strength on secreted FlgM levels. It was reported that high osmolarity increased Spi1 *invA* gene transcription in *S. enterica* and that Spi1-dependent type III secretion occurred only in bacteria grown under high-salt conditions (57). We tested the effects of either increased NaCl or KCl on secreted levels of FlgM produced from P_{araBAD}-*flgM*$^+$. FlgM secreted levels increased when the NaCl concentration was raised to 200 mM and then dropped at concentrations of 400 and 600 mM. At 200 mM NaCl, the level of secreted FlgM was 3.7-fold higher than the level at 100 mM NaCl (Fig. 8A and B), which is close to the NaCl concentration in our standard LB medium (86 mM). Thus, the NaCl concentration in LB medium is not optimal for FlgM secretion. KCl had a similar effect on secreted FlgM levels; 200 and 400 mM KCl produced the highest levels of secreted FlgM, at 3.2- and 3.6-fold compared to the secreted FlgM level at 100 mM NaCl. This could be due to effects on the solubility of FlgM in the cytoplasm. Increased ionic strength also had a positive effect on motility in soft agar, although this was suppressed under FlgM induction conditions, possibly due to inhibition of σ^{28}-dependent flagellar class 3 transcription (Fig. 8C).

Effect of multiple conditions on FlgM secretion. Individual results described above that enhanced the yield of secreted FlgM were combined in order to obtain a strain and conditions that maximized this yield under conditions of FlgM overexpression from P_{araBAD}::*flgM*$^+$ (Fig. 9A and B). Some of these strains, such as the *fljB*^{enx} *vh2* strain, strains containing *clpX* mutations, and strains containing *flhD8089*, provided the highest FlgM secretion levels. All of them produced secreted FlgM levels at about 5-fold that of the wild type. In these strains, only a trace amount of FlgM accumulated within the cell, suggesting that expression and stability of cellular FlgM were limiting.

Use of FlgM as a T3S signal to secrete δ-SVIE, MrVIA, AMA1, and NCR peptides. We tested the use of FlgM as a secretion signal to secrete and purify peptides and proteins that are otherwise difficult to purify. For this purpose, we asked collaborators to provide DNA clones of proteins they were unable to purify by standard methods. These include a malarial surface protein, AMA-1 (apical membrane antigen 1), synthesized by *Plasmodium falciparum*, in collaboration with Ray Norton, the defensin-like peptides from *Sinorhizobium meliloti*, NCR (nodule-specific cysteine-rich) peptides, in collaboration with Graham Walker, and the neuroac-

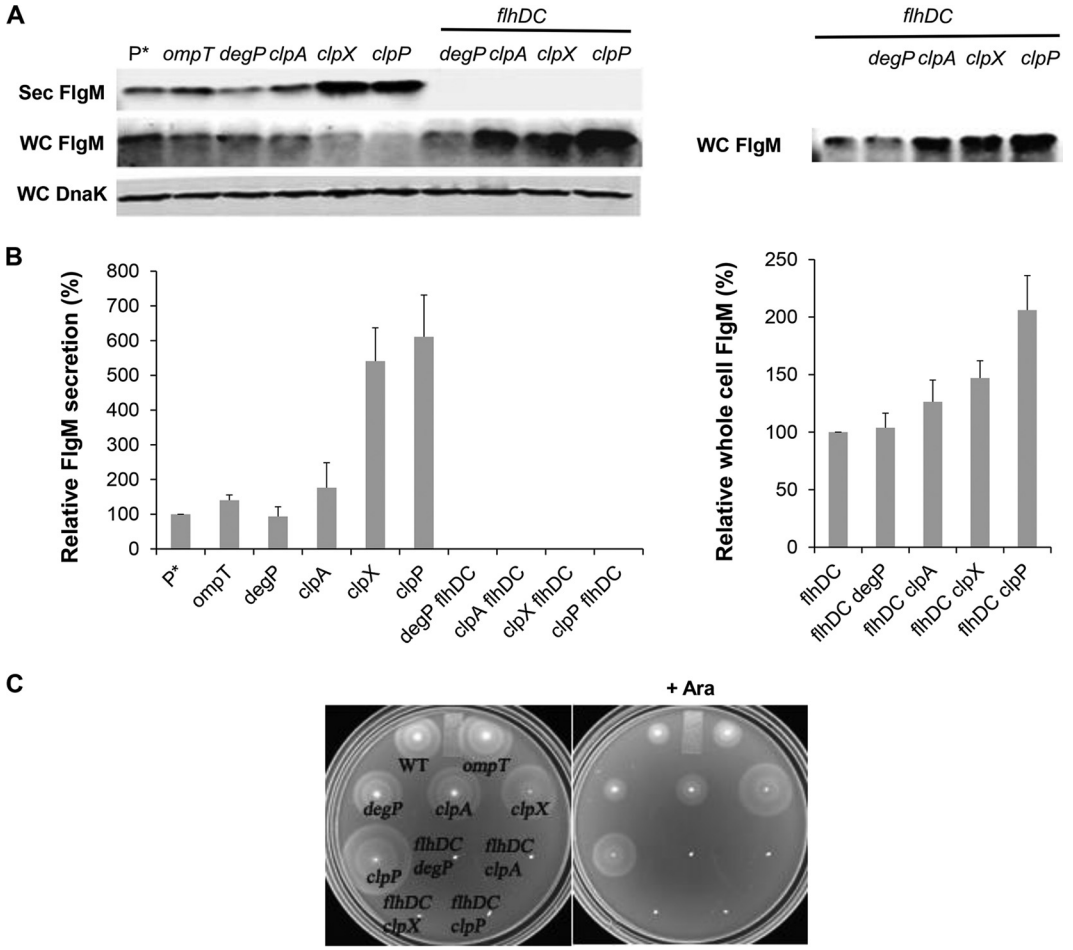

FIG 7 Effects of cellular protease mutant alleles on levels of secreted FlgM and cell motility. (A) Secreted levels of FlgM were determined for strains carrying deletion mutant alleles of the *ompT*, *degP*, *clpA*, *clpX*, or *clpP* gene with and without a functional *flhDC* operon by quantitative Western blot analysis using anti-FlgM antibody to detect FlgM in the supernatant of the spent growth medium. Sec FlgM, secreted FlgM; WC FlgM, whole cellular FlgM; WC DnaK, whole cellular DnaK. P*, parent strain, TH18500 (Δ*araBAD1156*::*flgM*$^+$). (B) Relative levels of secreted FlgM in the *flhD*$^+$*C*$^+$ and *flhDC* null backgrounds and relative whole-cell FlgM accumulation level in *flhDC* null background. (C) Motility assay in various mutant backgrounds, depicted with the Δ*araBAD1156*::*flgM*$^+$ strain (WT) in the absence and presence of added 0.2% arabinose.

tive conopeptides from *Conus* sp., δ-SVIE and MrVIA, in collaboration with Baldomero Olivera. The δ-SVIE protein is a small peptide produced by a venomous marine cone snail and inhibits sodium channels in vertebrate neuromuscular systems. This 31-amino-acid peptide (DGCSSGGTFCGIHPGLCCSEFCFLWCIT FID) is hydrophobic and contains 6 cysteine residues that form 3 pairs of intramolecular disulfide bonds. The hydrophobic nature of the peptide and requirement for multiple disulfide bond formation to produce an active conformation of δ-SVIE are an impediment to proper folding when this peptide is overexpressed in *E. coli*. We wanted to test whether δ-SVIE could be produced in an active form via FlgM-mediated secretion. We reasoned that because secretion from the cell initiates with the N terminus of FlgM, δ-SVIE sequence of the FlgM-δ-SVIE fusion would exit the cell as it exits the ribosome: from the N terminus to the C terminus. This might facilitate proper folding and disulfide formation of δ-SVIE into an active conformation. C-terminal fusions of δ-SVIE to hexa-His-tagged (6His) FlgM with either TEV (tobacco etch virus) or enterokinase (ETK) protease cleavage sites engineered between the two proteins were expressed from the chromosomal P_{araBAD} expression locus. The 6His tag facilitates purification using a Ni-agarose affinity column, and the TEV and ETK cleavage sites are recognized by TEV protease and enterokinase, respectively, allowing the secreted δ-SVIE to be separated from its FlgM secretion signal.

All of the selected single mutants increased FlgM-6His-TEV-δ-SVIE secretion (see Fig. 10A and B). As expected, the secreted levels of P_{araBAD}-expressed fusion protein levels were enhanced in mutant backgrounds that produced higher secreted FlgM levels. Secreted levels of FlgM-6His-TEV-δ-SVIE in the *flhD7793* (TH18880), *flhD8089* (TH18647), *fliT* (TH18769), *rcsB* (TH18830), *clpX* (TH18353), and *fliA* (CRBS ATG) (TH20056) strains were 16.8-, 10.9-, 9.4-, 6.3-, 16.7-, and 16.6-fold that of the wild type (TH17831). We also combined different mutations together to test FlgM-6His-TEV-δ-SVIE secretion levels. In the multiple mutant background *flhD7793 rcsB* (TH19675), *flhD7793 rcsB flgM* (TH20044), *rcsB fliT* (TH19673), *fljB*^{enx} *vh2 fliB-fliT* (TH20075), *fljB*^{enx} *vh2 fliC* (TH20050), *fljB*^{enx} *vh2 clpX* (TH20077), and *flhD8089 fljB*^{enx} *vh2 clpX* (TH20044) strains, levels were 34-, 17-, 49-, 16.7-, 11.7-, 28.2-, and 52.7-fold of that of the wild type. When 6His-

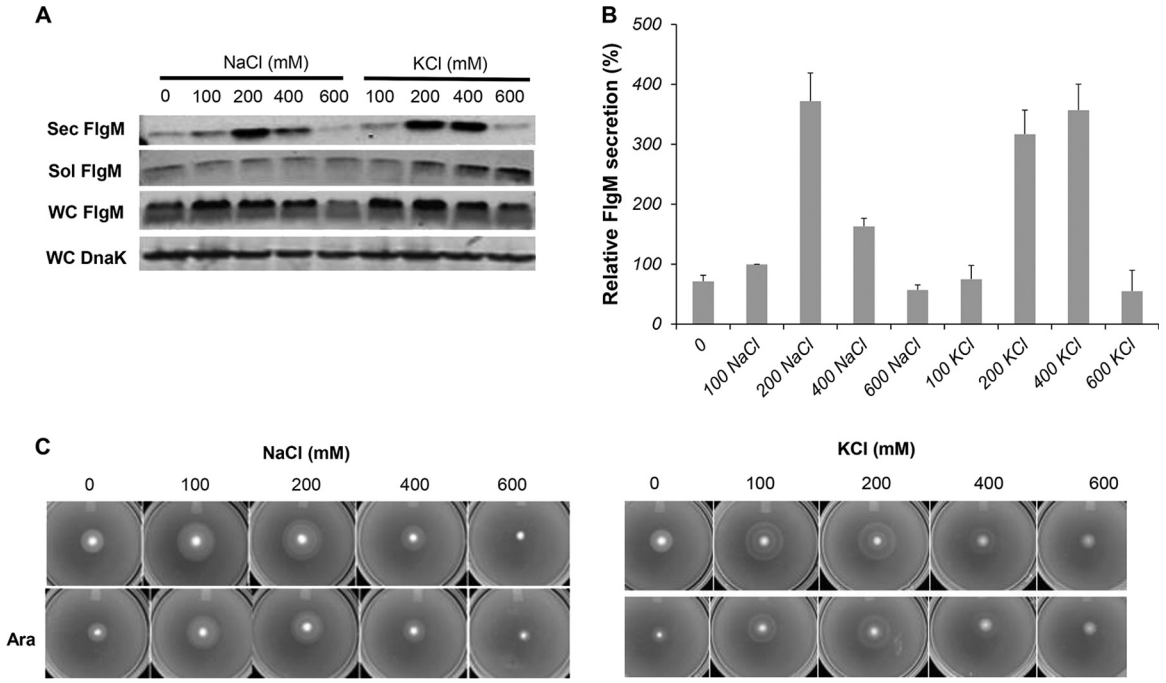

FIG 8 Effects of NaCl and KCl ionic strengths on levels of secreted FlgM and cell motility. (A) Secreted levels of FlgM were determined with strain P*, the parent strain, TH18500 (Δ*araBAD1156*::*flgM*$^{+}$), in LB-0.2% arabinose medium with NaCl and KCl added at concentrations depicted by quantitative Western blot analysis, using anti-FlgM antibody to detect FlgM in the supernatant of the spent growth medium. Sec FlgM, secreted FlgM; Sol FlgM, cellular soluble FlgM; Insol FlgM, cellular insoluble FlgM; WC DnaK, whole cellular DnaK. (B) Relative levels of secreted FlgM. (C) Motility assay using various concentrations of NaCl and KCl, depicted with strain TH18500, in the absence and presence of added 0.2% arabinose (Ara).

ETK was inserted between FlgM and SVIE, the *flhD7793* (TH19118), *flhD7793 flgM* (TH19122), *flhD7793 flgM clpX* (TH19145), and *flhD7793 lrhA fliB-fliT ydiV hin-fljA flgMN flgKL* (TH19120) strains increased FlgM-6His-ETK-δ-SVIE secretion levels up to 19-, 8.5-, 17-, and 110-fold of that of wild type. Except for the *flhD7793 lrhA fliB-fliT ydiV hin-flj*A *flgMN flgKL* strain (TH19120), addition of 100 mM NaCl resulted in increased secretion levels of the fusion protein.

Figure 11 shows the secretion of P_{araBAD}-expressed FlgM-6His-ETK-AMA1, FlgM-6His-ETK-NCR, and FlgM-6His-ETK-MrVIA constructs in the *flhDC7793*, *lrhA*, *ydiV fliB-fliT hin-fljBA*, *flgMN*, and *flgKL* mutant backgrounds. The culture supernatant was concentrated 20-fold by TCA precipitation and run on SDS-PAGE gels. Coomassie staining revealed the FlgM fusion constructs to be the prominent proteins present in the cell supernatant.

DISCUSSION

The type III secretion (T3S) systems of the flagellum and injectisome provide a conduit for the production and purification of recombinant proteins that are fused to the T3S signal. Although the nature of the T3S signal is not fully understood, there appears to be a universal requirement for an N-terminal disordered peptide signal and a coupling to the proton motive force as the secretion fuel (58, 59). Many but not all T3S substrates require cognate T3S chaperones for their stability in the cytoplasm and/or as secretion pilots for targeting the T3S substrate to the secretion apparatus in the cytoplasmic membrane. Another feature of T3S systems is the ability to undergo a secretion specificity switch from early to late secretion substrate specificity. In the flagellar system, this occurs upon completion of extracellular hook growth that is more than 40 nm in length, resulting in the transition to filament-type substrate secretion and assembly (60, 61). The FlgM protein is an integral part of the transition from hook completion to filament assembly. FlgM is a late, filament-type flagellar secretion substrate and an anti-σ^{28} factor. FlgM is a small, 97-amino-acid protein. The N-terminal half of FlgM includes the T3S signal, while the C-terminal half includes the anti-σ^{28} interaction domain. It was initially determined that either domain of FlgM could serve as a late flagellar secretion substrate. Later it was found that σ^{28} was the T3S chaperone for FlgM, which accounted for the ability for low-level C-terminal FlgM secretion to occur in the absence of the N-terminal secretion signal. It has been shown that fusion of recombinant proteins to the C terminus of FlgM would allow for their secretion and purification without a requirement to lyse cells prior to purification (16). In this study, we examined conditions that might facilitate FlgM production and secretion. These conditions were then applied to produce the recombination proteinsFlgM-6His-TEV-δ-SVIE,FlgM-6His-ETK-δ-SVIE,FlgM-6His-ETK-MrVIA, FlgM-6His-ETK-AMA1, and FlgM-6His-ETK-NCR peptides to produce and purify these proteins without cell lysis using FlgM as a vector to direct the secretion of the fused peptide/protein from the cell via the flagellar T3S system.

FlgM and FlgM fusion constructs were overexpressed from the chromosomal P_{araBAD} promoter by replacing the *araBAD* coding region with the *flgM* or FlgM fusion coding regions. By removing *araBAD*, we ensured that an arabinose inducer would not be consumed as a carbon source. This inducing system proved sufficient to produce FlgM in quantities in excess of what the cell could secrete (Fig. 2). We then manipulated the cells by introducing

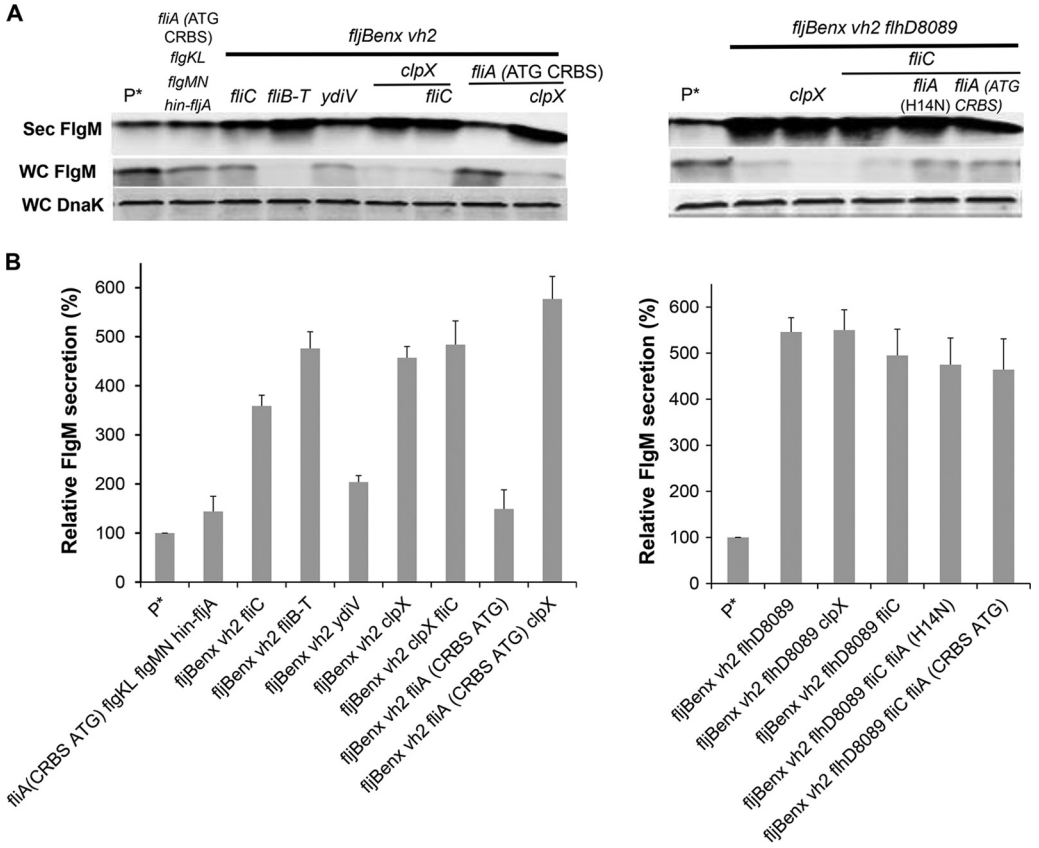

FIG 9 Effects of different combined mutations on levels of secreted FlgM. (A) Secreted levels of FlgM were determined for strains carrying different combined mutations, depicted by quantitative Western blot analysis using anti-FlgM antibody to detect FlgM in the supernatant of the spent growth medium (Sec FlgM, secreted FlgM; WC FlgM, whole cellular FlgM; WC DnaK, whole cellular DnaK). P*, parent strain, TH18500 (Δ*araBAD1156*::*flgM*$^{+}$). (B) Relative levels of secreted FlgM.

mutations that might increase FlgM stability and secretion in order to increase production of secreted FlgM.

Initially we examined the effect of mutations that were known to increase the number of flagellar T3S systems per cell on secreted FlgM levels. These included null mutations in known negative regulators of *flhDC* transcription (*ecnR*, *rcsB*, *lrhA*, and *dskA*) and *flhDC* promoter up alleles, P_{flhDC}*7793* and P_{flhDC}*8089*. We also tested null alleles of *fliT* and *ydiV* that inhibit $FlhD_4C_2$ function at a posttranscriptional level. Such mutations had been shown to increase the production and secretion of the flagellar hook protein into the periplasm. All mutant backgrounds tested resulted in increased levels of secreted FlgM. After determining the effects of different mutations on secreted levels of FlgM, we tried to combine our observations to construct an optimized secretion strain to maximize the amount of FlgM secreted from the cell. We found that all of the strains containing *fliB-fliT*, *clp*X, or *flhD8089* increased FlgM secretion by about 5-fold that of the wild-type strain, but the combination did not increase FlgM secretion further. This suggests that there is a limit on the number of flagellar secretion portals (hook-basal bodies) that can be achieved by increased $FlhD_4C_2$-dependent transcription and the maximal effect was achieved with the individual mutations. Under conditions of increased $FlhD_4C_2$-dependent transcription, it is possible that the cellular levels of FlgM became a limiting factor in FlgM secretion.

We explored the effects of various alleles of the FlgM T3S chaperone σ^{28}, encoded by the *fliA* gene. Any allele that resulted in increased cellular levels of σ^{28} resulted in a corresponding increase in FlgM secretion, especially under FlgM overexpression conditions. These results are consistent with the role of σ^{28} as the T3S chaperone for FlgM.

Removal of late secretion competitors of FlgM secretion or their cognate chaperones had mixed results. Of the 4 secretion competitors FlgK, FlgL, FliD, and FliC/FljB, only removal of the filament late secretion substrates FliC/FljB had a significant effect on FlgM secretion. This is not surprising, since the amount of filament subunits in the flagellum is about 1,000 times that of the other three components.

Removal of the filament T3S chaperone protein, FliS, resulted in a 3-fold increase in secreted FlgM levels, while removal of the FlgK and FlgL T3S chaperone FlgN had no effect. Recently, FliS was shown to bind FlgM, but FliS was not able to dislodge FlgM in complex with σ^{28} (44, 45). Since *fliS* and *fliT* are cotranscribed in the same operon (*fliS* is upstream of *fliT*), any deletion mutation of *fliS* may affect *fliT* gene expression. In this work, we could not conclude how removal of FliS might affect FlgM secretion. Unexpectedly, a flagellar phase variation mutant allele, *fljB*^{enx} *vh2*, showed a significant increase in secreted FlgM levels compared to results with the *hin-5717* allele.

We observed decreased secreted FlgM levels in a Spi1 deletion strain. This is in agreement with recent results demonstrating a

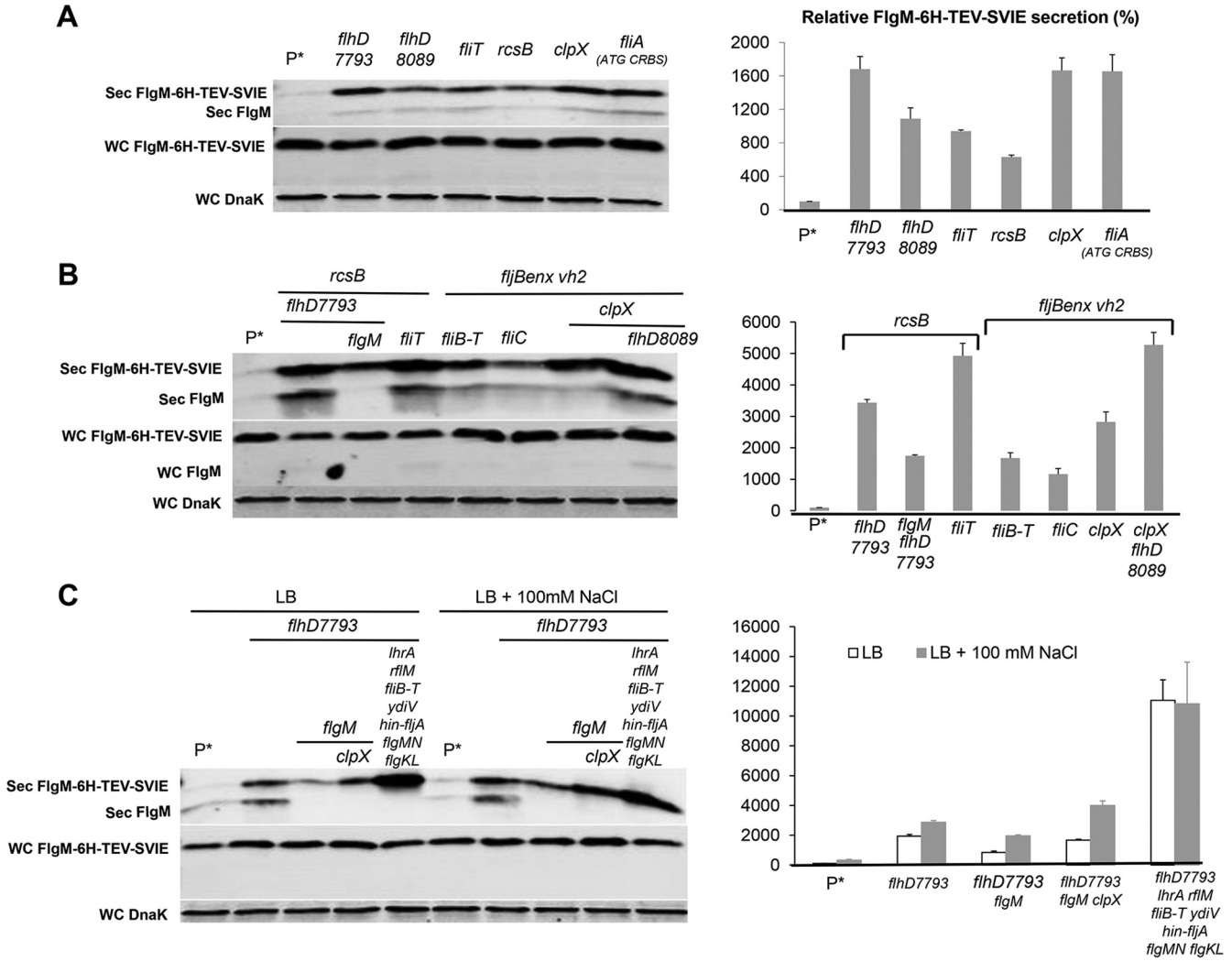

FIG 10 Effects of different combined mutations and added salt concentrations on levels of secreted FlgM-6His-TEV-δ-SVIE and FlgM-6His-ETK-δ-SVIE. (A, B, and C) Levels of secreted FlgM-6His-TEV-δ-SVIE and FlgM-6His-ETK-δ-SVIE were determined for strains carrying different combined mutations, depicted by quantitative Western blot analysis using anti-FlgM antibody to detected FlgM-6His-TEV-δ-SVIE and FlgM-6His-ETK-δ-SVIE in the supernatant of the spent growth medium (Sec FlgM-6His-TEV-SVIE, secreted FlgM-6His-TEV-δ-SVIE; Sec FlgM-6H-ETK-δ-SVIE, secreted FlgM-6His-ETK-δ-SVIE; Sec FlgM, secreted FlgM; WC DnaK, whole cellular DnaK). Relative levels of secreted FlgM-6His-TEV-δ-SVIE and FlgM-6His-ETK-δ-SVIE are presented at the right of the gels. P*, parent strain, TH17831 (Δ*araBAD1124*::*flgM*-6His-*TEV*-δ-*SVIE*).

positive effect of the Spi1 transcription factor HilD on *flhDC* transcription under Spi1-noninducing conditions (50, 51).

Removal of cellular proteases also produced mixed results on FlgM secretion. The ClpXP protease was known to regulate the number of flagella per cell by degradation of the $FlhD_4C_2$ complex, which is directed by the YdiV protein. YdiV is produced during poor nutrient growth conditions. YdiV binds the FlhD component of $FlhD_4C_2$, which prevents further interaction between $FlhD_4C_2$ and DNA. YdiV then targets $FlhD_4C_2$ to the ClpXP protease for degradation (32, 33). As expected, removal of either ClpX or ClpP resulted in increased FlgM secreted levels. Removal of the DegP or OmpT protease was also tested for effects on FlgM secretion, and no effect was observed.

The last variable tested for secreted levels of overexpressed FlgM was ionic strength. It was reported that type III secretion was induced by higher osmolarity (57). We tested the effect of NaCl and KCl concentrations on FlgM secretion and observed that addition of NaCl to 200 mM or KCl to 200 to 400 mM resulted in an about 4-fold increase in secreted FlgM levels compared to results with 100 mM NaCl, which is close to the concentration of NaCl in LB (0.5%, or 86 mM). It was possible that the NaCl effect was due to increased potential of the proton motive force. However, the same effect was observed with the addition of KCl, suggesting that it is ionic strength that controls secreted FlgM levels. It is possible that ionic strength simply results in increased stability of $FlhD_4C_2$, but this remains to be determined.

In summary, while many factors could be altered to increase FlgM secretion, combinations of these factors were not additive, and the rate of FlgM secretion appeared limited. The apparent rate of FlgM secretion was enhanced by increasing σ^{28} levels to facilitate FlgM delivery to hook-basal body (HBB) secretion conduits but again to a limited extent, since intracellular FlgM still accumulated. The effect of increased $FlhD_4C_2$-dependent transcription on the number of HBB structures did enhance secreted FlgM

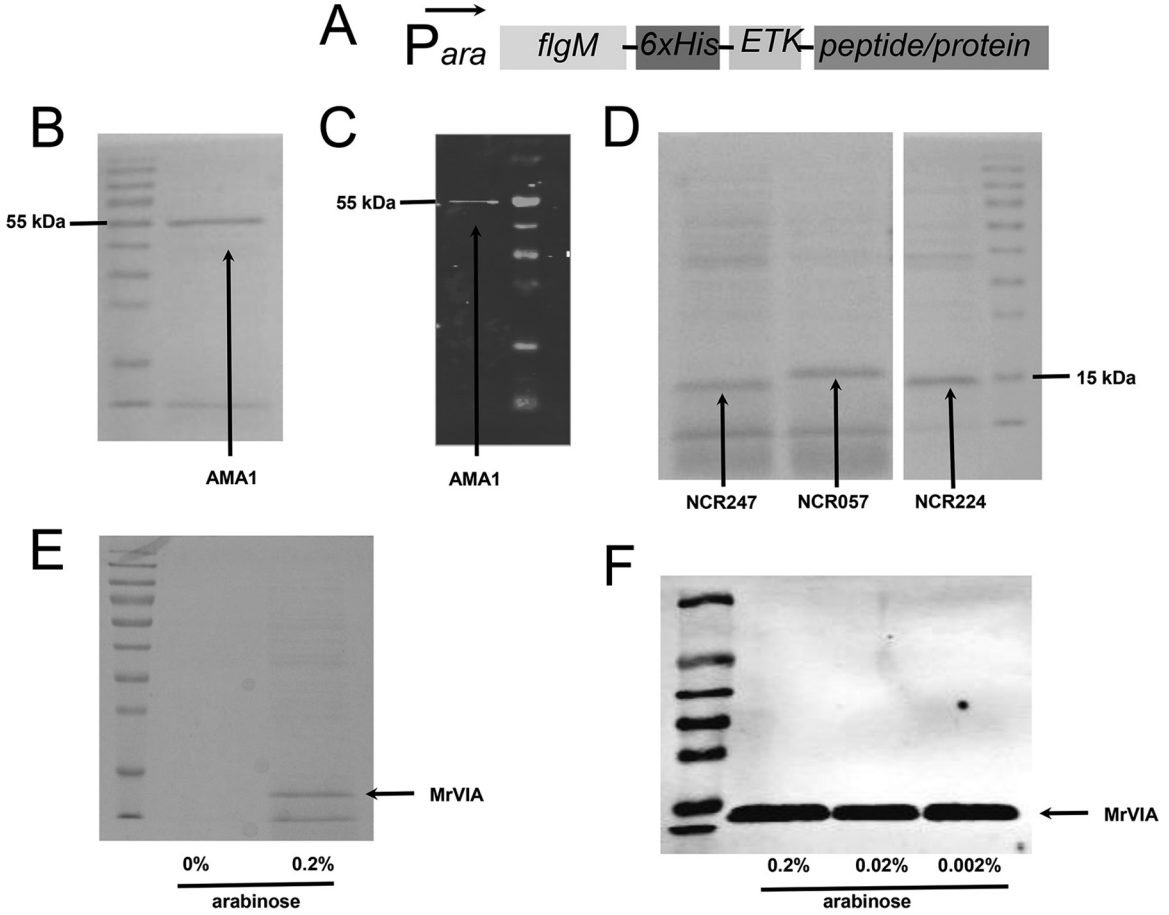

FIG 11 Secretion of AMA1, NCR, and MrVIA peptides fused to a His-tagged FlgM secretion signal. (A) Schematic diagram of the constructs tested. (B) Coomassie-stained SDS-PAGE gel of the supernatant from a culture expressing FlgM-His6-ETK-AMA1 in an optimized secretion strain (TH20685). The supernatant was concentrated 20-fold by TCA precipitation. (C) Western blot against 20 μl of nonconcentrated supernatant from panel B with anti-His antibody. (D) Coomassie-stained SDS-PAGE gel of the supernatant from cultures expressing three different NCR peptides fused to a His-tagged FlgM secretion signal in an optimized secretion strain background (NCR247, TH20221; NCR057, TH20222; NCR224, TH20223). The supernatants were concentrated 20-fold by TCA precipitation. (E) Coomassie-stained SDS-PAGE gel of the supernatant from a culture expressing conopeptide MrVIA fused to a His-tagged FlgM secretion signal in an optimized secretion strain background (TH20492). The supernatants were concentrated 20-fold by TCA precipitation. (F) Western blot against 20 μl of nonconcentrated supernatant from strain TH20492 (FlgM-His-ETK-MrVIA) with anti-His antibody grown under different arabinose induction concentrations.

levels but was also limited. This is consistent with previous results showing that increased $FlhD_4C_2$-dependent transcription produced at most a 2- to 3-fold increase in the number of HBB structures per cell (21). Increased ionic strength improved secreted FlgM levels but not when combined with other factors, suggesting that ionic strength contributed to other factors tested, such as enhanced FlhDC or σ^{28} levels or facilitation of σ^{28}-mediated FlgM secretion or $FlhD_4C_2$-dependent transcription.

Finally, we tested the practicality of the optimized FlgM secretion system to purify peptides which are otherwise difficult to produce by using standard bacterial expression systems. These included a cysteine-rich, hydrophobic peptide, contoxin δ-SVIE, nodule-specific, cysteine-rich (NCR) antimicrobial peptides produced by the root nodules of certain legumes, and a malaria surface antigen domain of apical membrane antigen AMA-1. Figure 10 shows the effects of different conditions on secretion of the P_{araBAD}-expressed FlgM-6His-ETK-δ-SVIE construct. As reported earlier, the *flhDC7793* promoter up allele resulted in a substantial increased in the level of secreted FlgM-6His-ETK-δ-SVIE. Removal of FlgM reduced the positive effect of the *flhDC7793* allele, probably due to increased expression of secretion competitors, which are upregulated in the absence of FlgM. A strain that combines the *flhDC7793* allele with mutations resulting in increased FlhDC stability (*lrhA*, *ydiV*, and *fliT* [within the *fliBCDST* deletion]) and mutations that remove secretion competitors (*fliC* [within the *fliBCDST* deletion], *fljB* [within the *hin-fljBA* deletion], *flgMN*, and *flgKL*) resulted in more than a 100-fold-increased secreted-protein level compare to that of the wild-type strain. For the FlgM-6His-ETK-MrVIA, FlgM-6His-ETK-AMA1, and FlgM-6His-ETK-NCR constructs, Coomassie-stained supernatants from cell cultures revealed the secreted FlgM-peptide fusions to be the prominent secreted proteins.

This work utilized the *Salmonella* Typhimurium strain LT2, which is attenuated for virulence. For commercial protein production, a similar strain background could be constructed and tested for *E. coli* K-12, or the Spi2 region of LT2 could simply be deleted to eliminate any virulence potential of *Salmonella* protein production strains using FlgM-mediated secretion.

ACKNOWLEDGMENTS

This work was supported by PHS grant GM056141 (to K.T.H.) from the National Institutes of Health and by a Technology Commercialization Project grant from the University of Utah.

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