INFECTION AND IMMUNITY, July 2008, p. 3131–3140 Vol. 76, No. 7
0019-9567/08/$08.00+0 doi:10.1128/IAI.01696-07
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# The *Streptococcus pneumoniae* Competence Regulatory System Influences Respiratory Tract Colonization▽

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Received 19 December 2007/Returned for modification 17 February 2008/Accepted 19 April 2008

**The *Streptococcus pneumoniae* ComDE two-component signaling system controls the development of genetic competence in the bacterium and affects virulence in models of pneumonia and bacteremia. We have investigated the impact of the competence pathway during colonization of the nasopharynx, the principal ecological niche of the pneumococcus. Previous work showed that deletion of the pneumococcal CiaRH signaling system inhibited colonization and increased expression of genes required for competence. We anticipated that signaling by the competence pathway might similarly reduce carriage. Consistent with this expectation, a *comE* deletion that blocked transformation increased colonization fitness such that the mutant outcompeted the wild type in an infant rat model of asymptomatic carriage. Deletion of *comD*—immediately upstream of *comE* and likewise required for competence—similarly increased colonization fitness if the orientation of the antibiotic resistance cassette inserted into the *comD* locus was such that it reduced transcription of *comE*. However, an alternative *comD* deletion mutation that caused an increase in *comE* transcription impaired colonization instead. Activation of the competence system through a *comE*(*D143Y*) mutation did not affect colonization, but an inability to secrete the competence-stimulating peptide due to deletion of *comAB* produced a density-dependent reduction in colonization fitness. These results suggest a model in which signaling by the unactivated form of ComE reduces colonization fitness compared to that of bacteria in which it is either activated or absent entirely, with the most substantial fitness gain accompanying deletion of *comE*. This observation demonstrates that the pneumococcus incurs a substantial fitness cost in order to retain a functional competence regulatory system.**

Natural competence for genetic transformation in the gram-positive pathogen *Streptococcus pneumoniae* is triggered by a peptide pheromone signaling system through a molecular pathway that has been well described (reviewed in reference 5). The role of this pathway in the overall biology of this pathogen, however, is less well characterized. Competence is triggered by activation of the ComDE two-component signaling system in response to accumulation of competence-stimulating peptide (CSP) outside the bacterium. CSP is produced as a prepeptide encoded by *comC* and is exported by the ComAB transporter and cleaved to generate the mature peptide (11, 12). The ComD histidine kinase is then activated by CSP and signals through its cognate response regulator, ComE, to induce the expression of an early group of genes (4, 26, 27, 35). These induced genes include *comAB* and *comCDE* themselves, resulting in a positive feedback loop that promotes synchronized development of competence throughout the population in response to increasing levels of CSP. ComE also induces expression of *comX*, which encodes an alternative sigma factor that then stimulates expression of a larger group of late-phase genes, including those required for DNA uptake and recombination (18, 20, 21).

Several pieces of evidence have recently suggested that the competence circuit regulates traits that may be important for aspects of pneumococcal physiology beyond genetic exchange. Microarray profiling revealed that activation of the competence pathway increased the expression of more than 120 genes, or approximately 6% of the bacterial genome (27). Fewer than 30 of these genes were required for transformation (27), suggesting that other phenotypic characteristics may be coregulated with the induction of competence. The competence pathway was recently linked to the virulence of this pathogen. Mutants lacking the ComD receptor were shown to be attenuated in models of both pneumonia and bacteremia (2, 16), while deletion of the ComAB peptide transporter decreased virulence in the bacteremia model but not in a pneumonia model (2, 16). Perturbation of the competence system in the opposite direction by administration of synthetic CSP has also been shown to produce a similar effect, improving survival of mice infected intravenously with *S. pneumoniae* (24). The competence pathway has been linked to several additional aspects of pneumococcal biology that are likely to be relevant to survival in vivo, including bacterial aggregation and biofilm formation (25, 27, 34).

The impact of the competence system on pneumococcal colonization of the upper respiratory tract, however, has not been well characterized. Because asymptomatic carriage in the upper respiratory tract represents the environment in which *S. pneumoniae* exists most commonly and from which the bacterium spreads to colonize new hosts, we investigated the effect of the competence pathway on colonization of this site. Our earlier work had demonstrated that deletion of the pneumococcal CiaRH two-component system strongly attenuates such

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▽ Published ahead of print on 28 April 2008.

TABLE 1. Pneumococcal strains used in this study

| Strain | Characteristics[a] | Reference |
|---|---|---|
| D39 | Serotype 2 clinical isolate | 1 |
| P143 | R6x laboratory strain, but spontaneous Sm$^r$ mutant | 30 |
| P1139 | Serotype 3 strain 0100993; *ciaRH*::*ermAM* | 33 |
| 706 | Nov$^r$ | 15 |
| P1301 | D39, but Nov$^r$ by transformation with 706 DNA | 30 |
| P1302 | D39, but Sm$^r$ by transformation with P143 DNA | This work |
| P1304 | D39, but Sm$^r$ by transformation with P1302 DNA | This work |
| P1310 | D39 recovered from murine intraperitoneal passage | Weiser[b] |
| P1474 | P143, but *comD*::Janus by transformation with ligated PCR products; Km$^r$ | This work |
| P1534 | P1310, but Sm$^r$ by transformation with P1304 *rpsL* PCR product | This work |
| P1535 | P1310, but $comD_{for}$::Janus by transformation with P1474 *comD* PCR product; Km$^r$ | This work |
| P1537 | P1310, but *comAB*::Janus by transformation with ligated PCR products; Km$^r$ | This work |
| P1538 | P1310, but *comE*::Janus by transformation with ligated PCR products; Km$^r$ | This work |
| JKP102 | P1310, but Janus inserted downstream of *comE* with ligated PCR products; Km$^r$ | This work |
| JKP105 | P1534, but Janus inserted downstream of *comE* with ligated PCR products; Km$^r$ | This work |
| JKP109 | P1310, but *ciaRH*::*ermAM* by transformation with P1139 *ciaRH* PCR product; Em$^r$ | This work |
| JKP114 | JKP109, but *comAB*::Janus by transformation with P1537 *comAB* PCR product; Km$^r$ Em$^r$ | This work |
| JKP116 | JKP109, but *comE*::Janus by transformation with P1538 *comE* PCR product; Km$^r$ Em$^r$ | This work |
| JKP158 | P1310, but $comD_{rev}$::Janus by transformation with ligated PCR products; Km$^r$ | This work |
| MSP110 | JKP105, but Janus replaced with PCR product carrying *comE*(*D143Y*); Sm$^r$ | This work |
| TMP133 | P1310, but *comDE*::Janus by transformation with ligated PCR products; Km$^r$ | This work |

[a] Em$^r$, erythromycin resistant; Km$^r$, kanamycin resistant; Nov$^r$, novobiocin resistant; Sm$^r$, streptomycin resistant.
[b] From the collection of J. Weiser, University of Pennsylvania.

colonization (29). Consistent with other studies of the CiaRH system (7, 8, 10), this *ciaRH* deletion also increased expression of genes in the competence regulon (29). Repression of competence genes by the CiaRH system involves the activity of the HtrA protease (30), which also contributes to the colonization potential of the organism (29). These observations motivated our further examination of the contribution of the competence system during colonization. Because the *ciaRH* and *htrA* mutations that impaired colonization derepressed competence, we sought to evaluate the effects of increased competence signaling as well as decreased competence during colonization.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Bacterial strains used in this study are described in Table 1. *S. pneumoniae* was grown in C+Y medium (14) at pH 7.4 or plated on tryptic soy agar spread with 5,000 U catalase (Worthington Biochemicals, Freehold, NJ) and incubated in candle extinction jars at 37°C. Antibiotics were used at the following concentrations: streptomycin, 500 μg/ml; kanamycin, 500 μg/ml; erythromycin, 1 μg/ml; and novobiocin, 5 μg/ml.

**Construction of mutant strains.** In order to analyze the contribution of the competence regulatory system to fitness for colonization, mutants were constructed with both decreased and increased activity of this signaling system. An isolate of the type 2 pneumococcal strain D39 that had been passaged by murine intraperitoneal infection was chosen as the wild-type background in which to conduct these experiments. A streptomycin resistance marker was introduced into this strain by transformation with a PCR product carrying an *rpsL*(*K56T*) mutation in order to distinguish this strain during mixed colonization assays. Deletions in the competence genes *comAB*, *comD*, and *comE* were constructed by inserting the Janus cassette (32) into these loci, using PCR ligation mutagenesis (17) and resulting in kanamycin resistance. In order to analyze polar effects of its insertion, the Janus cassette was inserted into *comD* in each direction by interchanging the restriction enzyme sites engineered into the primers used to amplify DNA regions flanking *comD*. A control strain was also constructed with the Janus cassette similarly inserted in the intergenic region downstream of the 3′ end of *comE*. A *ciaRH*::*ermAM* insertion-deletion mutation was transferred into the type 2 background by transformation with a PCR product carrying this construct. To activate the competence system, overlap-extension PCR was used to generate a *comE*(*D143Y*) PCR product, which was used to replace the Janus cassette that had been inserted downstream of *comE*; isolates were sequenced to screen for the *comE*(*D143Y*) mutation in the flanking region of DNA. PCR primers used in the construction of these strains are listed in Table 2. The presence of each mutation was confirmed by sequencing. Transformation assays in vitro confirmed that both *comD* deletions and the *comE* deletion prevented development of competence even in the presence of exogenous CSP. The Δ*comDE* double mutant was likewise unable to become competent in the presence of CSP, whereas the *comAB* deletion mutant was able to be transformed in response to exogenous CSP. Insertion of the Janus cassette downstream of *comE* did not affect the transformation profile of the bacterium.

**In vitro competitive growth assays.** *S. pneumoniae* strains were grown individually at 37°C in C+Y medium for 2 h after inoculation from colonies scraped off tryptic soy agar plates, after which time all cultures had entered log-phase growth. Strains were then diluted in fresh medium and mixed in a 1:1 ratio to achieve an overall optical density reading at 620 nm ($OD_{620}$) of approximately 0.04. Colony counts were performed on serial dilutions of aliquots taken after 0, 1.5, 3, and 4.5 h of growth in mixed cultures at 37°C. Plating on selective medium containing either kanamycin or streptomycin was used to distinguish competing strains.

**Nasal colonization model.** Each bacterial inoculum was prepared by growth to an $OD_{620}$ of approximately 0.3, washed, and resuspended in phosphate-buffered saline. Strains for use in competitive colonization experiments were mixed in a 1:1 ratio. Randomized groups of 10 to 14 newborn Sprague-Dawley rats (Taconic, Germantown, NY) were inoculated intranasally with $2 \times 10^6$ to $6 \times 10^6$ CFU of each strain. Colonization was monitored by serial washings of the nasopharynx of each animal on days 1, 2, 4, and 7 following inoculation, as previously described (29, 36). The density of bacteria in the recovered fluid was measured by colony counting; selective media were used to distinguish strains in competitive colonization assays. The relative strength of each competitive interaction was estimated by calculating a competitive index, defined as the ratio of CFU/ml of streptomycin-resistant to kanamycin-resistant bacteria recovered in nasal washings on day 7 for two competing strains divided by the ratio in the inoculum. All procedures were performed in accordance with institutional animal care guidelines.

**Transformation assays.** Pneumococcal strains were inoculated into C+Y medium from colonies scraped off tryptic soy agar plates and grown for 2 h at 37°C before being diluted in fresh medium to achieve an $OD_{620}$ of approximately 0.01. At target growth densities, aliquots were removed and mixed with P1301 DNA (conferring novobiocin resistance) at a final concentration of 2 μg/ml. Samples were incubated at 30°C for 40 min before the addition of 2 U/ml DNase I (Roche) and then at 37°C for 90 min. Transformation efficiency was calculated as the ratio of the number of colonies counted on serial dilutions plated in the presence of novobiocin to that in the absence of antibiotics. Where appropriate, CSP was added to samples at a final concentration of 1 μg/ml and samples were incubated for 10 min at 30°C before the addition of transforming DNA. Assays

TABLE 2. PCR primers used in this study

| Usage | Primer name | Primer sequence (5′–3′) |
| --- | --- | --- |
| PCR ligation mutagenesis | | |
| *comD*$_{for}$ 5′-flanking region | COMCDE3380R26 | TGTAAAAAATAGAGCCAATCTTTCTG |
| | COMCDE2781 (BamHI) | ACGAGGATCCAAAAATGAACAATAACCGTCCC |
| *comD*$_{for}$ 3′-flanking region | COMCDE1560 (ApaI) | AGCAGGGCCCTTAGAAACAGAGATGGAAGGCAG |
| | COMCDE798F23 | CTTGAAATAGGACAACGATGGT |
| *comD*$_{rev}$ 5′-flanking region | COMCDE3380R26 | TGTAAAAAATAGAGCCAATCTTTCTG |
| | COMCDE2781 (ApaI) | AGCAGGGCCCAAAAATGAACAATAACCGTCCC |
| *comD*$_{rev}$ 3′-flanking region | COMCDE1560 (BamHI) | ACGAGGATCCTTAGAAACAGAGATGGAAGGCAG |
| | COMCDE798F23 | CTTGAAATAGGACAACGATGGT |
| *comAB* 5′-flanking region | COMAB1786F23 | TTTTGTTTAGTGATTGGGGTAAG |
| | COMAB2467 (BamHI) | ACGAGGATCCGAGAGCAGACCATTTTTTTGTTC |
| *comAB* 3′-flanking region | COMAB4432 (ApaI) | AGCAGGGCCCAATACCAAGAAGGGGCAGAGGG |
| | COMAB5281R20 | TAGCGAACAGAATCACCGAC |
| *comE* 5′-flanking region | COMCDE2305R25 | TTTCGTTTCAGATATGGTAAGTACG |
| | COMCDE1441 (BamHI) | ACGAGGATCCATATTCTCTCTAGTCTCACTTGATGTTC |
| *comE* 3′-flanking region | COMCDE757 (ApaI) | AGCAGGGCCCTCTCAAAAGTGATTGACAATTAGC |
| | COMCDE6F25 | AATGCTATGGTACAATTACTGATGG |
| *comDE* 5′-flanking region | COMCDE3380R26 | TGTAAAAAATAGAGCCAATCTTTCTG |
| | COMCDE2781 (BamHI) | ACGAGGATCCAAAAATGAACAATAACCGTCCC |
| *comDE* 3′-flanking region | COMCDE757 (ApaI) | AGCAGGGCCCTCTCAAAAGTGATTGACAATTAGC |
| | COMCDE6F25 | AATGCTATGGTACAATTACTGATGG |
| 5′-Flanking region downstream of *comE* | COMCDE2305R25 | TTTCGTTTCAGATATGGTAAGTACG |
| | COMCDE733 (BamHI) | ACGAGGATCCTGCTAATTGTCAATCACTTTTGAG |
| 3′-Flanking region downstream of *comE* | COMCDE698 (ApaI) | AGCAGGGCCCAGAAGGTCCGTTGGTCAAGG |
| | COMCDE6F25 | AATGCTATGGTACAATTACTGATGG |
| Overlap-extension PCR | | |
| *comE*(*D143Y*) 5′ region | COMCDE2389R23 | GGAACTAGCCTATTTTGACGAGG |
| | COMCDE1073F34 | GAAATAATCTACAACATCTTCATTTTCAAGTAAC |
| *comE*(*D143Y*) 3′ region | COMCDE1106R74DY | GTTACTTGAAAATGAAGATGTTGTAGATTATTTCTAC TACAATTACAAGGGAAATGATTTAAAAATTCCT TACC |
| | COMCDE112F26 | AGGATAAGTATGATATGATTGAGCAC |
| Amplification of other loci | | |
| Janus cassette | JANUS765F32 | GATCGGATCCGTTTGATTTTTAATGGATAATG |
| | JANUS2109R30 | ACCTGGGCCCCTTTCCTTATGCTTTTGGAC |
| *rpsL* locus | RPSL871F25 | CGGTACTTTTTACTTTTGGTCTCTC |
| | RPSL1430R22 | TCTTTATCCCCTTTCCTTATGC |
| *ciaRH*::Janus construct | CIARH288F22 | AACGTCGTTTGTTGGCTGAGGC |
| | CIARH3977R21 | CGGTCCATCATATCTTGGTGC |

for the Trt (transformation resistant to trypsin) phenotype, which indicates competence activation, were performed as previously described (30). In these assays, transformation is measured in the presence of exogenous trypsin, which digests extracellular CSP and prevents induction of competence unless mutational activation of the pathway turns it on downstream of the detection of CSP (15).

**RNA isolation and RT.** RNA was prepared, using a hot phenol lysis protocol as previously described (29), from in vitro cultures grown in C+Y medium at pH 7.4 to an $OD_{620}$ of approximately 0.08. Purified RNA samples in 50 μl distilled $H_2O$ were digested with 1 U Turbo DNase (Ambion, Austin, TX) for 60 min at 37°C. An additional 1 U of DNase was added to each sample after the first 30 min of the incubation. DNase was inactivated using 6 μl DNase inactivation reagent (Ambion) according to the manufacturer's protocol. RNA aliquots were reverse transcribed by first mixing 2 μl of RNA with 1 μl random nonamers (50 μM; Sigma, Saint Louis, MO), 1 μl of 10 mM deoxynucleotide triphosphates (Invitrogen, Carlsbad, CA), and 9 μl distilled $H_2O$. The mixtures were heated to 70°C for 10 min and chilled on ice. To each sample was then added 1 μl Superscript III reverse transcriptase (Invitrogen), 4 μl 5× first-strand synthesis buffer, 1 μl 0.1 M dithiothreitol, and 1 μl RNase Out (Invitrogen). Replicate samples were processed without reverse transcriptase. For reverse transcription (RT), samples were incubated at room temperature for 5 min before being moved to a heat block equilibrated at 42°C. Immediately after transfer, the block setting was adjusted to 50°C for 60 min of incubation with an initial temperature gradient. Samples were finally heated to 70°C for 15 min, followed by digestion with 0.5 μl RNase H for 20 min at 37°C.

**Quantitative PCR.** cDNA samples were analyzed by quantitative PCR, using primers and probes designed for *comE*. Transcript levels for *comE* were normalized to those of *rpoA* as an internal control. The sequences of the primers and probes used for *comE* were 5′-AGCTCATTCGTCATTACAATCCTTACG-3′, 5′-CAAAATCTAGGGCTGATACCTGGT-3′, and 5′-6-carboxyfluorescein-TTTGCGACTCTAACATATAAAT-nonfluorescent quencher-3′. The sequences of the primers and probes used for *rpoA* were 5′-CAGAAGATGCTTTAGGGCTTTCAG-3′, 5′-CATCACTTCAGTTGACTTAGCAATCTCA-3′, and 5′-6-carboxyfluorescein-TCAAGATGTTCTGTCAAAATA-nonfluorescent quencher-3′. Dilutions of cDNA samples were mixed with 900 nM of each primer and 45 nM of probe in TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed using a model 7500 sequence detection system (Applied Biosystems). Serial dilutions of cDNA prepared from pneumococcal cultures stimulated with CSP to activate competence were used to generate standard curves for relative quantitation.

## RESULTS

**Construction of ComE activated mutant.** We sought to generate a pneumococcal strain containing a point mutation activating competence signaling for evaluation in a nasal colonization model. Such mutations in *comD* and *comE* have been described previously in the background of unencapsulated lab-

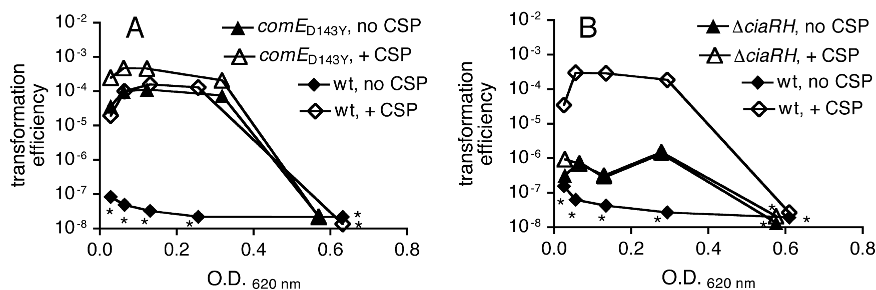


FIG. 1. Transformation efficiencies of wild-type *S. pneumoniae* strain P1534 and *comE*(*D143Y*) strain MSP110 (A) or Δ*ciaRH* strain JKP109 (B) in the presence and absence of exogenous CSP-1. No transformed bacteria were detected for the samples marked with asterisks; symbols for these data points represent the limit of detection of the assay, which decreases as the total number of bacteria in the sample rises.

oratory strains (7, 15, 22, 30) but not in an encapsulated background, as required for testing in vivo. During screening for a strain activated by a targeted *comE*(*K38E*) mutation, a clone was identified that displayed the Trt phenotype, indicating activation of the competence pathway despite degradation of CSP by trypsin. Although this isolate did not have the *comE*(*K38E*) change, sequencing revealed an alternative, *comE*(*D143Y*) mutation. This mutation is located just within the start of the predicted LytTR DNA binding domain of ComE (23) and does not involve the aspartate residue in the receiver domain on which phosphorylation of ComE is predicted to occur.

The *comE*(*D143Y*) mutation was generated again in the wild-type background, using overlap-extension PCR, in order to verify that the observed Trt phenotype arose from this mutation rather than from a change elsewhere in the genome. A *comE*(*D143Y*) PCR product was used to replace the counterselectable Janus cassette that had been placed downstream of *comE* because deletion of *comE* itself prevents subsequent transformation. Transformants were screened by sequencing to identify a clone in which the *comE*(*D143Y*) mutation had been incorporated through recombination within the flanking regions of the PCR product. This strain was designated MSP110 and displayed the predicted Trt phenotype (data not shown). Consistent with competence activation, the *comE*(*D143Y*) strain developed a high level of spontaneous competence during growth in vitro (Fig. 1A). In contrast, its wild-type parent strain became competent only in response to exogenous CSP. Both strains became refractory to stimulation with CSP at high densities. These data support the conclusion that the *comE*(*D143Y*) mutation causes a response similar to that triggered by exposure to CSP.

**Effects of competence deletion mutations on colonization.** The competence-inactivating *comAB*, *comD*, and *comE* mutations as well as the competence-activating *comE*(*D143Y*) mutation were tested in an infant rat model of colonization. A control strain in which the Janus cassette had been inserted into the intergenic region immediately downstream of *comE* was tested in parallel to assess potential fitness effects or downstream polar effects associated with this resistance marker. Inoculated individually, each of these strains was able to colonize at levels that were not substantially different from those seen with the wild type (data not shown). In order to assess more sensitively the differences in fitness for colonization, competitive assays were then performed in which strains were inoculated together with a reference strain.

In the competitive model, the Δ*comE* strain steadily outcompeted an isogenic streptomycin-resistant ($Sm^r$) wild-type strain (Fig. 2A). A kanamycin-resistant control strain with the Janus cassette downstream of *comE* ($Km^r$ $comE^+$) did not show a competitive effect of the same magnitude (Fig. 2G). The latter competition, between the $Km^r$ $comE^+$ strain and the $Sm^r$ wild-type strain, is hereafter referred to as the reference competition and served as a baseline for evaluation of the specific fitness effects associated with mutations in the competence pathway. Comparisons between strains were made on the basis of the log competitive index (Fig. 3A), where a value of zero would indicate no difference between a pair of strains. The log competitive index of −0.87 for the reference competition was significantly less than the neutral value of 0 ($P < 0.0001$; Wilcoxon signed-rank test) and indicates a small advantage for the $Km^r$ $comE^+$ strain over the $Sm^r$ wild type during colonization. This may reflect a fitness cost associated with the *rpsL* mutation conferring streptomycin resistance, as documented for other bacteria (3). The log competitive index of −2.15 seen with the Δ*comE* strain was significantly lower than that of the reference competition ($P < 0.001$) and indicates a fitness advantage attributable to deletion of *comE*.

To determine whether the apparent advantage of the Δ*comE* strain might be explained by differential recovery of the mutant and wild-type strains during the washing process, we dissected nasal tissues 8 days after inoculation to directly assess colonization levels. A log competitive index favoring the Δ*comE* strain (median, −2.38; range, −1.56 to −3.37) was also observed with tissue samples, with a value similar to that obtained from nasal washings. To assess the potential impact of in vivo recombination between strains during mixed colonization, we examined 96 colonies that were recovered from nasal washings on plates without either streptomycin or kanamycin throughout the course of the experiment. These colonies were tested to determine their resistance patterns. No colonies were found that were either sensitive to both antibiotics or resistant to both antibiotics. It is therefore unlikely that competition from an in vivo recombinant that may have gained fitness through the restoration of a streptomycin-sensitive *rpsL* allele explains the displacement of the streptomycin-resistant wild type during mixed colonization.

We expected that blockade of the competence pathway by

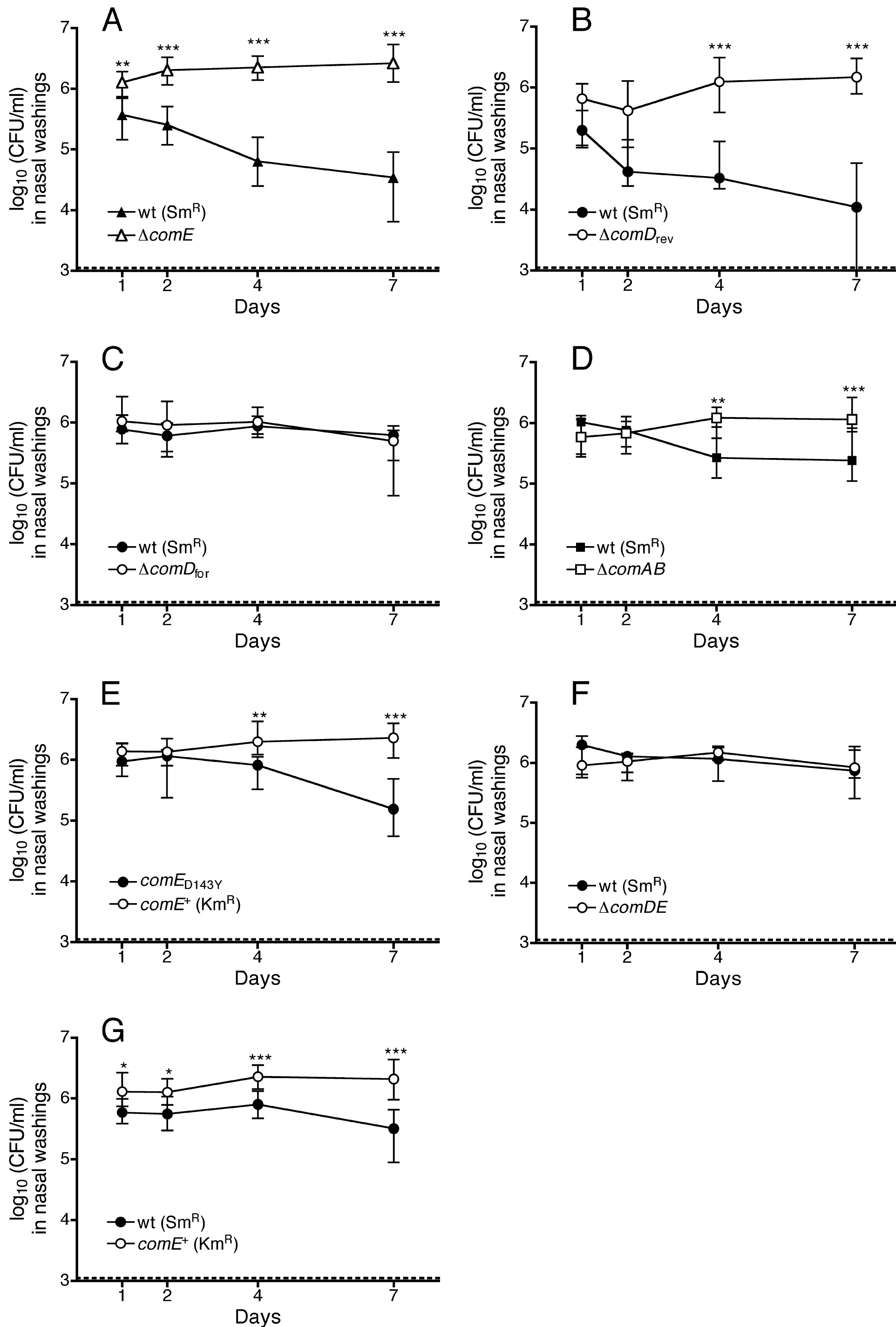


FIG. 2. Competitive colonization assays of wild-type *S. pneumoniae* strain P1534 (Sm$^r$) versus the competence mutant strains P1538 (Δ*comE*) (A), JKP158 (Δ*comD*$_{rev}$) (B), P1535 (Δ*comD*$_{for}$) (C), P1537 (Δ*comAB*) (D), and TMP133 (Δ*comDE*) (F). (E) Competitive colonization of the Sm$^r$ competence-activated *comE*(*D143Y*) strain MSP110 versus the Km$^r$ *comE*$^+$ control. (G) Reference competition of the Sm$^r$ wild-type strain P1534 versus the Km$^r$ *comE*$^+$ control strain JKP102, which has the Janus cassette in the intergenic region downstream of *comE*. Values are displayed as medians and interquartile ranges for colonization of at least 11 pups with each pair of strains. Differences in colonization levels between pairs of competing strains at each time point were assessed using the Kruskal-Wallis test with Dunn's correction (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). The dotted lines represent the limit of detection of the assay.

deletion of *comD*—located immediately upstream of *comE* and also required for competence—would result in an enhancement of fitness similar to that with *comE* deletion. This effect was not observed with a *comD* deletion mutant constructed with the Janus cassette inserted in the same direction as transcription of the *comCDE* locus (Δ*comD*$_{for}$) (Fig. 2C and 3A). Instead, the Δ*comD*$_{for}$ mutation caused a significant increase in competitive index relative to the reference competi-

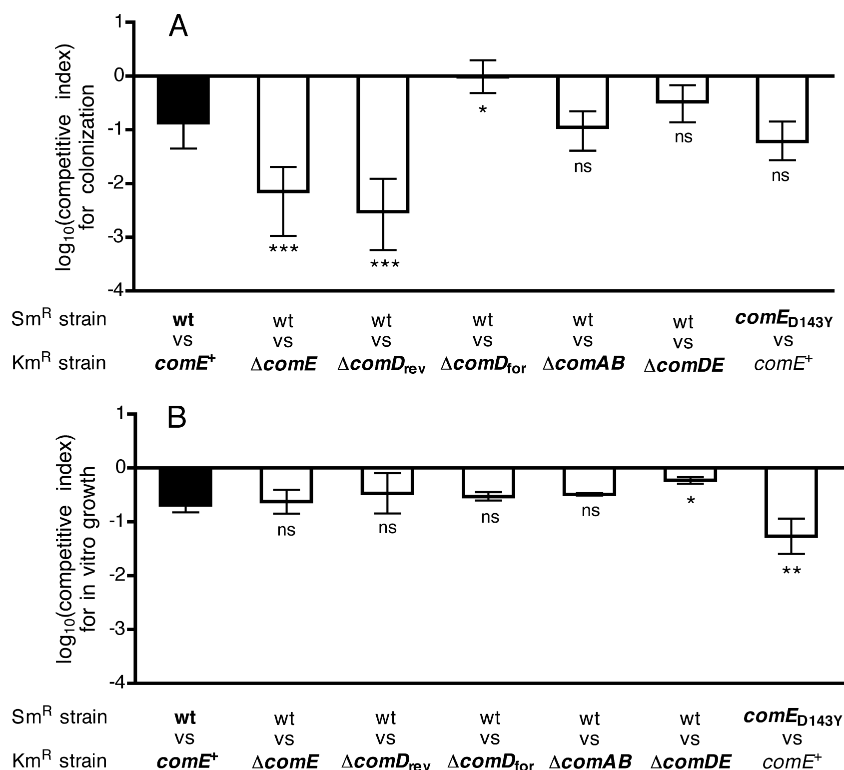


FIG. 3. (A) Competitive indices based on colonization levels at day 7 for the experiments shown in Fig. 2. Values are shown as medians and interquartile ranges. The competitive index for each pair of competing strains was compared with that for the reference competition (filled bar [wild type versus *comE*$^+$ strain]), using the Kruskal-Wallis test with Dunn's correction (*, $P < 0.05$; ***, $P < 0.001$; ns, not significant). (B) Competitive indices for growth in vitro. Values represent means ± standard deviations based on at least three independent determinations. The competitive index for each pair of strains was compared with that for the reference competition (filled bar [wild type versus *comE*$^+$ strain]), using analysis of variance with Dunnett's multiple comparison correction (*, $P < 0.05$; **, $P < 0.01$; ns, not significant).

tion value. Because the competitive index is calculated from the ratio of streptomycin-resistant to kanamycin-resistant bacteria, this elevated value indicates a loss of colonization fitness in the kanamycin-resistant Δ*comD*$_{for}$ strain. When the *comD* deletion was constructed with the Janus cassette in the reverse orientation (Δ*comD*$_{rev}$) (Fig. 2B and 3A), however, the resulting strain showed an enhanced colonization fitness similar to that of the Δ*comE* strain.

A strain with a deletion spanning both *comD* and *comE* was also tested in the colonization model. This Δ*comDE* mutation did not cause a change in the competitive index relative to the reference competition value (Fig. 2F and 3A). The interpretation of this result, however, is complicated by the fact that this mutation, unlike the *comD* and *comE* single mutations, also significantly slowed the growth of the bacteria in vitro, as described below (Fig. 3B).

**Impact of competence mutations on *comE* expression.** Because the difference observed between the Δ*comD*$_{for}$ and Δ*comD*$_{rev}$ strains suggested the likelihood of a polar effect on the downstream *comE* gene, *comE* transcript levels were measured by quantitative RT-PCR (Fig. 4). The Δ*comD*$_{for}$ mutation resulted in constitutive expression of *comE* at a level similar to that achieved by the wild type activated by CSP. The Δ*comD*$_{rev}$ mutation, in contrast, reduced the baseline level of *comE* transcripts to below that of the unstimulated wild type and prevented induction of *comE* expression in response to

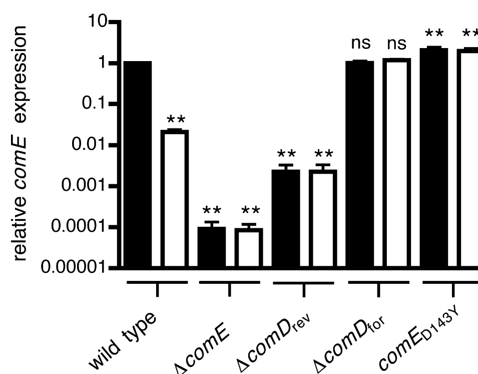


FIG. 4. Relative levels of *comE* transcripts in strains P1534 (wild type), P1538 (Δ*comE*), JKP158 (Δ*comD*$_{rev}$), P1535 (Δ*comD*$_{for}$), and MSP110 [*comE*(*D143Y*)], measured by quantitative RT-PCR. Values are normalized to transcript levels for the wild type after stimulation with exogenous CSP (assigned a value of 1) and represent means ± standard deviations for RNAs isolated from three independent cultures of each strain. No transcript was detected for the Δ*comE* strain; values shown for this strain represent the limit of detection of the assays. Expression levels were compared to that of the wild type after treatment with CSP, using analysis of variance with Dunnett's multiple comparison correction (**, $P < 0.01$; ns, not significant). CSP treatment (filled bars) caused a significant increase in *comE* expression for the wild type ($P < 0.01$) but did not significantly change *comE* transcript levels for any of the other strains.

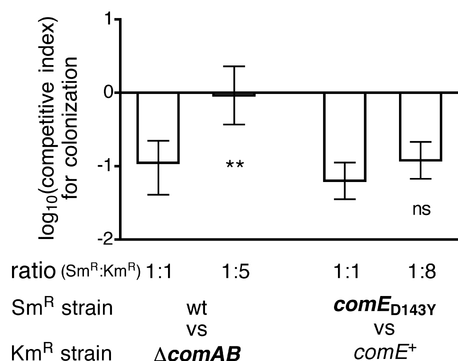


FIG. 5. Effect of inoculum ratio on competitive colonization with *S. pneumoniae*. Competitive indices are based on colonization levels at day 7 for the strain pairs P1534 (Sm$^r$ wild type)-P1537 (Km$^r$ Δ*comAB*) and MSP110 [Sm$^r$ *comE*(*D143Y*)]-JKP102 (Km$^r$ *comE*$^+$ control). Inoculum ratios were varied as indicated; data for 1:1 inoculation experiments are from experiments shown in Fig. 3. Values are shown as medians and interquartile ranges. Differences in competitive indices at the two inoculum ratios were assessed using the Kruskal-Wallis test with Dunn's correction (**, $P < 0.01$; ns, not significant).

CSP. As anticipated, the competence-activating *comE*(*D143Y*) mutation resulted in increased expression of *comE*, even in the absence of stimulation by exogenous CSP.

**Density-dependent interaction of Δ*comAB* and wild-type strains during colonization.** Deletion of *comAB* did not significantly affect colonization compared to that of the reference competition (Fig. 2D and 3A) when the Δ*comAB* and wild-type strains were inoculated at equivalent levels. Because *comAB* encodes the transporter required for export and processing of CSP, the Δ*comAB* strain does not develop spontaneous competence yet retains the ability to respond to CSP released by other bacteria. During cocolonization with bacteria that have the potential to develop competence, such stimulation might mask differences between the Δ*comAB* strain and the wild type. However, because the Δ*comAB* strain cannot participate in the autocatalytic loop by which activation of the competence system promotes further release of CSP, the potential for cross-stimulation should be reduced if the ratio of wild-type to Δ*comAB* bacteria is decreased. We therefore determined the competitive index for cocolonization with the wild-type and Δ*comAB* strains inoculated at a 1:5 ratio. Under these conditions, the competitive index was significantly higher than that observed with inoculation of the same strains at a 1:1 ratio (Fig. 5), corresponding to increased recovery of the streptomycin-resistant wild-type strain relative to that of the Δ*comAB* strain. This finding suggests that the Δ*comAB* mutation reduces colonization fitness unless stimulation from CSP released by the wild type is available to activate competence in the Δ*comAB* strain.

**Effects of competence-activating *comE*(*D143Y*) mutation on colonization.** Considering the competitive advantage associated with *comE* deletion, the possibility that the *comE*(*D143Y*) mutation might impair colonization was also investigated. Because the *comE*(*D143Y*) strain was resistant to streptomycin, the Km$^r$ *comE*$^+$ strain was used as a reference competitor for this assay (Fig. 2E). Comparison was again made to the reference competition between the Sm$^r$ wild-type strain and the Km$^r$ *comE*$^+$ control strain. The competitive index obtained with the *comE*(*D143Y*) strain was not significantly different from that of the reference competition (Fig. 3A).

Dual colonization with the *comE*(*D143Y*) strain and the Km$^r$ *comE*$^+$ control strain presents another situation in which cross-stimulation may reduce the differences between strains. In this case, activation of the competence circuit in the *comE*(*D143Y*) strain may cause enhanced release of CSP and thereby promote increased development of competence in the control strain. We therefore tested whether reducing the ratio of *comE*(*D143Y*) to control strain bacteria would impact the outcome of competitive colonization. Although a small increase in the competitive index was observed, this difference was not significant (Fig. 5). It is possible, however, that even at a reduced density the *comE*(*D143Y*) strain is able to stimulate competence in the control strain, because unlike the Δ*comAB* strain, for which a density-dependent effect was observed, the control strain has a normal competence circuit and should be able to participate in autocatalytic activation of competence once it begins to be triggered.

**Competence mutations and growth in vitro.** Competence activation through exposure to CSP is known to delay pneumococcal growth (24). We therefore tested the impact of our set of competence mutations during competitive growth in vitro to determine whether the effects seen during colonization might be attributed to general effects on growth rate. A competitive index for growth in vitro was defined as the ratio of CFU/ml of streptomycin-resistant versus kanamycin-resistant bacteria for the two strains after 4.5 h of growth divided by the ratio immediately after mixing. The competitive indices consistently showed that kanamycin-resistant strains containing the Janus cassette grew better in vitro than the isogenic streptomycin-resistant control strain (Fig. 3B). Deletion of *comAB*, *comD*, or *comE* did not significantly change the competitive index relative to that of the reference competition in vitro. The *comDE* deletion, however, was associated with a significant increase in the competitive index in vitro, reflecting reduced recovery of the kanamycin-resistant Δ*comDE* strain relative to the streptomycin-resistant control. The *comE*(*D143Y*) competence-activating mutation also significantly decreased recovery of the mutant strain (streptomycin resistant in this case) relative to the Km$^r$ *comE*$^+$ control strain following in vitro growth. Because the pattern of competitive indices measured in vitro did not mirror that seen in vivo, we concluded that the fitness effects of the *comE* and *comD* mutations are specific for colonization. The slow growth of the Δ*comDE* and *comE*(*D143Y*) strains in vitro, however, may have affected the ability of these two strains to compete during nasal colonization.

**Competence activation is not responsible for Δ*ciaRH* colonization defect.** Deletion of the CiaRH two-component system impairs pneumococcal colonization in the background of a serotype 3 strain and increases expression of the competence regulon (29). In the serotype 2 background used in this study, deletion of *ciaRH* also triggered spontaneous transformation that was not observed in the wild-type strain (Fig. 1B). The level of transformation measured in the Δ*ciaRH* strain, however, was substantially lower than the level achieved by the wild type in the presence of CSP. We then asked whether this intermediate level of competence activation contributes to the colonization defect seen with *ciaRH* deletion. Second mutations inactivating competence were constructed in the context

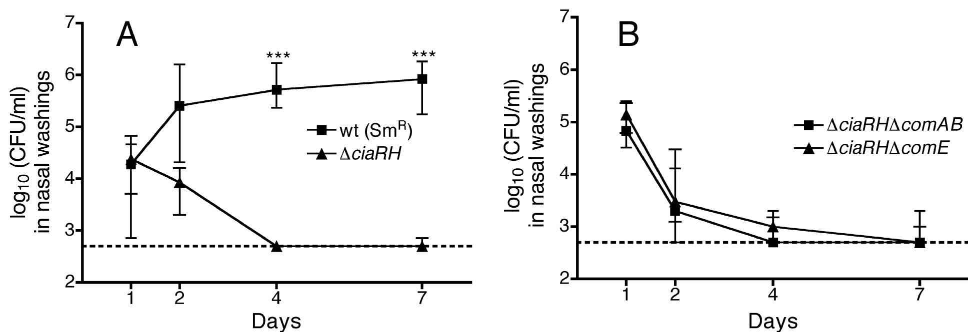


FIG. 6. Nasal colonization with single strains of *S. pneumoniae*. (A) Wild type (P1534) and Δ*ciaRH* (JKP109) strains; (B) Δ*ciaRH* Δ*comAB* (JKP114) and Δ*ciaRH* Δ*comE* (JKP116) strains. Values are shown as medians and interquartile ranges for colonization of groups of 10 to 13 pups with each strain. Differences in colonization levels between the wild-type and Δ*ciaRH* strains at each time point were assessed using the Kruskal-Wallis test with Dunn's correction (***, $P < 0.001$); neither of the double mutant strains showed significant differences from the Δ*ciaRH* strain at any of the time points. The dotted line represents the limit of detection of the assay.

of a *ciaRH* deletion. As seen with the serotype 3 strain studied previously, the Δ*ciaRH* mutation severely attenuated colonization in the serotype 2 background even in the absence of competition from another pneumococcal strain (Fig. 6A). Insertion of the Janus cassette to delete either *comAB* or *comE* did not correct the colonization defect caused by loss of *ciaRH* (Fig. 6B).

## DISCUSSION

We have investigated the influence of the pneumococcal competence signaling pathway on the ability of the bacterium to colonize the nasopharynx. Because asymptomatic carriage in the respiratory tract is the setting in which *S. pneumoniae* exists most frequently and from which it spreads to colonize new hosts, the impact of competence in this environment may be particularly important in defining the evolutionary pressures responsible for competence. We have found that deletion of the ComE response regulator, which is required for induction of competence, increases fitness for colonization above that of the wild type. This finding appears to contrast with earlier observations that competence inactivation through deletion of *comD* or *comAB* attenuated pneumococcal virulence in models of disease (2, 16) and may reflect differing requirements for asymptomatic carriage versus disease.

The magnitude of the competitive effect associated with the Δ*comE* mutation was substantial, corresponding to an increase in relative abundance of the Δ*comE* strain of 2.14 $\log_{10}$ units (nearly 140-fold) over 7 days. Of this increase, 1.28 $\log_{10}$ units (18.9-fold change) can be attributed directly to deletion of *comE*, as the excess effect beyond that seen with a control insertion of the resistance cassette directly downstream of *comE*. This change would correspond to a 50% daily increase in relative abundance of a *comE* null mutant in competition with wild-type *S. pneumoniae*. Pneumococcus therefore appears to incur a substantial fitness cost in order to maintain a functional *comE* gene compared to the potential advantage that might be gained by its elimination. The nature of the selective advantage for which ComE is nonetheless retained remains to be elucidated. These experiments, however, provide evidence that this function is unlikely to be utilization of the competence pathway for acquisition of extracellular DNA as a nutrient on the mucosal surface, as suggested for transformation in other bacteria (9, 28), because loss of this resource would be predicted to impair rather than improve colonization fitness.

The observation that *comD* deletion—also blocking competence—does not similarly enhance colonization unless the mutation is constructed in a manner that causes a polar decrease in *comE* transcription indicates that the signaling events that control fitness for colonization differ from those that regulate the development of competence. Several signaling models could be considered to explain such discordant effects of *comD* and *comE* deletions during colonization. (i) The unactivated form of ComE could regulate the transcription of an additional set of genes beyond those induced by activation of ComE in response to CSP. If such a set included genes affecting colonization, a deletion of *comE* causing loss of the ComE protein entirely would be predicted to impact colonization differently from a loss of *comD*, causing only inactivation of ComE. Although activation of the ComE response regulator is often presumed to correspond to its phosphorylation, this correspondence has not been demonstrated experimentally. Studies of two-component signaling in other organisms have revealed diversity in signaling architectures in which stimuli may regulate either kinase or phosphatase activities of sensor kinases and in which dephosphorylation occasionally may correspond to the "active" state of a response regulator (31). We therefore consider changes in ComE signaling in terms of its activation (i.e., the signaling state required for induction of genetic competence in response to detection of CSP by the ComD sensor kinase) rather than its phosphorylation. (ii) ComE could be activated by factors in vivo, independent of the ComD sensor kinase. In vitro, however, none of our mutants lacking *comD* displayed competence. Alternative phosphate donors that might activate ComE could include noncognate histidine kinases as well as the small-molecule phosphate donor acetyl phosphate (37). The efficiency of ComE activation through such an alternative pathway—if activation corresponds to phosphorylation—might be enhanced in the Δ*comD* strain if ComD possesses, in addition to its kinase activity, a phosphatase activity that is able to dephosphorylate its cognate response regulator, such as that described for other sensor kinases (13, 19). The lack of effect on colonization seen with

the competence-activating *comE*(*D143Y*) mutation, however, provides evidence against this model. (iii) ComD could interact with an alternative target, such as a noncognate response regulator. Such cross talk with another two-component signaling system could be facilitated in the absence of the cognate response regulator ComE. This model may be excluded because colonization enhancement is seen in the absence of ComD in the $\Delta comD_{rev}$ mutant.

Of these models, the first—involving effects of the unactivated form of ComE—is supported by the observation that the $\Delta comD_{rev}$ mutant displays enhanced colonization similar to that of the Δ*comE* mutant. This $\Delta comD_{rev}$ mutation causes a polar decrease in *comE* transcription. In contrast, the $\Delta comD_{for}$ mutation increases *comE* transcription and results in reduced colonization fitness. Because the $\Delta comD_{for}$ strain lacks the ComD histidine kinase, increased *comE* transcription in this background is expected to result in overexpression of the unactivated form of the ComE response regulator. Consistent with this prediction, the $\Delta comD_{for}$ strain does not display competence in either the presence or absence of CSP. Our results therefore suggest that increases in unactivated ComE impair colonization, while decreases in unactivated ComE enhance colonization.

Although this model would also predict enhanced colonization by the Δ*comDE* double mutant strain, this strain unexpectedly displayed an in vitro growth deficit not seen with any of the individual mutations in *comD* or *comE*. The basis for this growth deficit is uncertain, but as a consequence, comparisons of the impact of competence mutations on colonization are most appropriately focused on those strains that do not affect growth in vitro. We cannot currently determine whether the growth deficit of the Δ*comDE* strain counterbalances a colonization advantage associated with the loss of *comE* to produce the neutral result observed in vivo.

The density-dependent colonization defect associated with the Δ*comAB* strain provides further support for the hypothesis that unactivated ComE impairs colonization. In this strain, ComE activation would depend on cross-stimulation by CSP released by the cocolonizing wild-type strain. When the wild type is abundant, the potential for such stimulation would be increased, and under these conditions, colonization by the Δ*comAB* strain was strong. With the wild type being less abundant, however, the potential for ComE activation in the Δ*comAB* mutant would be reduced. Indeed, when the ratio of wild-type to Δ*comAB* bacteria in the inoculum was reduced, the mutant strain displayed decreased fitness for colonization. The density dependence of the Δ*comAB* colonization phenotype furthermore argues against the second signaling model presented above (i.e., in vivo activation of ComE through an alternative pathway). If activation of ComE during colonization were independent of CSP, a pattern consistent with cross-stimulation of the Δ*comAB* strain by CSP released by the wild type would not be expected. The observation of this density-dependent effect also provides indirect evidence that the competence system is activated at some level in the wild type during colonization, because release of CSP would be necessary for cross-stimulation of the Δ*comAB* strain. Although we have attempted to demonstrate this activation directly through transcriptome amplification and quantitative RT-PCR performed directly on nasal washing samples, the sensitivity of these assays has not been consistently high enough to measure transcript levels from these limited samples. It should be noted that similar cross-stimulation by extracellular CSP is unlikely to occur with the Δ*comD* and Δ*comE* mutants because these strains can neither respond to CSP nor participate in the autocatalytic loop that amplifies its production during competence.

The observation that the competence-activating *comE* (*D143Y*) mutation does not significantly impact colonization further supports the model that levels of the unactivated form of ComE, rather than the activated form, affect colonization potential. A density-dependent effect on fitness might also have been anticipated during cocolonization with the wild-type and *comE*(*D143Y*) strains if the latter increased the level of ComE activation in the former. Although the competitive index shifted slightly toward less strong colonization by the wild type when the abundance of the activated *comE*(*D143Y*) strain was reduced, this change was not significant. The potential to observe a density-dependent fitness effect may be reduced in this situation (compared to the competition between Δ*comAB* and wild-type strains) because CSP from the *comE*(*D143Y*) strain may trigger a positive feedback loop, amplifying its production by the cocolonizing wild type even with a reduced population of *comE*(*D143Y*) bacteria. Alternatively, the level of spontaneous competence activation in the wild type during colonization may be high enough that additional stimulation by the *comE*(*D143Y*) strain does not produce a substantial effect. Finally, the finding that secondary mutations blocking competence do not restore colonization fitness to the Δ*ciaRH* strain indicates that the colonization deficit of the Δ*ciaRH* strain is not due to its enhanced competence activation and is likewise consistent with colonization being affected by the unactivated form of ComE.

Although the cause of the colonization advantage conferred by loss of unactivated ComE is not currently known, it appears likely to stem from transcriptional effects on an uncharacterized portion of the ComE regulon governed by unactivated ComE. The colonization advantage appears to be specific to the in vivo environment, as competitive indices measured during growth in vitro did not mirror the results of competition during colonization. We have examined the impact of mutations in the competence pathway on adherence to cultured epithelial cell lines and have not observed substantial changes associated with deletion of *comE*. Pneumococcal biofilm formation was recently linked to the induction of competence as well (25). Our observation of robust colonization by the Δ*comE* strain, however, suggests either that such biofilms are not required for efficient colonization of the infant rat nasopharynx or that alternative pathways are sufficient to stimulate biofilm formation in vivo. *S. pneumoniae* has also been demonstrated to undergo allolysis, or fratricide, in which competent pneumococci lyse a subpopulation of noncompetent pneumococci present in the same culture (reviewed in reference 6). While this process is likely to be important in generating the pool of extracellular DNA available for transformation, our study suggests that such allolysis does not determine the outcome of competition between strains during colonization. Further work will be required to define the regulatory events controlled by the unactivated form of ComE and the impact of these events on colonization.

## ACKNOWLEDGMENTS

We thank J. Weiser for valuable discussions during the course of these experiments, S. King and J. Weiser for providing pneumococcal strain P1310, and T. Miller for technical assistance during the construction of strain TMP133.

This work was supported by Public Health Service grant K08 AI052129 from the National Institute of Allergy and Infectious Diseases to M.E.S. as well as by institutional funding from the Children's Hospital of Philadelphia.

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*Editor:* A. Camilli