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A Membrane-Embedded Amino Acid Couples the SpoIIQ Channel Protein to Anti-Sigma Factor Transcriptional Repression during *Bacillus subtilis* Sporulation

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ABSTRACT

SpoIIQ is an essential component of a channel connecting the developing forespore to the adjacent mother cell during *Bacillus subtilis* sporulation. This channel is generally required for late gene expression in the forespore, including that directed by the late-acting sigma factor σ^G. Here, we present evidence that SpoIIQ also participates in a previously unknown gene regulatory circuit that specifically represses expression of the gene encoding the anti-sigma factor CsfB, a potent inhibitor of σ^G. The *csfB* gene is ordinarily transcribed in the forespore only by the early-acting sigma factor σ^F. However, in a mutant lacking the highly conserved SpoIIQ transmembrane amino acid Tyr-28, *csfB* was also aberrantly transcribed later by σ^G, the very target of CsfB inhibition. This regulation of *csfB* by SpoIIQ Tyr-28 is specific, given that the expression of other σ^F-dependent genes was unaffected. Moreover, we identified a conserved element within the *csfB* promoter region that is both necessary and sufficient for SpoIIQ Tyr-28-mediated inhibition. These results indicate that SpoIIQ is a bifunctional protein that not only generally promotes σ^G activity in the forespore as a channel component but also specifically maximizes σ^G activity as part of a gene regulatory circuit that represses σ^G-dependent expression of its own inhibitor, CsfB. Finally, we demonstrate that SpoIIQ Tyr-28 is required for the proper localization and stability of the SpoIIE phosphatase, raising the possibility that these two multifunctional proteins cooperate to fine-tune developmental gene expression in the forespore at late times.

IMPORTANCE

Cellular development is orchestrated by gene regulatory networks that activate or repress developmental genes at the right time and place. Late gene expression in the developing *Bacillus subtilis* spore is directed by the alternative sigma factor σ^G. The activity of σ^G requires a channel apparatus through which the adjacent mother cell provides substrates that generally support gene expression. Here we report that the channel protein SpoIIQ also specifically maximizes σ^G activity as part of a previously unknown regulatory circuit that prevents σ^G from activating transcription of the gene encoding its own inhibitor, the anti-sigma factor CsfB. The discovery of this regulatory circuit significantly expands our understanding of the gene regulatory network controlling late gene expression in the developing *B. subtilis* spore.

Cellular development requires that complex molecular and morphological events occur in a precisely controlled spatiotemporal manner. Gene regulatory networks underlie and orchestrate these events, ensuring that the appropriate suites of developmental genes are activated or repressed at the right time and place (1). Endospore formation (sporulation) by the bacterium *Bacillus subtilis* is an ancient differentiation process and premier model system for studies of how gene regulatory networks drive prokaryotic development. Under favorable conditions, *B. subtilis* demonstrates vegetative growth by binary fission; however, when nutrients are depleted, *B. subtilis* cells embark upon an alternate cellular differentiation pathway toward quiescence, sporulation (2). Early in sporulation, an asymmetric cell division creates two compartments: a smaller forespore, which becomes the spore, and a larger mother cell, which aids in the development of the forespore but ultimately dies. The mother cell then engulfs the forespore in a phagocytosis-like process that results in a cell-within-a-cell configuration. As a consequence of engulfment, the forespore is separated from the mother cell by two membranes, the inner forespore membrane and the outer engulfing mother cell membrane. A protective peptidoglycan cortex and a protein coat are then deposited around the engulfed forespore, which is finally released as a mature spore into the environment upon lysis of the mother cell.

The aforementioned molecular and morphological events of sporulation, like those of developmental pathways in higher organisms, are orchestrated by a complex gene regulatory network (3, 4). At the core of the sporulation gene regulatory network are four RNA polymerase sigma (σ) subunits that coordinate distinct

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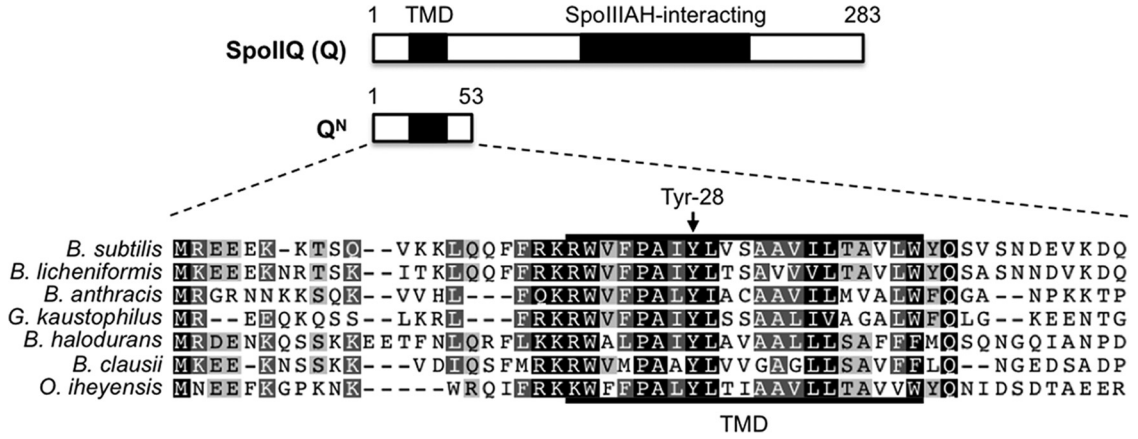

FIG 1 A highly conserved amino acid (Tyr-28) in SpoIIQ. (Top) Cartoon of the *B. subtilis* SpoIIQ (Q) protein, indicating its transmembrane domain (TMD) and the extracellular, SpoIIIAH (AH)-interacting domain. Q N-terminal residues 1 to 53 correspond to the Q^N truncation (middle) used in this study. (Bottom) A multiple-sequence alignment of the Q N terminus is shown for Q orthologs from various *Bacillus* and related species. Accession numbers for protein sequences are listed in Materials and Methods. Similar or identical residues are shaded light gray (60% identity), dark gray (80% identity), or black (100% identity). The conserved tyrosine (Tyr-28) residue is indicated.

programs of gene expression in the two developing cells. σ^F and σ^E direct gene expression at early times in the forespore and mother cell, respectively; at later times, σ^G replaces σ^F in the forespore, while σ^K replaces σ^E in the mother cell. This study is focused upon the gene regulatory circuitry that orchestrates the transition from early, σ^F-directed gene expression to late, σ^G-directed gene expression in the developing forespore. This σ^F-to-σ^G switch is tightly regulated such that overlap between the activities of the two sigma factors has not been detected (5); however, the molecular mechanisms that control the switch are not fully understood.

σ^F is made early in sporulation (prior to cell division) but is held inactive by the anti-sigma factor SpoIIAB (6, 7). Upon asymmetric cell division, σ^F is released from SpoIIAB inhibition in the forespore via a complex circuit involving the anti-anti-sigma factor SpoIIAA (8) and the membrane-embedded phosphatase SpoIIE, which dephosphorylates and activates SpoIIAA (9). SpoIIE also plays an earlier role in asymmetric division prior to σ^F activation (10, 11) and has further been observed to directly associate at later times with the forespore membrane protein SpoIIQ (Q), which is produced under the control of σ^F, though the function of this interaction is unknown (12). In addition to *spoIIQ* (*Q*), σ^F directs the transcription of other genes required for early forespore development, as well as *sigG*, the gene that encodes σ^G (*sigG* is also transcribed at later times in an autoregulatory loop by σ^G itself) (13). Another member of the σ^F regulon, *csfB*, encodes the anti-sigma factor CsfB (also called Gin, for σ^G inhibitor), which helps to delay σ^G activity until the early phase of σ^F-directed gene expression is complete (14–16). Still, deletion of *csfB* does not generally lead to premature/elevated activation of σ^G in the majority of sporulating cells (17, 18), indicating that other redundant regulatory mechanisms are also in place to keep σ^G activity in check at early times.

Upon the completion of engulfment, the early, σ^F-directed program of developmental gene expression is replaced by the late, σ^G-directed program. The inhibition of σ^F prior to this switch, among other unidentified mechanisms, is mediated by a small protein called Fin (previously called YabK) (19). To complete the switch, σ^G must escape direct inhibition by CsfB, though how this occurs is unknown. Interestingly, previous work has predicted that this involves, at least in part, a mechanism to prevent σ^G from activating further transcription of *csfB* during sporulation (20).

σ^G-directed gene activation also requires the assembly of a channel apparatus that connects the two cells and is comprised of the eight mother cell proteins SpoIIIAA-AH (AA-AH) and the forespore protein Q (21–26). In contrast to CsfB, this AA-AH·Q channel does not specifically regulate σ^G but, rather, is required more generally for any gene expression (i.e., even that directed by the heterologous phage T7 RNA polymerase) in the forespore (21). These findings, as well as the shrunken and collapsed forespores observed in mutants lacking channel genes (22), have led to a model in which the AA-AH·Q channel functions as a feeding tube through which the mother cell provides the forespore with essential nutrients and osmolytes at later stages of development.

As mentioned above, the forespore membrane protein Q is an essential component of the AA-AH·Q channel required for late, σ^G-directed gene expression in the forespore. The assembly of Q into this channel relies upon its extracellular C-terminal domain, which directly interacts with the AH channel protein anchored in the opposing mother cell membrane (24, 27). Additional interactions that have been experimentally detected between Q and other mother cell proteins await further characterization (28, 29). Q is anchored in the forespore membrane by an N-terminal transmembrane domain (TMD) that harbors several conserved amino acids (Fig. 1). One of these amino acids, Tyr-28, is conserved with 100% identity among *Bacillaceae* species with annotated Q orthologs. We previously found that phage T7 RNA polymerase (T7 RNAP), expressed in the forespore, was significantly more active during times coinciding with σ^G activity when Q Tyr-28 was replaced by Ala (Q^{Y28A}) (21). Remarkably, this Q^{Y28A} mutant phenotype was complemented with just an N-terminal fragment of Q that spans the TMD but lacks the C-terminal, AH-interacting domain (Q^N) (21). This finding suggested that the Q TMD and Tyr-28 in particular perform a secondary function that is distinct from the primary role of Q in channel formation and to which T7 RNAP is especially sensitive. However, the normal, physiological role for Q Tyr-28 in sporulation remains unclear.

Here we report that Q Tyr-28 participates in a previously unknown regulatory circuit required to prevent σ^G-dependent transcription of the gene encoding the σ^G inhibitor, CsfB. Consistent with the overproduction of CsfB, a Q^{Y28A} mutant fails to activate σ^G to wild-type (WT) levels. Importantly, other σ^F-dependent promoters do not display aberrant σ^G-dependent activation in the Q^{Y28A} mutant, indicating that Q Tyr-28 specifically regulates *csfB* expression. Bioinformatic and mutational analyses of the *csfB* promoter reveal a conserved promoter element that is both necessary and sufficient for Q Tyr-28-mediated inhibition. Together, these results support a model in which Q is a bifunctional protein that promotes the switch to σ^G activity at late times in the forespore in two ways: generally, as a component of the AA-AH·Q intercellular channel apparatus, and specifically, as a component of a regulatory circuit that represses *csfB* expression. Our data further suggest that Q may execute the latter function in collaboration with the multifunctional SpoIIE phosphatase, thereby coupling anti-sigma factor gene regulation not only to the AA-AH·Q channel but also to the earlier, SpoIIE-dependent processes of asymmetric cell division and σ^F activation.

MATERIALS AND METHODS

General methods. *B. subtilis* strains were maintained with Luria-Bertani (LB) medium in liquid cultures or on solid plates with 1.6% agar. *Escherichia coli* DH5α strains, each with a specific plasmid, were similarly maintained with LB medium including ampicillin (100 μg/ml). For all experiments in which sporulating cells were analyzed for *lacZ* reporter expression (i.e., assays for β-galactosidase activity) or for SpoIIE-green fluorescent protein (GFP) content (i.e., Western blotting), sporulation was induced by the resuspension method (30, 31). Cells were collected by centrifugation at hourly intervals and stored at −80°C for further processing. β-Galactosidase activity was measured as previously described (21). The results of all β-galactosidase reporter assays are graphed as the averages from three experiments, unless stated otherwise in the corresponding figure legend.

Strain and plasmid construction. All *B. subtilis* strains were derived from the laboratory strain PY79 (32). Details of the strain and plasmid sources and construction are given in the supplemental material. The full genotypes of the experimental strains used in this study are listed in Table S1 in the supplemental material, the plasmids used in this study are listed in Table S2 in the supplemental material, and the primers and gene fragments used in this study are listed in Tables S3 and S4 in the supplemental material, respectively.

Sequence analysis. Plasmid, genomic, and protein sequences were visualized, edited, and aligned with Geneious software. The following SpoIIQ reference sequences were selected as representative orthologs (GenBank accession numbers are given in parentheses): *B. subtilis* (NP_391536.1), *Bacillus licheniformis* (WP_003185941.1), *Bacillus anthracis* (NP_847683.1), *Geobacter kaustophilus* (WP_011232806.1), *Bacillus halodurans* (WP_010899871.1), *Bacillus clausii* (WP_011248679.1), and *Oceanobacillus iheyensis* (WP_011067358.1). The multiple-sequence alignment of SpoIIQ was generated with the ClustalW program in Geneious software. Full-length $P1P2_{csfB}$ sequences (where P1 is the σ^F/σ^G-dependent promoter and P2 is the σ^K-dependent promoter) were identified by NCBI BLAST analysis using the Geneious Linnaeus search function. $P1P2_{csfB}$ sequences were recovered from the following species: *B. subtilis* (query sequence), *Bacillus amyloliquefaciens*, *Bacillus atrophaeus*, *B. licheniformis*, and *Bacillus methylotrophicus.* A nonredundant consensus $P1P2_{csfB}$ sequence from each of these species was extracted and used as input in a multiple-sequence Geneious alignment in order to produce the sequence logo for the 37-nucleotide (nt) repressor sequence.

Microscopy. General microscopy methods have been previously described (19). Briefly, cells expressing the *spoIIE-gfp* fusion gene were collected at hours 2.5 and 3.5 of sporulation to acquire images of each forespore engulfment stage. Harvested samples were resuspended in phosphate-buffered saline containing 1 μg/ml of the membrane stain FM 4-64 (Life Technologies) and mounted on 3% agarose pads. Fluorescence microscopy was performed with an Olympus BX61 microscope fitted with filter sets U-M41001 and U-MWG2 for GFP and FM 4-64 detection, respectively. Images were captured with an Orca-R2 digital charge-coupled-device camera using Simple PCI software, v6.0 (Hamamatsu Corp.). Images were falsely colored, overlaid, and identically adjusted for brightness and contrast with Fiji software (33).

Western blot analysis. Cell pellets, each from 1 ml sporulating culture, were resuspended in B-PER lysis buffer (with DNase and lysozyme; Pierce) and 1× HALT protease inhibitor (Pierce). Lysis buffer volumes were normalized to cell densities using measurements of the optical density at 600 nm acquired during cell collection. Samples were incubated for 15 min at 37°C, followed by addition of 4× reducing sodium dodecyl sulfate (SDS) sample buffer (Amresco). Samples in 1× sample buffer were heated for 10 min at 80°C, and equal volumes were separated by SDS-PAGE on precast TGX gels (Bio-Rad). Resolved proteins were transferred to a polyvinylidene difluoride membrane using Turbo RDF transfer kits (Bio-Rad). The membranes were blocked with 5% bovine serum albumin and incubated first with rabbit polyclonal anti-GFP antibody (diluted 1:15,000; Abcam) and then with an anti-rabbit immunoglobulin peroxidase-conjugated secondary antibody (diluted 1:2,000; Immunostar) in between washes in Tris-buffered saline with 0.1% Tween 20 (TBST). Chemiluminescence was provided by the West Pico chemiluminescent substrate (Pierce) and visualized with an LAS-3000 image reader (Fujifilm). Densitometry was completed with Fiji software with quantified averages ($n = 3$) after background (hour 1) subtraction (33).

RESULTS

The highly conserved Q Tyr-28 is required for maximal σ^G activity and sporulation. We previously demonstrated that alteration of a highly conserved tyrosine (Tyr-28) in the Q TMD (Fig. 1) significantly stimulates the activity of T7 RNAP engineered to be expressed in the *B. subtilis* forespore (21). However, the relevance of this phenotype to the normal progression of sporulation remained unclear. To begin, we asked whether Q Tyr-28 is required for the normal activity of the late-acting forespore sigma factor σ^G. Interestingly, we observed a modest but significant reduction in the expression of *lacZ* reporters fused to the σ^G-dependent *sspB* promoter (Fig. 2A) and *spoVT* promoter (see Fig. S1 in the supplemental material) (34, 35). More specifically, Q^{Y28A} strains produced only 70 to 75% of the β-galactosidase levels seen in isogenic Q^+ strains, as measured at hour 5 of sporulation. This reduction in σ^G activity was reversed in Q^{Y28A} strains into which a second, wild-type copy of *Q* was introduced (data not shown). Interestingly, full complementation was also observed with an N-terminal fragment encoding only amino acids 1 to 53 of the product of *Q* (Q^N) (Fig. 1), which includes the TMD but lacks the C-terminal AH interaction domain of Q (21) (Fig. 2B). Introduction of the Y28A mutation in either context (in the full-length or N-terminal [$Q^{N,Y28A}$] fragment) prevented complementation (data not shown and Fig. 2B). Together, these results indicate that Q Tyr-28 is required for maximal activation of σ^G at late times in the forespore and that a fragment of Q containing merely its TMD can restore this function.

Given the reduction of σ^G activity in the Q^{Y28A} mutant, we predicted that this strain should also be less capable of forming heat-resistant spores. We previously reported that Q^{Y28A} cells exhibit apparently wild-type levels of spore formation after single-round sporulation assays (21). However, a subtle defect may be undetectable in a single round of sporulation. We therefore per-

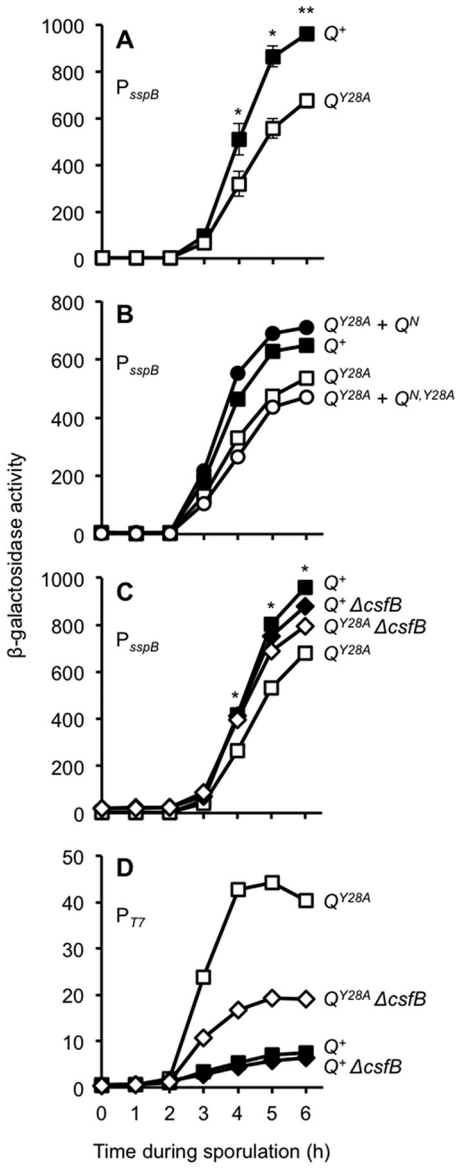

FIG 2 Q Tyr-28 regulates σ^G activity in a *csfB*-dependent manner. (A) Q Tyr-28 is required for maximal levels of σ^G activity. The σ^G-dependent activation of a P_{sspB}-*lacZ* reporter at the *ywrK* locus (also used in the assay for which the results are shown in panel C) was monitored during sporulation of Q^+ cells and Q^{Y28A} cells (strains AHB1586 and AHB1589, respectively). In each of these strains, the native *Q* gene was deleted and either wild-type Q (Q^+) or the Q Tyr-28 mutant (Q^{Y28A}) was expressed from the *sacA* locus. Significance was determined by a *post hoc* Student's *t* test. *, $P < 0.05$; **, $P < 0.001$. Error bars indicate SEMs ($n = 7$). (B) The Q^{Y28A} defect in σ^G activity can be complemented by the Q N terminus. The σ^G-dependent activation of a P_{sspB}-*lacZ* reporter at the *amyE* locus was monitored during sporulation of Q^+ cells, Q^{Y28A} cells, or Q^{Y28A} cells harboring complementation constructs encoding Q^N ($Q^{Y28A} + Q^N$) or $Q^{N,Y28A}$ ($Q^{Y28A} + Q^{N,Y28A}$) (strains KF26, KF27, KF32, and KF33, respectively). In each of these strains, the native *Q* gene was deleted and either Q^+ or Q^{Y28A} was expressed from the *lacA* locus. The Q^N or $Q^{N,Y28A}$ complementation constructs were inserted at the *sacA* locus, encode the 53-residue N terminus of Q, and have either tyrosine or alanine at position 28, respectively. (C) The reduction of σ^G activity exhibited by the Q^{Y28A} strain is partially reversed by removing the anti-σ^G factor CsfB. β-Galactosidase production from the P_{sspB}-*lacZ* reporter was monitored during sporulation of Q^+ cells, Q^{Y28A} cells, Q^+ cells from which *csfB* was deleted (Q^+ Δ*csfB*), or Q^{Y28A} cells from which *csfB* was deleted (Q^{Y28A} Δ*csfB*) (strains AHB1586, AHB1589, AHB1801, and AHB1802, respectively). In these strains, the native *Q* gene was deleted and either Q^+ or Q^{Y28A} was encoded at the *sacA* locus. *, $P < 0.05$, *post hoc* Student's *t* test. (D) The hyperactivity of T7 RNAP in the Q^{Y28A} mutant forespore is also partially reversed by deletion of *csfB*. T7 RNAP-directed P_{T7}-*lacZ* expression was monitored during sporulation of Q^+ cells, Q^{Y28A} cells, Q^+ cells from which *csfB* was deleted (Q^+ Δ*csfB*), or Q^{Y28A} cells from which *csfB* was deleted (Q^{Y28A} Δ*csfB*) (strains AHB1542, AHB1543, AHB1799, and AHB1800, respectively). In these strains, the P_{spoIIQ}-T7 RNAP was inserted at the *ylnF* locus, the T7 RNAP reporter P_{T7}-*lacZ* was inserted at the *ywrK* locus, the native *Q* gene was deleted, and Q^+ or Q^{Y28A} was encoded at the *sacA* locus.

formed competition assays in which the Q^{Y28A} mutant was competed directly with control Q^+ cells through multiple rounds of growth and sporulation. At the end of each round, the ratio of surviving heat-resistant spores of each genotype (assessed by use of a *lacZ* reporter in one of the strains) was determined, and surviving spores were back-diluted into fresh medium for another round of growth and sporulation. As predicted, the Q^{Y28A} mutant was unable to compete efficiently with the Q^+ strain, evidenced by an ~7% decrease in the mutant population per round (see Fig. S2A in the supplemental material). Importantly, strains with identical *Q* genotypes competed equally well (see Fig. S2B in the supplemental material), and the Q^{Y28A} defect was observed regardless of which strain (the Q^{Y28A} or Q^+ strain) was marked with *lacZ* (see Fig. S2A in the supplemental material). Finally, the defect was specific to sporulation, given that the Q^+ and Q^{Y28A} strains grew equally well in a growth medium that does not induce sporulation (see Fig. S2C in the supplemental material). We therefore conclude that Q Tyr-28 is ordinarily required for maximal σ^G activation and sporulation efficiency.

Decreased σ^G activity in the absence of Q Tyr-28 is partially dependent on CsfB. We hypothesized that the reduction in σ^G activity in Q^{Y28A} cells might be attributed to the inappropriate activity of a σ^G inhibitor. We first considered whether the reduction in σ^G activity might be due to inhibition by SpoIIAB. Due to the structural similarity of σ^F and σ^G (36, 37), the anti-σ^F factor SpoIIAB is also capable of binding and inhibiting σ^G (38, 39), although previous experiments have argued against a role for SpoIIAB-mediated inhibition of σ^G in the forespore during sporulation (37). Nevertheless, to test for the involvement of SpoIIAB, we introduced a mutation (E156K) into the gene (*sigG*) encoding σ^G that renders it insensitive to SpoIIAB-mediated inhibition (37). This strategy allowed us to dissect the role of SpoIIAB specifically in σ^G inhibition without confounding effects on the earlier, primary role of SpoIIAB in σ^F inhibition. We found that σ^G activation of the P_{sspB}-*lacZ* reporter was unaltered by the $sigG^{E156K}$ genotype in either *Q* background (see Fig. S3A in the supplemental material). The hyperactivity of the T7 RNAP in Q^{Y28A} cells (as monitored by use of a T7 RNAP-dependent *lacZ* reporter) was similarly unaffected by introduction of the $sigG^{E156K}$ mutation (see Fig. S3B in the supplemental material). As such, we conclude that σ^G activity in the Q^{Y28A} mutant is reduced (and T7 RNAP activity is increased) by a mechanism that does not involve the anti-sigma factor SpoIIAB.

We next tested whether the reduction of σ^G activity in the Q^{Y28A} strain was caused by inappropriate activity of the σ^G inhibitor, CsfB. Remarkably, we found that the Q^{Y28A}-associated reduction in σ^G activity was significantly (albeit not completely) rescued by the deletion of *csfB* (Fig. 2C). Deletion of *csfB* in a Q^+ background did not increase σ^G activity, suggesting that removal of this anti-sigma factor does not simply cause a generalized disinhibition of σ^G. We also found that *csfB* deletion significantly

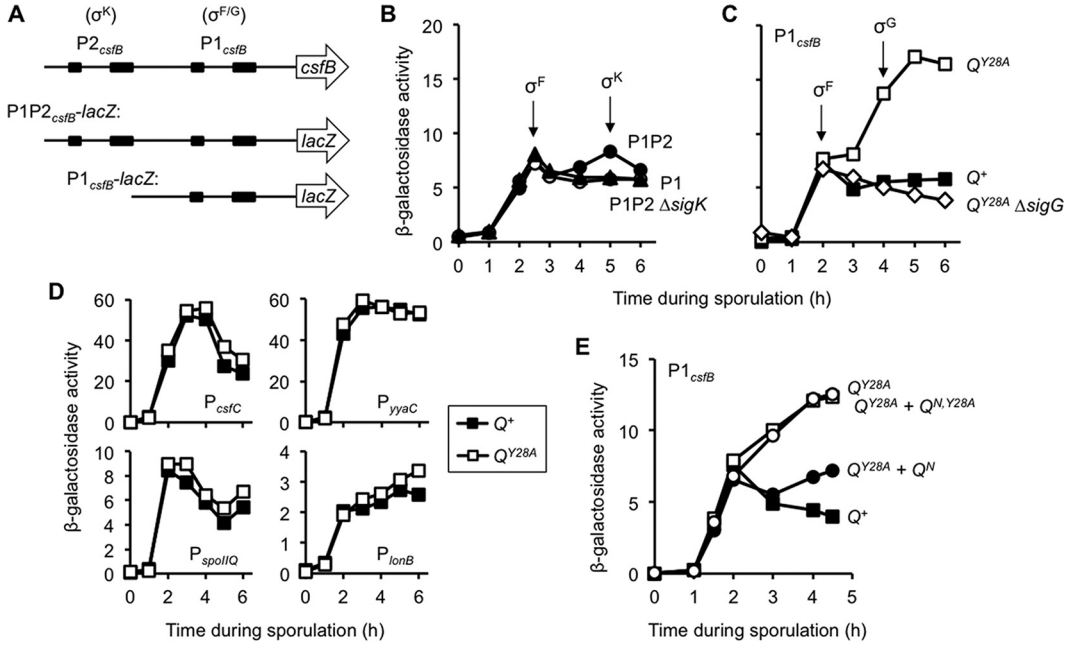

FIG 3 Q Tyr-28 is required to prevent σ^G-dependent activation of the *csfB* promoter during sporulation. (A) Cartoon of the *B. subtilis csfB* upstream regulatory region. The −35 and −10 promoter elements recognized by σ^K ($P2_{csfB}$) or σ^F and σ^G ($P1_{csfB}$) are depicted as black boxes. The $P1P2_{csfB}$-*lacZ* and $P2_{csfB}$-*lacZ* reporter constructs are drawn to scale below. (B) $P1_{csfB}$ and $P2_{csfB}$ are activated by σ^F and σ^K, respectively, during sporulation. The accumulation of β-galactosidase from $P1P2_{csfB}$-*lacZ* (P1P2; closed circles) and $P1_{csfB}$-*lacZ* (P1; triangles) was measured during sporulation of otherwise wild-type cells. The activity of the $P1P2_{csfB}$-*lacZ* reporter was also monitored in a strain lacking σ^K (P1P2 Δ*sigK*; open circles). Reporters were inserted at the *amyE* locus. P1P2, P1P2 Δ*sigK*, and P1 were carried by strains AHB1702, JDC5, and JDC138, respectively. (C) σ^G-dependent activation of P_{csfB} is unmasked in the Q^{Y28A} mutant. Activation of the $P1_{csfB}$-*lacZ* reporter was monitored during sporulation of strains harboring Q^+ or Q^{Y28A} (strains JDC142 and JDC143, respectively). $P1_{csfB}$-*lacZ* activity was also measured in a Q^{Y28A} strain lacking σ^G (Q^{Y28A} Δ*sigG*; strain JDC150). In each of these strains, the native *Q* gene was deleted and either Q^+ or Q^{Y28A} was inserted at the *sacA* locus. Note that the *y* axis in panel C is the same as that in panel B. (D) The Q Tyr-28 substitution does not unmask σ^G activation of other σ^F-activated promoters. Four representative σ^F-dependent promoters (those of *csfC*, *yyaC*, *spoIIQ*, and *lonB*) were fused to *lacZ* and assayed for expression in both wild-type Q^+ and Q^{Y28A} mutant strains. Q^+ and Q^{Y28A} strains are strains EBM49 and EBM50, respectively, for P_{csfC}; strains EBM44 and EBM47, respectively, for P_{yyaC}; strains EBM42 and EBM45, respectively, for P_{spoIIQ}; and strains EBM43 and EMB46, respectively, for P_{lonB}; in all of these strains, the *lacZ* reporter genes were inserted at *amyE*. Data are from representative, single experiments. (E) The 53-residue wild-type Q N terminus (Q^N) can restore proper $P1_{csfB}$ expression in the Q^{Y28A} mutant. $P1_{csfB}$-*lacZ* activity was monitored in strains harboring either Q^+ or Q^{Y28A} encoded at the *lacA* locus and was also monitored in Q^{Y28A} strains that were complemented with either wild-type Q^N (Q^{Y28A} + Q^N) or mutant $Q^{N,Y28A}$ (Q^{Y28A} + $Q^{N,Y28A}$) at the *sacA* locus (strains KF5, KF6, KF11, and KF12, respectively).

reduced the hyperactivity of T7 RNAP in Q^{Y28A} cells (Fig. 2D). These data support a model in which σ^G is subject to excessive inhibition by CsfB in Q^{Y28A} cells. Moreover, we speculate here that T7 RNAP hyperactivity, the very phenotype that originally drew our attention to the Q^{Y28A} mutant, may be an indirect consequence of reduced σ^G activity. In this engineered strain, T7 RNAP (expressed under σ^F control) and σ^G coexist in the forespore and compete for finite resources (21). Although we cannot exclude other models, it seems plausible that a modest reduction in σ^G activity, when multiplied across the ~100 endogenous σ^G target genes (4, 40, 41), would free up limiting resources and therefore highly stimulate T7 RNAP activation of its one target gene, the P_{T7}-*lacZ* reporter.

SpoIIQ Tyr-28 prevents σ^G-dependent activation of the *csfB* promoter. The genetic interaction between Q^{Y28A} and *csfB* suggests that Q Tyr-28 may promote maximal σ^G activity in the developing forespore by a mechanism that downregulates CsfB expression and/or activity. We first tested whether Q Tyr-28 influences *csfB* expression. The *csfB* gene is expressed under the control of σ^F at early times in the forespore and later by σ^K in the mother cell (15, 42). The transcription of *csfB* is also activated by σ^G during vegetative growth (20). This complex regulation appears to be mediated by two promoters in the regulatory region upstream of the *csfB* gene (Fig. 3A): one matching the consensus sequence for both σ^F and σ^G and a second, upstream promoter that matches the consensus sequence for σ^K (42).

Given the complexity of *csfB* expression, we built and characterized two different P_{csfB}-*lacZ* reporter constructs. The first included the σ^F/σ^G-dependent promoter (P1) and the σ^K-dependent promoter (P2) (see the P1P2 construct in Fig. 3A), while the second was shortened such that it harbored only the σ^F/σ^G-dependent promoter (see the P1 construct in Fig. 3A). As shown in Fig. 3B, both reporter constructs displayed nearly identical activity at early times of sporulation (at about hour 2), consistent with the σ^F-dependent activation of the P1 σ^F/σ^G-dependent promoter. We also observed a weaker, second phase of β-galactosidase production at about hour 5 of sporulation in strains harboring the $P1P2_{csfB}$-*lacZ* reporter but not the $P1_{csfB}$-*lacZ*, reporter, consistent with the σ^K-dependent activation of $P2_{csfB}$. This later activity was eliminated by deletion of *spoIVCB*, which is part of the composite gene, *sigK*, that encodes σ^K (Fig. 3B). Importantly, our data revealed no evidence for σ^G-mediated activation of the P1 σ^F/σ^G-dependent promoter during sporulation, in accordance with the fact that *csfB* activation by σ^G has been reported only during veg-

etative growth (20). Given our specific interest in *csfB* function in the forespore (i.e., the compartment in which σ^G is ordinarily active), as well as to exclude the potentially confounding σ^K-driven activation, we used the $P1_{csfB}$-*lacZ* reporter construct or variations thereof for all subsequent experiments, unless otherwise noted.

To determine whether Q Tyr-28 was necessary for proper *csfB* expression in the forespore, we monitored $P1_{csfB}$-*lacZ* expression in Q^+ and Q^{Y28A} strains. As expected, β-galactosidase produced from $P1_{csfB}$-*lacZ* in Q^+ cells peaked at hour 2 of sporulation (coinciding with the timing of σ^F activation), after which time there was no additional β-galactosidase production (Fig. 3C). The Q^{Y28A} mutant demonstrated levels of σ^F-dependent activation of $P1_{csfB}$-*lacZ* similar to those of the Q^+ control strain but also displayed a robust second wave of expression such that by hour 5 of sporulation, the Q^{Y28A} mutant had 3-fold higher levels of β-galactosidase than the Q^+ control strain (Fig. 3C). We confirmed by immunoblot analysis that a functional GFP-CsfB fusion protein under the control of the $P1_{csfB}$ promoter also accumulated to approximately 40% higher levels in the Q^{Y28A} strain than the Q^+ strain at later times during sporulation (data not shown). Together, these results indicate that aberrant late activation of the $P1_{csfB}$ promoter occurs in the absence of Q Tyr-28, leading to an increase in the steady-state levels of the anti-σ^G factor CsfB.

We next wondered whether the late activation of $P1_{csfB}$ during sporulation in Q^{Y28A} cells was due to σ^G, the very target of CsfB inhibition. Supporting this idea, the timing of the late activity coincided with the known timing of σ^G activity in the forespore (Fig. 2A). Indeed, we found that the aberrant activity of $P1_{csfB}$ in Q^{Y28A} cells (after hour 3) disappeared when *sigG* was deleted (Fig. 3C). We therefore conclude that the σ^F-dependent $P1_{csfB}$ promoter can be recognized and activated by σ^G during sporulation but that σ^G is ordinarily prevented from doing so by a mechanism that requires Q Tyr-28.

The ability of σ^G to activate *csfB* expression in the Q^{Y28A} mutant could be the result of the general misregulation of σ^F target promoters or a specific misregulation of $P1_{csfB}$. To distinguish between these possibilities, each of the σ^F-controlled promoters of *spoIIQ*, *lonB*, *yyaC*, and *csfC* (15, 40, 41, 43) was fused to the *lacZ* reporter gene and assayed in sporulating cells of the Q^+ and Q^{Y28A} strains. As shown in Fig. 3D, none of these reporters were aberrantly expressed in the Q^{Y28A} strain. As such, the activation of $P1_{csfB}$ by σ^G in the absence of Q Tyr-28 is not due to a general misregulation of σ^F target genes but is instead specific to *csfB*.

Finally, given that the N-terminal fragment of Q (Q^N; Fig. 1) restored wild-type levels of σ^G activity to the Q^{Y28A} mutant (Fig. 2B), we hypothesized that Q^N might also restore proper regulation of $P1_{csfB}$ in this mutant. As shown in Fig. 3E, *csfB* misexpression by σ^G was significantly reduced when Q^N (but not $Q^{N,Y28A}$) was introduced into Q^{Y28A} cells. Altogether, these results support a model in which Q Tyr-28 specifically prevents σ^G from inappropriately activating the transcription of a gene (*csfB*) that encodes its own inhibitor.

A conserved sequence within the *csfB* promoter is required to prevent σ^G-dependent activation. The ability of the Q^{Y28A} mutation to unmask the σ^G-dependent activation of *csfB* suggested to us that the *csfB* promoter itself may harbor features that make it a target for Q Tyr-28-dependent regulation. We reasoned that such features would likely be conserved among other spore-forming bacteria related to *B. subtilis*. At least four species have a *csfB* promoter arrangement similar to that found in *B. subtilis* (i.e., a σ^K-dependent promoter followed by a σ^F/σ^G-dependent promoter): *Bacillus amyloliquefaciens*, *Bacillus atrophaeus*, *Bacillus licheniformis*, and *Bacillus methylotrophicus*. Using sequences from all these species, we created a consensus logo sequence for the *csfB* regulatory region corresponding to $P1_{csfB}$ in these species (Fig. 4A). As expected, the −35 and −10 binding sites for σ^F/σ^G, as well as the ribosome binding site, displayed significant conservation. Sequences between these functional elements were less conserved, with the notable exception of an ~37-nt stretch mostly upstream of but also including 3 nt downstream of the −35 element of the σ^F/σ^G-dependent promoter.

To ascertain if this conserved region contributes to the regulation of *csfB* expression, we first made progressive deletions from the 5′ end of our $P1_{csfB}$-*lacZ* reporter construct to produce two $P1_{csfB}$ truncations ($^{trunc1}P1_{csfB}$ and $^{trunc2}P1_{csfB}$; Fig. 4A). As shown in Fig. 4B, the levels of β-galactosidase produced from the $^{trunc1}P1_{csfB}$-*lacZ* and $^{trunc2}P1_{csfB}$-*lacZ* fusions were similar to those produced from $P1_{csfB}$-*lacZ* at hour 2 of sporulation, indicating normal σ^F-dependent activation. At later times, however, we observed a substantial 2- to 3-fold increase in expression in the $^{trunc1}P1_{csfB}$-*lacZ* and $^{trunc2}P1_{csfB}$-*lacZ* reporter fusions (Fig. 4B). The more extensive deletion of the 5′ end ($^{trunc2}P1_{csfB}$) promoted the highest level of β-galactosidase expression. We next generated a new $P1_{csfB}$-*lacZ* variant in which seven of the most highly conserved nucleotides that were removed in $^{trunc2}P1_{csfB}$-*lacZ* were mutated ($^{mut1}P1_{csfB}$-*lacZ*; Fig. 4A). The $^{mut1}P1_{csfB}$-*lacZ* reporter gene was also aberrantly expressed during late sporulation in a manner that was indistinguishable from that of the $^{trunc2}P1_{csfB}$-*lacZ* reporter (Fig. 4B). As expected, the late activity of the $^{mut1}P1_{csfB}$-*lacZ* and $^{trunc2}P1_{csfB}$-*lacZ* reporters was significantly reduced when *sigG* was deleted (Fig. 4C and data not shown), confirming dependence upon σ^G, as in the Q^{Y28A} mutant strain. Finally, we wondered whether conserved nucleotides closer to the $P1_{csfB}$ −35 element might also be necessary to prevent σ^G-dependent activation. Indeed, we found that substitution of nucleotides upstream or downstream of the −35 element ($^{mut2}P1_{csfB}$ or $^{mut3}P1_{csfB}$, respectively; Fig. 4A) caused aberrant late activation to the same extent that $^{trunc2}P1_{csfB}$ and $^{mut1}P1_{csfB}$ did (see Fig. S4 in the supplemental material). Altogether, these results suggest that conserved nucleotides mostly upstream of but also downstream of the −35 element of $P1_{csfB}$ are required to prevent its activation by σ^G during late sporulation.

Q Tyr-28 and the *csfB* promoter element operate in the same genetic pathway. Our data thus far indicate that σ^G can activate *csfB* expression in the forespore under two circumstances: (i) when we substitute Tyr-28 of Q (as in Q^{Y28A}) and (ii) when we mutate a conserved sequence near the σ^F/σ^G −35 binding site (as in $^{mut1}P1_{csfB}$). These findings may reflect two separate mechanisms of *csfB* regulation or could indicate that Q Tyr-28 and the conserved $P1_{csfB}$ regulatory element operate together in a single pathway to block σ^G activation of P_{csfB} during sporulation. To distinguish these possibilities, we generated a double mutant strain harboring the $^{mut1}P1_{csfB}$-*lacZ* reporter in a Q^{Y28A} mutant background. As shown in Fig. 4D, the $^{mut1}P1_{csfB}$-*lacZ* Q^{Y28A} double mutant produced the same levels of β-galactosidase produced by both single mutants alone. The nonadditivity of these phenotypes argues against separate mechanisms and instead supports a model in which Q Tyr-28 and the conserved promoter element operate in the same pathway to block σ^G activation of $P1_{csfB}$.

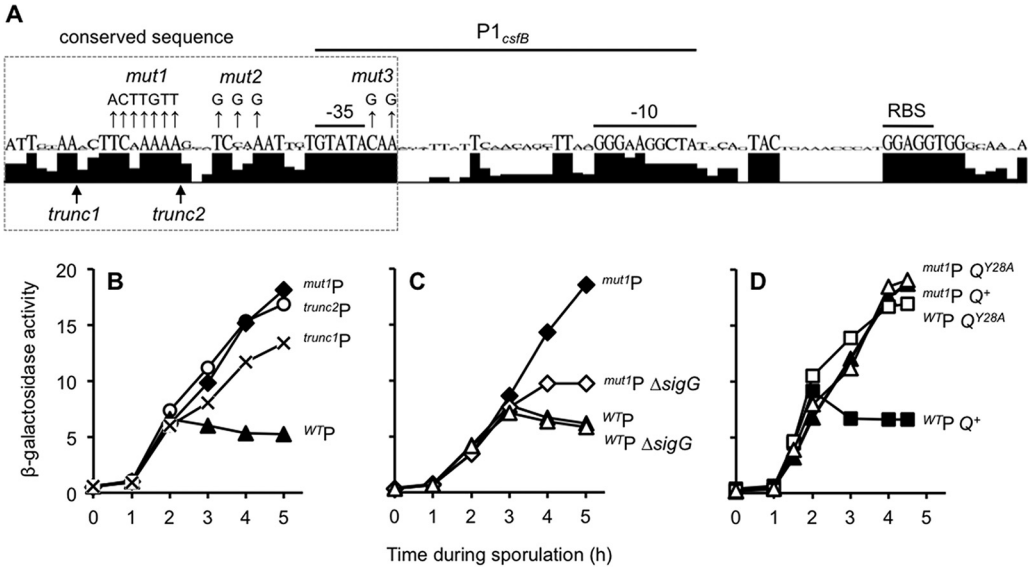

FIG 4 A regulatory element near $P1_{csfB}$ is necessary to prevent its activation by σ^G during sporulation. (A) A 37-nt sequence adjacent to the *B. subtilis* $P1_{csfB}$ −35 element is well conserved. The *csfB* regulatory sequences corresponding to the $P1_{csfB}$-*lacZ* construct from *B. subtilis*, *B. amyloliquefaciens*, *B. atrophaeus*, *B. licheniformis*, and *B. methylotrophicus* were analyzed by the use of Geneious software to generate a sequence logo depiction of nucleotide conservation. Shown below the sequence logo are the 5′ boundaries of the $^{trunc1}P1_{csfB}$-*lacZ* and $^{trunc2}P1_{csfB}$-*lacZ* constructs. Nucleotides mutated in the $^{mut1}P1_{csfB}$-*lacZ*, $^{mut2}P1_{csfB}$-*lacZ*, and $^{mut3}P1_{csfB}$-*lacZ* constructs are indicated by *mut1*, *mut2*, and *mut3*, respectively. Note that the data obtained for the $^{mut2}P1_{csfB}$-*lacZ* and $^{mut3}P1_{csfB}$-*lacZ* constructs are presented in Fig. S4 in the supplemental material. RBS, ribosome binding site. (B) Activity of $P1_{csfB}$ variants during sporulation. β-Galactosidase production was monitored during the sporulation of strains harboring $P1_{csfB}$-*lacZ* (^{WT}P), $^{trunc1}P1_{csfB}$-*lacZ*, $^{trunc2}P1_{csfB}$-*lacZ*, and $^{mut1}P1_{csfB}$-*lacZ* reporter constructs integrated at *amyE* (strains KF76, KF159, AHB2088, and KF13, respectively). (C) The late activity of $^{mut1}P1_{csfB}$-*lacZ* is due to inappropriate activation by σ^G. β-Galactosidase production from the $^{mut1}P1_{csfB}$-*lacZ* reporter was assessed in a strain with native *sigG* (^{mut1}P) or a strain from which *sigG* was deleted (^{mut1}P Δ*sigG*). As a control, the wild-type $P1_{csfB}$-*lacZ* reporter was also assessed in a strain with a native *sigG* (^{WT}P) or a strain from which *sigG* was deleted (^{WT}P Δ*sigG*). Reporter genes were inserted at the *amyE* locus in these strains (strains KF13, KF15, JDC138, and KF109, respectively). (D) Mutation of the *csfB* promoter element and substitution of Q Tyr-28 misregulate *csfB* expression to a similar extent and in a nonadditive manner. β-Galactosidase activity from the $P1_{csfB}$-*lacZ* or $^{mut1}P1_{csfB}$-*lacZ* reporter constructs was measured in strains harboring Q^+ or Q^{Y28A}. The strains were $P1_{csfB}$-*lacZ* Q^+ (^{WT}P Q^+), $P1_{csfB}$-*lacZ* Q^{Y28A} (^{WT}P Q^{Y28A}), $^{mut1}P1_{csfB}$-*lacZ* Q^+ (^{mut1}P Q^+), and $^{mut1}P1_{csfB}$-*lacZ* Q^{Y28A} (^{mut1}P Q^{Y28A}) (strains KF1, KF2, KF3, and KF4, respectively). In each of these strains, the native Q gene was deleted and either Q^+ or Q^{Y28A} was inserted at the *sacA* locus; *lacZ* reporters were inserted at the *amyE* locus. Note that the *y* axes for panels C and D are the same as the *y* axis for panel B.

The conserved *csfB* promoter element is sufficient to repress promoter activation by σ^G in late forespore development. Our data indicate that a conserved promoter element near the −35 element bound by σ^F in $P1_{csfB}$ is necessary to block subsequent σ^G-dependent expression of *csfB* during sporulation. We were curious if this conserved sequence might also be sufficient to repress σ^G activity in a promoter ordinarily activated by both σ^F and σ^G. We chose as our test promoter that of the putative σ^F inhibitor gene *fin* (previously *yabK*) (19). Not only has P_{fin} been demonstrated to be activated by both σ^F and σ^G (19), but P_{fin} also harbors a −35 element identical to that found in $P1_{csfB}$ (GTATA). This identity facilitated the construction of chimeric promoters (see below) in which surrounding nucleotides were swapped without altering core promoter nucleotides.

To begin, we constructed a P_{fin}-*lacZ* promoter fusion that harbored 29 nt upstream of the σ^F/σ^G-dependent −35 element, the same number of nucleotides found upstream of the matching −35 element in our $P1_{csfB}$-*lacZ* promoter fusion (Fig. 5A). As expected, this P_{fin}-*lacZ* promoter fusion was activated first during the height of σ^F activity (approximately hour 2) and then later under the control of σ^G (approximately hours 3 to 5) (Fig. 5B). We then replaced the 5′ 35 nt of P_{fin} with the corresponding 35 nt of $P1_{csfB}$, yielding a chimeric promoter, $P_{csfB\text{-}fin}$ (Fig. 5A). (The final 2 nt of the 37 nt conserved sequence were already identical in P_{fin}.) As shown in Fig. 5B, the chimeric $P_{csfB\text{-}fin}$-*lacZ* reporter displayed significantly reduced σ^G-dependent activation compared to that of the original P_{fin}-*lacZ* reporter. Importantly, as shown in Fig. 5B, σ^G-dependent activation of $P_{csfB\text{-}fin}$ was restored when we mutated the same seven highly conserved nucleotides altered in $^{mut1}P_{csfB}$ (Fig. 4A). Altogether, these data indicate that these 37 nt, which include the $P1_{csfB}$ −35 element, are sufficient to interfere with late σ^G activity when placed in the context of another σ^F/σ^G-dependent promoter.

Finally, we tested whether the 37-nt repressor sequence derived from $P1_{csfB}$ was sufficient to confer sensitivity to Q Tyr-28. In other words, is the σ^G-dependent activation of $P_{csfB\text{-}fin}$ unmasked in a Q^{Y28A} mutant like that of P_{csfB} was? As shown in Fig. 5C, P_{fin}-*lacZ* activity is ordinarily slightly diminished in the presence of the Q^{Y28A} mutation (i.e., relative to that in the control Q^+ strain), consistent with the ~25 to 30% reduction in σ^G activity that we have reported here (Fig. 2A). In contrast, the $P_{csfB\text{-}fin}$ reporter, which was engineered to harbor the 37-nt repressor sequence, demonstrated an increase in expression in the Q^{Y28A} strain relative to that in the Q^+ strain (Fig. 5D). We therefore conclude that a conserved 37-nt sequence found near $P1_{csfB}$ is necessary and sufficient to prevent late, σ^G-dependent promoter activation by a mechanism that requires Q Tyr-28.

Misregulation of *csfB* expression is insufficient to explain the Q^{Y28A}-mediated reduction in σ^G activity. We have demonstrated that Q^{Y28A} mutant forespores exhibit diminished σ^G activity and

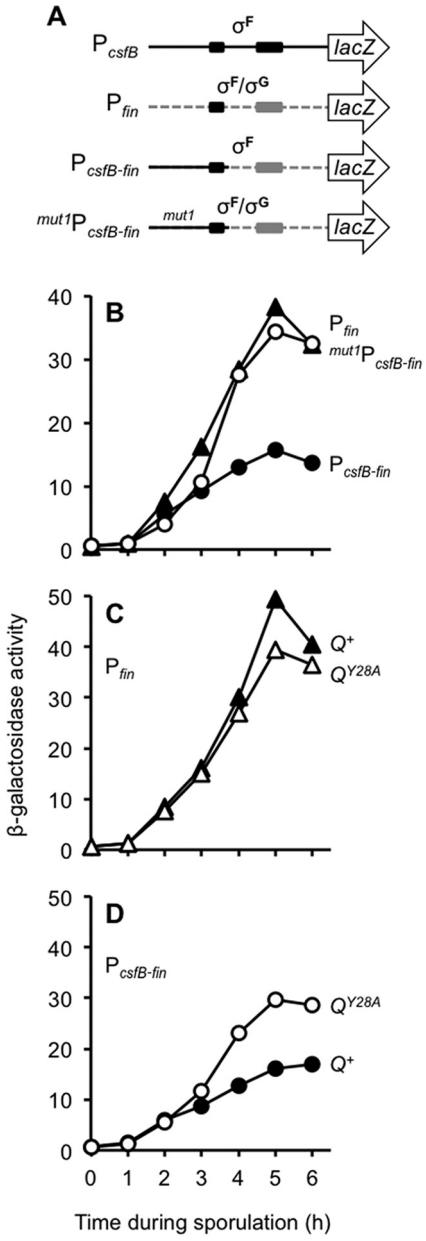

FIG 5 The conserved 37-nt sequence from $P1_{csfB}$ is sufficient to reduce σ^G-dependent activation of a promoter ordinarily recognized by σ^F and σ^G. (A) Cartoon of $P1_{csfB}$, P_{fin}, and a chimeric promoter ($P_{csfB\text{-}fin}$) in which the 37-nt conserved sequence from $P1_{csfB}$ was substituted for the corresponding 37 nt in P_{fin}. Also shown is the $^{mut1}P_{csfB\text{-}fin}$ promoter construct in which the 7-nt sequence mutated in $^{mut1}P1_{csfB}$ is introduced into $P_{csfB\text{-}fin}$ (marked by *mut1*). The −35 and −10 elements for each promoter are indicated as shaded boxes; note that P_{csfB} and P_{fin} have identical −35 elements. (B) The $P_{csfB\text{-}fin}$ promoter but not $^{mut1}P_{csfB\text{-}fin}$ displays significantly reduced activation by σ^G. β-Galactosidase production from P_{fin} (triangles), $P_{csfB\text{-}fin}$ (closed circles), and $^{mut1}P_{csfB\text{-}fin}$ (open circles) fusions to *lacZ* (inserted at the *amyE* locus) was measured during sporulation of otherwise wild-type strains (strains KF76, KF129, KF130, and KF166, respectively). (C) Q Tyr-28 is required for maximal levels of σ^G activation of P_{fin}. P_{fin}-*lacZ* reporter gene expression was monitored in strains harboring Q^+ or Q^{Y28A} (strains KF146 and KF152, respectively). (D) σ^G-dependent activation of $P_{csfB\text{-}fin}$ is unmasked in the Q^{Y28A} mutant. $P_{csfB\text{-}fin}$-*lacZ* reporter gene expression was monitored in strains harboring Q^+ or Q^{Y28A} (strains KF147 and KF153, respectively). In all strains for which the results are shown in panels C and D, the native *Q* gene was deleted and either Q^+ or Q^{Y28A} was encoded at the *sacA* locus. Reporters were inserted at *amyE*.

that this is partially reversed by *csfB* deletion. Moreover, we found that the Q^{Y28A} mutation causes *csfB* to be aberrantly expressed by σ^G, which likely contributes to higher steady-state levels of the *csfB*-encoded σ^G inhibitor, CsfB. We therefore hypothesized that the diminished σ^G activity in the Q^{Y28A} mutant is due at least in part to misexpression of *csfB* by σ^G. A key prediction of this hypothesis is that expression of *csfB* from a mutant promoter activated by both σ^F and σ^G should cause a reduction in σ^G activity similar to that caused by the Q^{Y28A} mutation. To test this hypothesis, we directly engineered the misexpression of *csfB* by mutating seven highly conserved nucleotides upstream of the $P1_{csfB}$ (σ^F/σ^G) −35 element (the same mutations found in the $^{mut1}P1_{csfB}$-*lacZ* construct) at the native *csfB* locus (i.e., without disrupting any other aspect of the *csfB* promoter or coding sequence). Surprisingly, however, altering *csfB* expression in this manner failed to detectably reduce the activity of σ^G (see Fig. S5A in the supplemental material). Moreover, expression of *csfB* from wild-type or mutated promoters at both the native locus and an additional, ectopic locus had no observable effect on σ^G activity (see Fig. S5B in the supplemental material), nor did any of these alterations to *csfB* expression cause late hyperactivity of T7 RNAP in the forespore, which we speculate may be a sensitive indicator of subtle changes in σ^G activity (data not shown). We therefore conclude that the misexpression of the anti-σ^G gene *csfB* cannot fully explain the reduction of σ^G activity in the Q^{Y28A} mutant and, as such, that *csfB* expression is unlikely the only target of Q Tyr-28 regulation.

Q Tyr-28 is required for the proper localization and stability of the phosphatase SpoIIE. Our data support a model in which an amino acid within the Q TMD, Tyr-28, somehow blocks σ^G-dependent expression of *csfB*. One possibility is that Q Tyr-28 exerts this effect by modulating the activity of the AA-AH·Q channel, which globally promotes late forespore gene expression, including that directed by σ^G (21). We find this scenario unlikely, however, given the specificity of *csfB* misregulation, i.e., given that no other tested promoters were similarly misregulated in the Q^{Y28A} mutant. Instead, we posit here that Tyr-28 executes a separate function of Q, by which it modulates the expression of specific genes (including *csfB*) to maximize the activity of σ^G. We further predict that Q does so in collaboration with one or more intermediary proteins that topologically link the membrane-embedded Tyr-28 to regulatory events occurring in the cytosol. An intriguing candidate for such an intermediary protein is the membrane phosphatase SpoIIE. It has been reported previously that SpoIIE and Q interact in the forespore membrane and that the relocalization of SpoIIE to the forespore septal membrane during engulfment requires Q (12). These events occur after the well-established functions of SpoIIE in polar septum formation and σ^F activation, suggesting that SpoIIE and Q together carry out an undetermined function in the forespore at later times.

Given the earlier requirement for SpoIIE in σ^F activation, we were unable to delete *spoIIE* to directly test for a later role in proper P_{csfB} regulation. Nevertheless, to explore a possible link between Q Tyr-28-mediated gene regulation and SpoIIE, we tested whether Tyr-28 is required for the dynamic localization of SpoIIE in the forespore membrane. As previously reported, a functional SpoIIE-GFP fusion protein localized almost exclusively to the septal membrane during engulfment in wild-type cells but mislocalized around the entire forespore membrane in the absence of *Q* (Fig. 6A). When Δ*Q* cells were complemented with Q^+

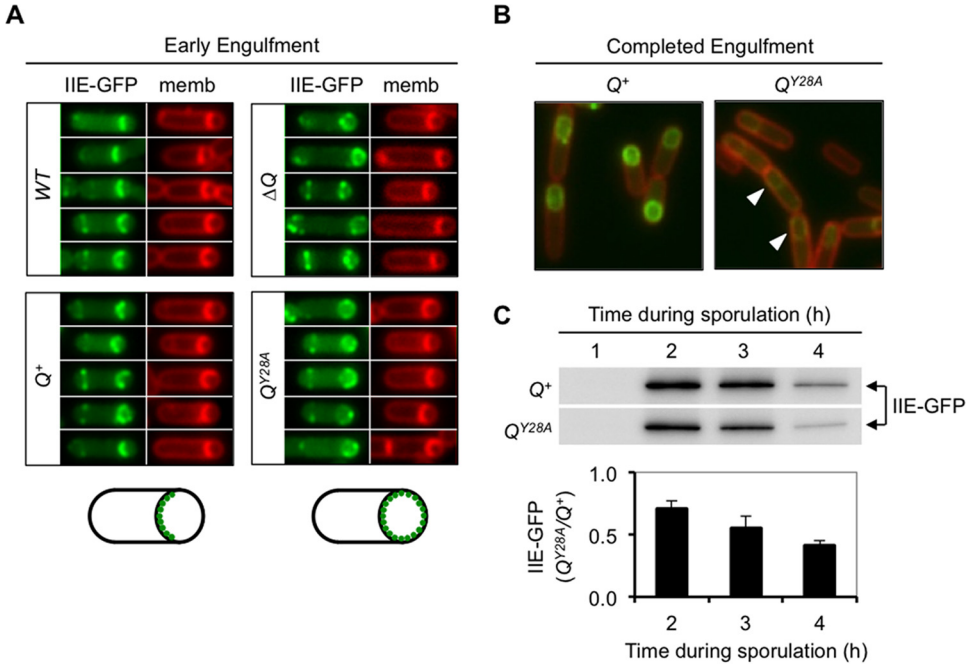

FIG 6 Q Tyr-28 is required for the proper localization and stability of SpoIIE-GFP. (A) SpoIIE-GFP is mislocalized in Q^{Y28A} mutant forespores during engulfment. The localization of a functional SpoIIE-GFP fusion protein (encoded at the native *spoIIE* locus) was monitored by fluorescence microscopy during sporulation (hour 2.5) of a strain harboring wild-type *Q* at its native locus (WT), a strain in which *Q* was deleted (Δ*Q*), or strains in which *Q* was deleted and complemented with either Q^+ or Q^{Y28A} at the *sacA* locus (strains AHB1648, AHB1649, AHB1650, and AHB1651, respectively). A representative set of cells at an early stage of engulfment is shown for each strain, with both SpoIIE-GFP (IIE-GFP) fluorescence (green) and FM 4-64 membrane (memb) fluorescence (red) being shown in pseudocolor. (Bottom) Two cartoons illustrate SpoIIE-GFP localization to the septal forespore membrane in strains with wild-type Q (left) and SpoIIE-GFP mislocalization around the forespore membrane in strains lacking either Q or Q Tyr-28 (right). (B) Q^{Y28A} forespores demonstrate diminished SpoIIE-GFP signal intensity (arrowheads) compared to Q^+ cells. Representative images of Q^+ and Q^{Y28A} cells (strains AHB1650 and AHB1651, respectively) at hour 3.5 of sporulation, after engulfment was complete, are shown. SpoIIE-GFP fluorescence (green) and FM 4-64 membrane fluorescence (red) are shown merged. Membranes surrounding engulfed forespores are not stained because the FM 4-64 dye is not able to permeate the membrane. GFP images for each strain were captured and processed identically. (C) SpoIIE-GFP steady-state protein levels are quantifiably reduced in the Q^{Y28A} mutant. Immunoblot analysis of whole-cell extracts from sporulating Q^+ or Q^{Y28A} cells (strains AHB1650 and AHB1651, respectively) was performed using anti-GFP antibodies. The time during sporulation is indicated. Equal protein loading was confirmed by Coomassie staining (not shown). Densitometry was used to quantify the average levels of SpoIIE-GFP in the Q^{Y28A} mutant as a fraction of those in the Q^+ strain at each time point after the background was subtracted. Error bars are SEMs ($n = 3$).

at an ectopic locus, proper SpoIIE-GFP localization to the forespore septal membrane was restored (Fig. 6A). In contrast, when Δ*Q* cells were complemented with Q^{Y28A}, SpoIIE-GFP localization failed to be restored, with the majority of the protein remaining distributed throughout the forespore membrane throughout engulfment (Fig. 6A). We therefore conclude that the SpoIIE·Q complex at the forespore septal membrane during engulfment requires Q Tyr-28.

In addition to the mislocalization of SpoIIE-GFP during engulfment, we also observed a notable decrease in the intensity of SpoIIE-GFP fluorescence in Q^{Y28A} forespores relative to that in Q^+ forespores after engulfment was complete (Fig. 6B). Consistent with the microscopy findings, the steady-state levels of SpoIIE-GFP measured by immunoblot analysis were consistently lower in the Q^{Y28A} cells than Q^+ cells, with the difference becoming more pronounced at later times such that by hour 4 of sporulation, the Q^{Y28A} cells harbored only ~40% of the SpoIIE-GFP levels present in Q^+ cells (Fig. 6C). Altogether, these results indicate that Q Tyr-28 is required for the localization and stability of SpoIIE-GFP in the forespore membrane, raising the intriguing possibility that Q Tyr-28 modulates the expression of *csfB* by a mechanism that involves the multifunctional SpoIIE phosphatase.

DISCUSSION

In the developing *B. subtilis* spore, the transition from early, σ^F-directed gene expression to late, σ^G-directed gene expression is precisely orchestrated but still relatively poorly understood. One key player in the σ^F-to-σ^G switch is CsfB, an anti-sigma factor capable of inhibiting σ^G (14, 16). The *csfB* gene is transcribed early in the forespore under σ^F control (15). Interestingly, σ^G can also direct *csfB* transcription during vegetative growth (i.e., in nonsporulating cells) from the same promoter utilized by σ^F during sporulation ($P1_{csfB}$) (Fig. 3A) (20, 42). The ability of σ^G to activate *csfB* expression in vegetative cells is proposed to serve a quality control purpose, to shut down inappropriate σ^G activity under nonsporulation conditions (20). However, these findings raise an important question: if σ^G is able to recognize $P1_{csfB}$ (i.e., like it does during vegetative growth), why does it not also do so in the forespore during sporulation, at which point a negative-feedback loop would be detrimental? In this study, we report the discovery of a regulatory circuit involving amino acid Tyr-28 of the forespore membrane protein Q and a conserved element in the *csfB* promoter that blocks the σ^G-dependent activation of $P1_{csfB}$ in the forespore. Removing either Q Tyr-28 or the *csfB* promoter element leads to inappropriate σ^G-dependent *csfB* activation during

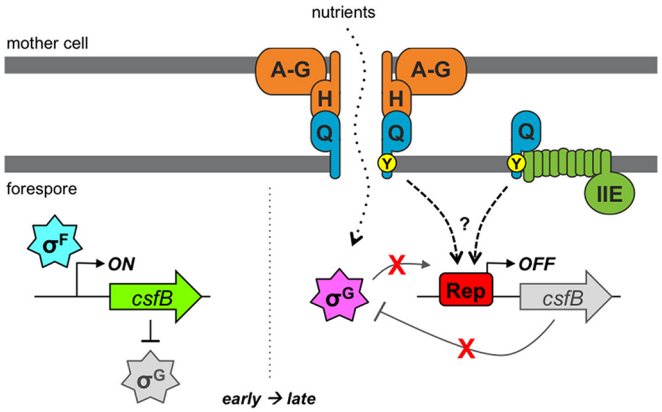

FIG 7 A model for the Q Tyr-28-mediated *csfB* regulatory pathway. After asymmetric division and during engulfment (early, left), transcription of the *csfB* gene is activated by σ^F in the forespore. In turn, the produced CsfB protein acts as an inhibitor of σ^G, helping to prevent its premature activation. After the completion of forespore engulfment (late, right), the transcription of *csfB* by σ^G is prevented by a conserved *csfB* promoter element, which we propose is a binding site for a repressor protein (Rep). The cessation of *csfB* transcription limits CsfB production to promote maximal σ^G activation. A highly conserved tyrosine (Tyr-28; Y) in the TMD of Q (whose gene is also activated by σ^F [not shown]) is also required for the inhibition of σ^G-dependent *csfB* transcription. Q is known to assemble into a channel apparatus with the mother cell proteins AA-AH (A-H) and to form a direct interaction with AH (H). This channel is generally required for gene expression at late times in the forespore, including that directed by σ^G; as such, it has been proposed to serve as a feeding tube through which the mother cell provides nutrients required for macromolecular synthesis (as shown). A separate subpopulation of Q is also known to interact with the SpoIIE phosphatase (IIE), an interaction that we have shown in the current study to be dependent specifically on Q Tyr-28. We propose that this subpopulation of Q, in collaboration with SpoIIE, positively regulates the *csfB* repressor protein by an unknown mechanism. However, we cannot rule out other scenarios, including the possibility that SpoIIE is not involved and/or that Q interacts with yet another partner via Tyr-28 to effect *csfB* regulation. Regardless, our data support the conclusion that Q is a bifunctional protein that (i) generally activates σ^G, through its assembly into the AA-AH·Q channel, and (ii) specifically maximizes the activity of σ^G, through participation in a gene regulatory circuit that represses expression of the gene encoding the anti-σ^G factor CsfB.

sporulation. The nonadditivity of these two mutant phenotypes is consistent with Q Tyr-28 and the *csfB* promoter element operating in the same pathway to ensure that *csfB* is transcribed exclusively under σ^F control at early times in the forespore.

The Q Tyr-28–*csfB* regulatory circuit. By what mechanism might Q Tyr-28 and the *csfB* promoter element prevent σ^G-dependent activation of *csfB*? Figure 7 provides our working model for this regulatory pathway. First, we propose that the conserved *csfB* promoter element is a binding site for an unidentified DNA-binding repressor protein (Rep in Fig. 7). Given the proximity of the conserved nucleotides to the −35 element of $P1_{csfB}$ (Fig. 4A), as well as our findings that alterations to either side of the −35 element cause promoter misregulation, we speculate that a protein docked at this site could simply prevent RNA polymerase holoenzyme binding due to obstruction of the −35 element. But then how does binding of a repressor specifically block σ^G-mediated activation of $P1_{csfB}$ without also interfering with its earlier activation by σ^F? To account for this, we predict that the putative repressor protein is present and active specifically in the forespore only after the earlier phase of σ^F activity is complete. One possibility is that the gene encoding the repressor protein is expressed under the control of σ^F or σ^G. Alternatively, the repressor protein may be differentially regulated by a posttranslational modification or some other protein-protein interaction in the forespore at later times. Efforts are ongoing in our laboratory to identify this putative *csfB* transcriptional repressor through candidate and unbiased approaches. To date we have excluded the possibility of the involvement of several candidates (our unpublished results), including two DNA-binding proteins known to regulate gene expression in the forespore, RsfA and SpoVT (35, 44).

The mechanism by which Q Tyr-28 blocks σ^G-dependent *csfB* expression is less clear. The best-understood function of Q is its assembly with the mother cell proteins AA-AH into a channel apparatus that connects the forespore and mother cell (23). This AA-AH·Q channel is generally required for gene expression at late times in the forespore, including that directed by σ^F, σ^G, or the heterologous T7 RNAP (21). The current model suggests that the channel serves as a portal (feeding tube) through which the mother cell delivers small-molecule nutrients required for transcription and/or translation (21, 22). One possibility is that the misexpression of *csfB* by σ^G in Q^{Y28A} mutant cells is the result of altered channel activity. For example, the AA-AH·Q^{Y28A} mutant channel may have enhanced activity that generally stimulates gene expression in the forespore, including that directed by σ^G. We find this explanation unlikely for several reasons. First, and in contrast to the activity of $P1_{csfB}$, the well-characterized σ^G-dependent promoters P_{sspB} and P_{spoVT} are not more active in the Q^{Y28A} mutant but, rather, display an ~25 to 30% reduction in expression. Second, no other tested σ^F-target promoter displayed evidence of misexpression by σ^G in the absence of Q Tyr-28. Together, these findings indicate that *csfB* expression is specifically misregulated in Q^{Y28A} cells, in contrast to the general effect on forespore gene expression expected from altered channel activity. Finally, the misexpression of *csfB* in Q^{Y28A} cells is complemented by an N-terminal fragment of Q entirely lacking the C-terminal domain required to interact with mother cell channel components. As such, we propose here that the best explanation for our data is that the Q^{Y28A} variant is defective for a second function of Q that specifically regulates expression of *csfB* in the forespore and for which the N-terminal TMD region of Q is sufficient.

If Q Tyr-28 does not affect *csfB* expression via AA-AH·Q channel activity (as we have argued against above), then by what mechanism does it do so? We speculate that Q Tyr-28 is part of a mechanism required to activate the *csfB* repressor protein (Fig. 7, dashed arrows). We expect that the repressor protein is regulated such that it is present and active only at late times in the forespore. Given that Q itself is expressed under σ^F control, its involvement in this pathway could provide a delay function, creating a window of time during early sporulation in which the repressor protein is not yet active and σ^F-mediated activation of *csfB* can occur. It is tempting to speculate further that the involvement of Q in this pathway could also serve a checkpoint function, coupling the status of the AA-AH·Q channel function to σ^G activation. For example, perhaps the forespore stops the synthesis of the σ^G inhibitor CsfB only once the channel, which provides general resources required for gene expression, is assembled and active.

A role for SpoIIE? One question raised by our model for *csfB* regulation is a topological one: how can Q Tyr-28, an amino acid buried within the forespore membrane, regulate gene expression in the forespore cytosol? Only the N-terminal ~20 amino acids of Q are predicted to be exposed to the forespore cytosol, and these are followed by the Q TMD within which Tyr-28 is embedded.

Given this topology, as well as the relative lack of conservation among its cytosolic N-terminal amino acids (Fig. 1), it seems unlikely that any part of the full-length, membrane-anchored Q protein interacts directly with any cytosolic components of the *csfB* regulatory circuit. As such, we envision two possible explanations for Q Tyr-28-mediated *csfB* regulation in the cytosol. The first is that Q may undergo a change in topology such that Tyr-28 gains access to the cytosol. Intriguingly, Q is subject to proteolysis by the secreted protease SpoIVB at a site not far downstream of the TMD (after Val-72), resulting in an N-terminal fragment that becomes cytosolic (45, 46). However, we have found that *csfB* expression is not misregulated in a strain in which *spoIVB* is deleted (our unpublished results), arguing against a model in which a SpoIVB-generated fragment of Q is responsible for *csfB* regulation. Nevertheless, it remains a formal possibility that Q (or a subpopulation of Q) undergoes another change in topology that has yet to be characterized.

The second possible explanation for Q Tyr-28-mediated *csfB* regulation in the cytosol, which we currently favor, is that Q laterally interacts (via Tyr-28) with an intermediary protein in the membrane and that this intermediary protein has a cytosolic domain that either regulates the putative *csfB* repressor or acts directly as the repressor. The multifunctional, membrane-anchored sporulation protein SpoIIE, which plays critical roles in earlier sporulation events, including asymmetric division and σ^F activation (9–11), is an intriguing candidate for this hypothetical intermediary protein linking Q Tyr-28 to *csfB* promoter regulation. The SpoIIE protein has the expected topology, with both an N-terminal membrane-spanning region (with 10 TMDs) (47) and two cytosolic domains: a central regulatory domain (48) and a C-terminal protein phosphatase 2C domain (9, 47). SpoIIE persists and dynamically localizes to the forespore membrane throughout and after engulfment (12) and is therefore appropriately positioned to participate in the regulatory circuit controlling *csfB* expression. Moreover, this late forespore population of SpoIIE interacts with a subpopulation of Q (apparently distinct from the subpopulation forming channel interactions), as evidenced by biochemical copurification, microscopic colocalization, and the loss of SpoIIE dynamic localization in a Δ*Q* mutant (12). These data indicate that SpoIIE, in cooperation with Q, performs a third function (in addition to its roles in asymmetric division and σ^F activation) at late times in the forespore.

Could it be that *csfB* gene regulation in collaboration with Q Tyr-28 is the mysterious third function of SpoIIE? Consistent with this idea, we found here that SpoIIE was mislocalized and unstable in the Q^{Y28A} mutant, thus correlating proper SpoIIE localization and stability with proper regulation of the *csfB* promoter. On the other hand, the mislocalization and instability of SpoIIE in the Q^{Y28A} strain were not detectably complemented by the wild type N-terminal domain of Q (Q^N) (our unpublished results), in contrast to the robust restoration of σ^G activity and *csfB* repression found using the same complementation strategy. This lack of correlation may argue against a role for SpoIIE in *csfB* regulation or could indicate that, for example, only a small subpopulation of properly localized and/or stable SpoIIE is sufficient to fully restore *csfB* regulation and σ^G activity. Unfortunately, a direct test for a causal link between SpoIIE and proper *csfB* regulation is complicated by the earlier functions of SpoIIE in sporulation: a Δ*spoIIE* mutant is unable to progress to the stage of sporulation at which this regulatory circuit operates (10, 49). Efforts to find SpoIIE variants that are specifically defective in *csfB* regulation (for example, by identifying and substituting highly conserved amino acids in the SpoIIE TMDs) have so far been unsuccessful. Current work in our laboratory is focused on other approaches, such as targeted protein degradation, to determine the role, if any, of SpoIIE in *csfB* regulation. In the event that SpoIIE is found to not participate in *csfB* regulation, our findings here will instead serve as evidence of another function of Q Tyr-28 (in partnership with SpoIIE) at later times in the developing forespore.

Other targets for Q Tyr-28? Our identification of the regulatory circuit controlling *csfB* expression was serendipitous, given that this study began as an investigation of a puzzling variant of Q lacking the amino acid Tyr-28. We were first able to link Q^{Y28} to *csfB* through our finding that the 25 to 30% reduction in σ^G activity in the Q^{Y28A} mutant was significantly alleviated in the absence of *csfB*. Based on this genetic interaction, we hypothesized that σ^G was subject to excessive inhibition by CsfB in the absence of Q Tyr-28. This in turn led us to discover that Q Tyr-28 participates in a regulatory circuit that ordinarily prevents σ^G-dependent activation of $P1_{csfB}$ in the forespore. The simplest explanation for the Q^{Y28A} mutant phenotype, then, is that σ^G activity is reduced due to excessive synthesis of *csfB* by σ^G itself; in other words, it is reduced due to an inappropriately timed negative-feedback loop in which σ^G directs the synthesis of its own inhibitor. The ability of Δ*csfB* to partially restore σ^G activity to Q^{Y28A} cells is consistent with this model. A second prediction of this model is that expression of *csfB* itself from a mutant promoter activated by both σ^F and σ^G (such as the $^{mut1}P_{csfB}$ promoter) should also cause a reduction in σ^G activity, similar to that caused by the Q^{Y28A} mutation. However, we observed no detectable defect in σ^G activity in cells expressing $^{mut1}P_{csfB}$-*csfB*. One explanation could be that the alterations to the *csfB* promoter in the strain with $^{mut1}P_{csfB}$ do not cause misexpression to the same extent that they do in the Q^{Y28A} mutant, although we find this unlikely, given that misexpression appears to be equivalent in the two strains, as measured from *lacZ* reporters. Instead, we interpret these data to indicate that the misexpression of *csfB* is not sufficient to explain the reduction of σ^G activity in Q^{Y28A} cells.

By what other mechanisms might Q Tyr-28 promote maximal σ^G activity? One possibility is that Q Tyr-28 also regulates CsfB at the posttranscriptional level (CsfB activity or protein stability, for example). In addition, we predict that there must be other targets of Q Tyr-28 regulation aside from *csfB*, given that the deletion of *csfB* does not fully restore σ^G activity to Q^{Y28A} cells. It is tempting to speculate that these targets are other genes whose expression is similarly regulated by Q Tyr-28 in the forespore; in this scenario, the reduction of σ^G activity in the Q^{Y28A} mutant would be due to the collective misregulation of several genes and, as such, would not be phenocopied by misexpression of any one individual gene. We anticipate that transcriptional profiling of wild-type *Q* versus Q^{Y28A} strains will help to identify any additional targets of Q Tyr-28 transcriptional regulation that, like *csfB*, modulate σ^G activity in the forespore.

Q is a multifunctional protein. One final conclusion from this study is that Q is a bifunctional protein. In addition to its role as a component of the AA-AH·Q feeding tube channel, which generally promotes late gene expression in the forespore, we have found here that Q also functions in a gene regulatory circuit to specifically limit forespore expression of *csfB* (Fig. 7). This finding adds Q to a growing list of bi-/multifunctional proteins that serve to

coordinate two or more events or processes during bacterial cell growth and development (50). For example, during the switch from flagellum-mediated motility to sessile growth in biofilms, the *B. subtilis* protein EpsE functions as both a flagellar clutch that halts cellular motility and an extracellular polysaccharide (EPS) biosynthetic enzyme, helping to form the matrix that binds the biofilm community together (51, 52). In another example, *Caulobacter crescentus* CtpS functions as both a CTP biosynthetic enzyme and a regulator of cell shape via interaction with the cytoskeletal protein crescentin (53). Our discovery that Q is also a bifunctional protein helps to further highlight this important class of proteins and raises the intriguing idea that other critical regulatory mechanisms and pathways driving *B. subtilis* sporulation (or any number of other biological processes for that matter) are hidden in plain sight within some of the most well-studied proteins.

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