Competence in *Streptococcus pneumoniae* Is Regulated by the Rate of Ribosomal Decoding Errors

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ABSTRACT Competence for genetic transformation in *Streptococcus pneumoniae* develops in response to accumulation of a secreted peptide pheromone and was one of the initial examples of bacterial quorum sensing. Activation of this signaling system induces not only expression of the proteins required for transformation but also the production of cellular chaperones and proteases. We have shown here that activity of this pathway is sensitively responsive to changes in the accuracy of protein synthesis that are triggered by either mutations in ribosomal proteins or exposure to antibiotics. Increasing the error rate during ribosomal decoding promoted competence, while reducing the error rate below the baseline level repressed the development of both spontaneous and antibiotic-induced competence. This pattern of regulation was promoted by the bacterial HtrA serine protease. Analysis of strains with the *htra* (S234A) catalytic site mutation showed that the proteolytic activity of HtrA selectively repressed competence when translational fidelity was high but not when accuracy was low. These findings redefine the pneumococcal competence pathway as a response to errors during protein synthesis. This response has the capacity to address the immediate challenge of misfolded proteins through production of chaperones and proteases and may also be able to address, through genetic exchange, upstream coding errors that cause intrinsic protein folding defects. The competence pathway may thereby represent a strategy for dealing with lesions that impair proper protein coding and for maintaining the coding integrity of the genome.

IMPORTANCE The signaling pathway that governs competence in the human respiratory tract pathogen *Streptococcus pneumoniae* regulates both genetic transformation and the production of cellular chaperones and proteases. The current study shows that this pathway is sensitively controlled in response to changes in the accuracy of protein synthesis. Increasing the error rate during ribosomal decoding induced competence, while decreasing the error rate repressed competence. This pattern of regulation was promoted by the HtrA protease, which selectively repressed competence when translational fidelity was high but not when accuracy was low. Our findings demonstrate that this organism is able to monitor the accuracy of information used for protein biosynthesis and suggest that errors trigger a response addressing both the immediate challenge of misfolded proteins and, through genetic exchange, upstream coding errors that may underlie protein folding defects. This pathway may represent an evolutionary strategy for maintaining the coding integrity of the genome.

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Quality control processes during protein biosynthesis ensure the production of functional cellular proteins and prevent the accumulation of toxic aggregates (1) of misfolded proteins. Starting with DNA replication and continuing through ribosomal synthesis of nascent polypeptides, proofreading mechanisms monitor the accurate transmission of information required for protein production (2–4), while cellular chaperones and proteases facilitate proper protein folding and degradation of misfolded proteins (5–9). To deal with conditions of elevated protein folding stress, coordinated responses, including the unfolded protein response (5), heat shock response (8, 10), and extracytoplasmic stress response (9), are employed by both bacteria and eukaryotes to promote production of chaperones and proteases. Although these responses address the downstream folding aspects of protein biosynthesis, they do not address the quality of the upstream information that drives protein production.

The competence response of *Streptococcus pneumoniae* is intriguing in this regard because it combines chaperone and proteinase production with genetic recombination. Although characterized initially as regulating transformation, the *S. pneumoniae* competence signaling pathway controls a broader phenotype, recently also designated the pneumococcal X-state (11), that includes induction of such stress response proteases and chaperones as those encoded by *clpL, htra, grpE, dnaK, dnaJ, groEL*, and *groES* (12). Competence in *S. pneumoniae* is induced in response to detection by a two-component signaling system of a peptide pheromone (CSP) secreted by the bacterium. The resulting sharp peak of competence expression during early or mid-exponential-phase
growth has often been considered an example of quorum sensing, or a prokaryotic display of a density-dependent multicellular behavior. This view recently has been questioned (11, 13) based on observations showing that the development of competence is modulated by other factors beyond density, including activation by the antibiotics streptomycin and kanamycin (14). Although the mechanism of this antibiotic effect was uncertain, these agents increase the rate of decoding errors during translation (15). We therefore tested whether the effect of these agents on competence was due specifically to the induction of ribosomal errors. Our observations support a role for competence as a multifaceted response to errors during protein biosynthesis.

RESULTS
Modulation of ribosomal decoding errors and competence by antibiotics. Competence was monitored using an ssrB'-luc transcriptional fusion, activation of which correlates with transformability of pneumococcal cultures (14). Development of spontaneous competence in untreated, wild-type cultures is sensitively dependent on pH, and use of permissive conditions (pH near 7.30; Fig. 1C) or nonpermissive conditions (pH near 7.20; Fig. 1A and B) facilitates assays for either enhancement or repression of competence. Streptomycin induced competence at concentrations of 3 or 6 μg/ml (Fig. 1A), while a similar effect was seen with 6 or 12 μg/ml of kanamycin (Fig. 2A).

Prudhomme et al. previously reported that a comA deletion mutant failed to induce competence in response to streptomycin (14). This observation suggested that antibiotic induction of competence resembled the spontaneous pneumococcal competence displayed under permissive conditions in that it depended on secretion of CSP by the ComAB transporter system. Consistent with a role for CSP in streptomycin-induced competence, we found that stimulation of a sensitive strain by streptomycin could indirectly activate expression of the ssrB'-luc reporter in a streptomycin-resistant strain when grown in mixed culture (Fig. 3). Together these observations suggest that induction of competence by streptomycin proceeds through the peptide pheromone system rather than bypassing this pathway.

To measure the rate of translational errors in S. pneumoniae, we utilized a nonsense suppression assay in which a premature stop codon interrupts a constitutive lacZ reporter. Streptomycin induced translational misreading of 2 stop codons (TAA and TAG) at the concentrations required to trigger competence (Fig. 1G and H). The third stop codon (TGA) displayed an elevated error rate back nearly to the wild-type level and restored induction of competence by streptomycin (see Fig. S1 in the supplemental material).

Effects of ribosomal mutations on decoding accuracy and competence regulation. Intrinsic decoding accuracy is affected by mutations in components of the 3OS ribosomal subunit. These mutations may produce either a restrictive phenotype with increased translational fidelity or a ram (ribosomal ambiguity) phenotype with an elevated rate of decoding errors (21–23). The S12 ribosomal protein, encoded by rpsL, is a frequent site of restrictive mutations that confer streptomycin resistance and increase decoding accuracy (21, 24). The rpsL(K56T) mutation in S. pneumoniae (homologous to K42T in E. coli numbering) reduced nonsense suppression to 4.7% of the wild-type level (Fig. 4B) and repressed development of spontaneous competence (Fig. 4A). Although ram mutations have not been previously identified in S. pneumoniae, ram mutations in other organisms frequently map to the interface between the S4 and S5 proteins (25). A pneumococcal rpsD(K201T) mutation [homologous to rpsD(K205T) in E. coli numbering] produced a weak ram phenotype with elevation of nonsense suppression by only 15% and did not affect competence (data not shown). A small C-terminal deletion in the S4 protein [rpsD(ΔK201-L203)], however, produced a strong ram effect (Fig. 4E) and promoted development of competence even under nonpermissive conditions (Fig. 4C). In the restrictive rpsL(K56T) background, this ram mutation increased the translational error rate back nearly to the wild-type level and restored competence under permissive conditions, which had been lost in the restrictive mutant (Fig. 4D).

Error-sensitive modulation of competence by the HtrA protease. Having observed the sensitivity of the pneumococcal competence pathway to translational errors, we considered how this pattern of regulation might be generated and in particular whether the pneumococcal HtrA protease might be involved. This surface-associated serine protease is the sole member of the HtrA family found in S. pneumoniae, and activity of this protease has been shown to repress competence, although the molecular target of its activity is uncertain (26). The observation that DegP (one of
three HtrA proteases in *E. coli* degrades and refolds denatured periplasmic proteins by means of its dual protease and chaperone activities (27–29) suggested that HtrA might interact with misfolded proteins in addition to regulating competence. If processing of misfolded proteins were to compete with the ability of HtrA to repress competence, we reasoned that this interaction might result in derepression of competence in response to errors during protein synthesis, some of which would produce proteins with intrinsic folding defects.

We predicted, therefore, that the ability of HtrA to repress competence would decline as the frequency of translational errors rose. Strains with wild-type *htrA* were compared under conditions of low, medium, and high error rates (Fig. 5D) with isogenic strains in which an *htrA* (*S234A*) mutation (26) inactivates the catalytic site of the protease. As anticipated, wild-type *htrA* caused repression of competence when decoding errors were rare but had less effect as decoding errors became more common (Fig. 5A to C; see also Fig. S2 in the supplemental material). At the highest level...
of mistranslation, competence profiles of the mutant and wild-type strains were nearly identical. Similar results were seen when an htrA deletion strain was examined (see Fig. S3).

We observed, furthermore, that the htrA(S234A) strain displayed a growth defect compared to the wild type in the presence of streptomycin (Fig. 5E to G) under permissive conditions in which both strains developed competence with similar kinetics. This conditional reduction in the growth rate became evident only when the concentration of streptomycin was increased from 1 to 3 μg/ml (P = 0.13, 0.017, and 0.0009 at 1, 2, and 3 μg/ml of streptomycin, respectively). The faster growth of the wild-type strain indicates that in addition to its role as a regulator of competence, htrA functions to alleviate the stress imposed by mistranslated proteins. This finding is consistent with a model of dual regulatory and stress-response activities for pneumococcal HtrA, and competition between such dual activities may be responsible for the derepression of competence observed in response to translational errors.

To exclude the possibility that streptomycin might induce competence by lowering HtrA expression rather than by modulating its proteolytic effects, we assessed the impact of this antibiotic on HtrA protein levels. Neither streptomycin nor other antibiotics

![Diagram](image-url)
competition-inducing antibiotics reduced HtrA levels (see Fig. S4 in the supplemental material), indicating that under high translational error conditions this protease continues to be expressed but is no longer effective in repressing competence.

**DISCUSSION**

In this work, we have shown that development of competence in *S. pneumoniae* is sensitively modulated in response to the rate of biosynthetic errors during protein production. This pattern of activation in response to decoding errors provides an explanation for the observation that cellular chaperones and proteases are induced as part of the pneumococcal competence regulon and suggests that competence functions as a response to misfolded proteins arising from such synthetic errors. Although this response may help the bacterium prevent the accumulation of misfolded proteins, generalized stress per se from aberrant protein production does not appear to be required for competence induction. Rather, this pathway is able to monitor changes in translational fidelity that even represent improvements over the performance of the wild type and to activate competence before the level of miscoding is sufficient to impair growth [in Fig. 4A, compare the enhanced growth of the competent wild-type *rplL* strains with that of the *rpsL*(K56T) strain that does not develop competence].

We have observed that the pneumococcal HtrA protease represses competence selectively under conditions where the translational error rate is low. This protease appears thereby to function as an indirect sensor to modulate the development of competence in response to the level of mistranslated proteins. Because the molecular target through which HtrA represses competence is uncertain, the mechanism of this error-sensitive competence modulation remains the subject of investigation. A model that appears consistent with our observations without invoking the existence of new components of the signaling pathway would involve direct degradation of CSP by HtrA. In addition to its parsimony, this model is attractive because the hydrophobic patch on CSP that is required for its signaling function (30) appears likely to resemble the hydrophobic regions of unfolded proteins that become substrates for the DegP protease when exposed to a solvent by denaturation (31). The conditional reduction in the growth rate of the *htrA*(*S234A*) mutant when exposed to increasing concentrations of streptomycin suggests that, like DegP, pneumococcal HtrA may also serve to degrade generic misfolded proteins. Competition among substrates for limited HtrA proteolysis may then cause derepression of competence as a consequence of reduced degradation of CSP when the abundance of mistranslated proteins rises. This model would be consistent with the observation that streptomycin-induced competence requires the CSP transport protein ComA (14) as well as our finding that streptomycin induces competence in a manner that is capable of spreading between cells in a mixed culture. At a more general level, it is conceivable that HtrA regulates competence through a substrate other

![Figure 4](https://mbio.asm.org/figure/2011/11/09/7101/RibosomalErrorsInduceS_pneumoniaeCompetence.png)

**FIG 4** Ribosomal mutations affecting translational accuracy and competence. (A) A strain with an *rpsL*(K56T) allele (KSP86) displays repressed luciferase activity (triangles) compared to isogenic strains with wild-type (wt) *rpsL* before the K56T mutation (R895) or after reversion (KSP107). OD$_{620}$s of cultures are shown with squares of matching colors. (B) The *rpsL*(K56T) mutation decreases suppression of a TAA codon. (C and D) An *rpsD*($\Delta$K201-L203) *rpsL* mutant strain (DCP32 in panel C and DCP31 in panel D) increases luciferase activity (triangles) compared with that of wild-type *rpsD* (strains DCP19 in panel C and KSP190 in panel D) in the background of wild-type *rpsL* (C) or *rpsL*(K56T) (D) alleles. All strains in these panels express the *ssbB*-luc competence reporter. (E) Effects of *rplL* and *rpsD* mutations on suppression of a TAA codon. *, P < 0.001 by ANOVA with Bonferroni’s correction.
than CSP but that an analogous competitive mechanism (i.e., between competence-related and generic substrates for HtrA) may still account for the induction of competence by translational errors.

The localization of HtrA to the cell envelope of *S. pneumoniae*, where it fractionates primarily with the membrane and to a lesser degree with the cell wall (26), suggests that HtrA interacts selectively with surface-associated or secreted proteins and regulates competence in response to mistranslation of these proteins. Factors in addition to HtrA, however, must participate in the regulation of competence in response to decoding errors because changes in the error rate impact competence even when *htrA* is inactivated, although the response is less steep. One candidate for mediating this residual response is the multisubunit cytoplasmic Clp protease, which also degrades aberrant proteins (32) and represses competence through degradation of the competence sigma factor ComX (33). Direct investigation of the role of Clp proteases in the error-induced competence response has been impaired by a severe growth defect observed following deletion of *clpP*, encoding the catalytic subunit, and the acquisition of a secondary mutation(s) that affects competence development.

An alternative to the model of conditional degradation of CSP by HtrA is the possibility that HtrA might function exclusively to relieve the stress of misfolded surface proteins and that accumulation of these misfolded proteins when not cleared by HtrA might trigger competence through another pathway that has yet to be described. Although this second model cannot be excluded, we favor the former mechanism for the reasons discussed above and because the loss of *htrA* activity stimulated competence even under conditions where the mutation was not associated with a growth defect (Fig. 5A). This observation suggested that induction of competence in the *htrA* mutant is unlikely to be an indirect effect of stress from accumulation of misfolded proteins. Whether misfolded proteins stimulate competence directly or by reducing degradation of CSP, these findings also raise the question of whether external stresses that damage and denature surface proteins might activate competence in a manner analogous to what we have seen with translational errors. The possibility that such external interactions—with either the immune system or other bacteria—may trigger competence remains to be investigated. It should also be noted that this work has been conducted using the unencapsulated laboratory strain R6 and that more definitive conclusions regarding the biological relevance of these observations will require confirmation with wild-type strains.

In addition to streptomycin and kanamycin, it was previously reported that pneumococcal competence could be induced by ei-
Ribosomal Errors Induce S. pneumoniae Competence

Such divergent functions should perhaps not be surprising considering that the protease domain of pneumococcal HtrA has no more sequence similarity with either DegS (56%) or DegP (64%) than these proteases have with each other (65%) or with the third E. coli protease in the HtrA family, DegQ (63% similar to pneumococcal HtrA, 67% to DegS, and 86% to DegP). The broad distribution of the HtrA family of proteases, however, in both bacteria and eukaryotes—where HtrA proteases have been linked to protein misfolding diseases such as Alzheimer’s (44) and Parkinson’s (45, 46) diseases—raises the question of whether HtrA proteases in other organisms may also function as biosynthetic error sensors, albeit with outputs potentially distinct from transformation.

The finding that competence is induced by ribosomal decoding errors additionally suggests a mechanism by which genetic damage—in the form of miscoding at the level of the genome rather than the ribosome—may trigger the same competence response. A pathway sensing such informational lesions in the genome would contrast with previously characterized DNA damage responses that are activated by physical damage. The pneumococcal competence system may thereby function to address the stress of protein misfolding both by activating proteases and chaperones and by initiating transformation to repair underlying genetic damage.

MATERIALS AND METHODS

Bacterial growth conditions, strains, and mutagenesis. Both cultures of S. pneumoniae strain R6 and derivatives were grown in C+Y medium prepared using 2 distinct recipes that have both been described by that name. An early reference to this medium used Difco Casamino Acids in its formulation (47), whereas modifications later substituted individual amino acids among other changes (48, 49). We have designated the first recipe as C+YCA (for Casamino Acids) and the second as C+YVA (for its increased concentration of yeast extract, as well as bovine serum albumin). The compositions of these media are given in Table S1 in the supplemental material. We found that C+YVA but not C+YCA supported development of spontaneous competence in the microtiter plate assay described below. The final pH of C+YVA medium was adjusted to near 7.30 or 7.20 to produce medium either permissive or nonpermissive, respectively, for the development of spontaneous competence in untreated, wild-type cultures. S. pneumoniae was grown in C+YCA medium for mutagenesis and routine propagation. Antibiotics were used in the following concentrations for selection: erythromycin, 2 µg/ml (S. pneumoniae) and 500 µg/ml (E. coli); streptomycin, 500 µg/ml; and kanamycin, 500 µg/ml.

S. pneumoniae and E. coli strains used in this study are described in Tables S2 and S3 in the supplemental material, respectively. Targeted mutations were introduced into S. pneumoniae by PCR ligation mutagenesis (50) using primers listed in Table S4. Unmarked point mutations and deletions in htrA and bgaA were produced using the counterselectable Janus cassette (51). Because disruption of rpsD with the Janus cassette would have been lethal, mutations in this gene were generated by introducing aph3 (conferring kanamycin resistance) downstream of rpsD by PCR ligation mutagenesis using primers designed to introduce changes into the 3’ end of rpsD adjacent to aph3. Control strains were generated with aph3 in the same location downstream of wild-type rpsD. Mutations in rpsL were isolated by direct screening or selection for changes in streptomycin susceptibility following transformation.

lacZ reporters for measuring nonsense suppression were constructed by amplifying the promoterless copy of lacZ from pEV35 (52) using primers encoding the pneumococcal amiA promoter. This ampiclon was cloned into the BamHI site of the shuttle plasmid pMU1328 (53). Premature stop codons were generated using QuickChange mutagenesis (Agilent...
Technologies, Santa Clara, CA). Plasmids with lacZ reporters were transformed into S. pneumoniae strains with deletions in bgaA, inactivating this endogenous galactosidase and reducing background activity in LacZ assays.

Nonsense suppression assays. Nonsense suppression was measured by determining beta-galactosidase activity in cultures of pneumococcal strains bearing lacZ reporters with and without premature stop codons early in the coding sequence. The ratio of LacZ activity in the presence of the stop codon compared to that from an uninterrupted reporter provided a measure of ribosomal accuracy. Strains to be tested were initially inoculated into C+Y medium and grown to an optical density at 620 nm (OD620) of 0.26 before being concentrated by centrifugation and resuspended in 0.75 volumes fresh C+Y medium with 16% glycerol. Aliquots were frozen at −75°C for later use. From these frozen stocks, cultures were diluted 1:50 into C+Y medium containing antibiotics as indicated and grown at 37°C to an OD620 near 0.25. Bacteria were then lysed by adding Triton X-100 to a concentration of 0.1% and incubating for 10 min at 37°C. To each sample, 0.25 volumes of a reaction buffer containing 5 mM MgCl2, 50 mM KCl, 0.3 M Na2HPO4, 0.2 M NaHPO4, 4 mg/ml 2-nitrophenyl-β-D-galactopranoside, and 250 mM 2-mercaptoethanol was then added. Samples were incubated at room temperature, after which color development was stopped by addition of 0.025 M Na2HPO4, 0.005 M NaCl, 4 mM 2-mercaptoethanol, and 0.001 M 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. After incubation for 1 h at 37°C, absorbance was measured at 420 and 550 nm, and Miller units were calculated as previously described (54). Statistical comparisons were made by analysis of variance (ANOVA) testing, with posttests for linear trends for analyses involving multiple doses of the same antibiotic, using the Prism 4.0 software program (GraphPad Software, La Jolla, CA).

Competence assays. Competence was determined by measuring the activity of a luciferase reporter in strains with an sbbB-luc transcriptional fusion (55). Pneumococcal sbbB is induced specifically during competence, and activity of this fusion has been shown to reflect competence for transformation (14). Strains to be tested were initially inoculated into CAT medium (56) and grown to an OD620 of 0.26 before being concentrated by centrifugation and resuspended in 0.75 volumes fresh CAT medium with 16% glycerol. Aliquots were frozen at −75°C for later use. From these frozen stocks, cultures were diluted 1:400 into C+Y medium containing 0.65 mM 1,2-luciferin. Antibiotics were added as indicated. Samples were grown in 200-μl aliquots in white NBS 96-well microplates (Corning Inc., Corning, NY). Luminescence and the OD620 were measured by a plate reader (Bio-Tek, Winooski, VT). Activity of the luciferase reporter in strains with a luc+ ssbB fusion (55) was used as a negative control.

SUPPLEMENTAL MATERIAL

SUPPLEMENTAL MATERIAL for this article may be found at http://mbio.asm.org.

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