Deletion of the Zinc Transporter Lipoprotein AdcAII Causes Hyperencapsulation of *Streptococcus pneumoniae* Associated with Distinct Alleles of the Type I Restriction-Modification System

Claire Durmort, a Giuseppe Ercoli, b Elisa Ramos-Sevillano, b Suneeta Chimalapati, b Richard D. Haigh, c Megan De Ste Croix, c Katherine Gould, d Jason Hinds, d Yann Guerardel, e Thierry Vernet, a Marco Oggioni, c Jeremy S. Brown b

a Institut de Biologie Structurale (IBS), Univ. Grenoble Alpes, CEA, CNRS, Grenoble, France
b Centre for Inflammation and Tissue Repair, Department of Medicine, Royal Free and University College Medical School, Rayne Institute, London, United Kingdom
c Department of Genetics and Genome Biology, University of Leicester, Leicester, United Kingdom
d Institute for Infection and Immunity, St. George’s University of London, London, United Kingdom
e Univ. Lille, CNRS, UMR 8576 – UGSF-Unité de Glycobiologie Structurale et Fonctionnelle, Lille, France

Giuseppe Ercoli and Elisa Ramos-Sevillano contributed equally to the manuscript.

**ABSTRACT** The capsule is the dominant *Streptococcus pneumoniae* virulence factor, yet how variation in capsule thickness is regulated is poorly understood. Here, we describe an unexpected relationship between mutation of *adcAII*, which encodes a zinc uptake lipoprotein, and capsule thickness. Partial deletion of *adcAII* in three of five capsular serotypes frequently resulted in a mucoid phenotype that biochemical analysis and electron microscopy of the D39 *adcAII* mutants confirmed was caused by markedly increased capsule thickness. Compared to D39, the hyperencapsulated Δ*adcAII* mutant strain was more resistant to complement-mediated neutrophil killing and was hypervirulent in mouse models of invasive infection. Transcriptome analysis of D39 and the Δ*adcAII* mutant identified major differences in transcription of the Sp_0505-0508 locus, which encodes an SpnD39III (ST5556II) type I restriction-modification system and allelic variation of which correlates with capsule thickness. A PCR assay demonstrated close linkage of the SpnD39III C and F alleles with the hyperencapsulated Δ*adcAII* strains. However, transformation of Δ*adcAII* with fixed SpnD39III alleles associated with normal capsule thickness did not revert the hyperencapsulated phenotype. Half of hyperencapsulated Δ*adcAII* strains contained the same single nucleotide polymorphism in the capsule locus gene cps2E, which is required for the initiation of capsule synthesis. These results provide further evidence for the importance of the SpnD39III (ST5556II) type I restriction-modification system and allelic variation of which correlates with capsule thickness. A PCR assay demonstrated close linkage of the SpnD39III C and F alleles with the hyperencapsulated Δ*adcAII* strains. However, transformation of Δ*adcAII* with fixed SpnD39III alleles associated with normal capsule thickness did not revert the hyperencapsulated phenotype. Half of hyperencapsulated Δ*adcAII* strains contained the same single nucleotide polymorphism in the capsule locus gene cps2E, which is required for the initiation of capsule synthesis. These results provide further evidence for the importance of the SpnD39III (ST5556II) type I restriction-modification system and allelic variation of which correlates with capsule thickness. Further investigation will be needed to characterize how mutation of *adcAII* affects SpnD39III (ST5556II) allele dominance and results in the hyperencapsulated phenotype.

**IMPORTANCE** The *Streptococcus pneumoniae* capsule affects multiple interactions with the host including contributing to colonization and immune evasion. During infection, the capsule thickness varies, but the mechanisms regulating this are poorly understood. We have identified an unsuspected relationship between mutation of *adcAII*, a gene that encodes a zinc uptake lipoprotein, and capsule thickness. Mutation of *adcAII* resulted in a striking hyperencapsulated phenotype, increased resistance to complement-mediated neutrophil killing, and increased *S. pneumoniae* virulence in mouse models of infection. Transcriptome and PCR analysis linked the hyperencapsulated phenotype of the Δ*adcAII* strain to specific alleles of the...
SpnD39III (ST5556II) type I restriction-modification system, a system which has previously been shown to affect capsule thickness. Our data provide further evidence for the importance of the SpnD39III (ST5556II) type I restriction-modification system for modulating capsule thickness and identify an unexpected link between capsule thickness and ΔAdcAll, further investigation of which could further characterize mechanisms of capsule regulation.

**KEYWORDS** Streptococcus pneumoniae, capsule expression, virulence, AdcAll, restriction modification, SpnD39III

*Streptococcus pneumoniae* (the pneumococcus) is a Gram-positive bacterial commensal of the human nasopharynx (1) and also a common invasive pathogen causing pneumonia, septicemia, and meningitis (2). *S. pneumoniae* has multiple virulence factors which facilitate disease pathogenesis (3), the most important of which is the capsule. The capsule is an extracellular polysaccharide layer which plays a crucial role in *S. pneumoniae* immune evasion by inhibiting complement recognition, phagocytosis, and bacterial entrapment by mucus (4). Variation in *S. pneumoniae* capsule structure results in multiple different biochemical and antigen structures, with at least 98 distinct capsule polysaccharide serotypes recognized at present (5). This diversity is mainly related to genetic variation in the multigene *cps* locus (6) and correlates closely with strain phenotypes such as invasive potential, duration of colonization, and ability to evade complement-mediated neutrophil phagocytosis (7, 8). The degree of capsule expression by *S. pneumoniae* is also affected by phase variation at different sites of infection (9, 10). Opaque-phase *S. pneumoniae* has increased thickness of the capsule layer and is associated with invasive infections such as septicemia, whereas transparent-phase *S. pneumoniae* has thinner capsule layers and is associated with colonization and biofilm formation (11–13). Despite the importance of capsule expression during *S. pneumoniae* interactions with the host, the molecular mechanisms underpinning phase variation and capsule thickness remain relatively poorly understood.

One mechanism that has been recently described to control capsule expression is epigenetic regulation by phase-variable control of DNA methylation driven by the type I restriction-modification system SpnD39III (ST5556II) (14). The SpnD39III (ST5556II) system consists of multiple genes that can be shuffled by recombination on inverted repeats to create enzymes capable of methylation at six different recognition sites. Capsule expression and thickness (opaque versus transparent) have been correlated with different SpnD39III alleles (14–16), and this system may be involved in regulating at least some aspects of *S. pneumoniae* phase variation. As yet, both the environmental conditions influencing allele distribution and how the effects of methylation patterns on gene expression lead to changes in capsule thickness have not been resolved.

Within mammalian hosts, the available concentrations of several cations are strictly controlled. As a consequence, cation ABC transporters of iron, manganese, and zinc are essential for *S. pneumoniae* growth and survival in the host (17–19). ABC transporters consist of a membrane-attached lipoprotein substrate binding protein and membrane permease(s) and ATPase proteins. Zinc acquisition is mediated by two ABC transporters identified by their lipoprotein components as AdcA and AdcAll (20, 21). Adjacent to *adcAll* is *phtD*, which encodes the surface protein PhtD, a member of the Pht histidine triad surface protein family that are involved in *S. pneumoniae* virulence. The histidine triad motifs of Pht proteins have a high affinity for zinc, and these proteins may provide a surface reservoir of zinc for import into *S. pneumoniae* via AdcA and AdcAll ABC transporters (22–24). We have previously demonstrated that deletion of *adcA* partially attenuates virulence, and deletion of both *adcA* and *adcAll* had a profound effect on *S. pneumoniae* physiology under low zinc conditions and strongly attenuated virulence (19, 25). In contrast, the virulence of the single *adcAll* deletion mutant was significantly increased. Here, we describe this unexpected consequence of partial deletion of *adcAll* in detail and show that the hypervirulence of the D39 ΔadcAll mutant strains is...
associated with a mucoid phenotype and increased capsule expression and is correlated closely with specific SpnD39III alleles and a point mutation in the \( csp2E \) capsule locus gene.

RESULTS

Deletion of \( adc\text{A}II \) in the \( S. \ pneumoniae \) D39 strain results in a markedly increased expression of the capsule. During our previous investigation of the functional roles of the AdcA and AdcAII zinc ABC transporter systems, a single deletion mutant of the \( adc\text{A}II \) gene was made by partial replacement of the \( adc\text{A}II \) gene with the chloramphenicol resistance cassette \( \text{cat} \) (Fig. 1A). The resulting \( \Delta adc\text{A}II \) mutant strains displayed a visibly increased mucoid colony morphology (Fig. 1B). Capsule thicknesses

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**FIG 1** Creation and macroscopic phenotype of the \( \Delta adc\text{A}II \) mutant. (A) Gene map of the \( adc\text{A}II \) locus showing the bp 201 to 750 deletion and replacement with an antibiotic resistance cassette (\( \text{cat} \) or \( \text{kana} \)) present in the \( \Delta adc\text{A}II \) mutant. (B) Colony morphology on Columbia blood agar plates of wild-type (WT) D39 strain and the \( \Delta adc\text{A}II \) mutant. (C) Relative amount of monosaccharides in capsule extracts of WT D39 strain and \( \Delta adc\text{A}II \) mutant determined by GC-MS. All monosaccharide derivatives were identified according to their specific retention times and EI-MS fragmentations, as described in reference 26. (D) Example of measuring the volume of D39 and \( \Delta adc\text{A}II \) bacterial pellets using microcapillary tubes. (E) Height (mm) of bacterial pellets for the WT and mutant strains in the indicated strains measured using microcapillary tubes. Each point represents data for independent clones containing the indicated mutation, and bars represent mean values for independently derived colonies for each mutant strain. \( P \) values were calculated using unpaired \( t \) test. **, \( P < 0.01 \); ***, \( P < 0.001 \).
were compared between the D39 and ΔadcAll mutant strains using a range of assays. Initially, colony volume was assessed by transferring single colonies to a capillary tube and measuring the height of visible bacterial material. This demonstrated an increased volume of the ΔadcAll strain compatible with a thicker capsule layer (Fig. 1D and E). Capsule width was then directly visualized for the ΔadcAll and wild-type D39 strains using electron microscopy (EM), which demonstrated that the bacterial cells of the ΔadcAll mutant had a considerably enlarged capsule layer compared to D39 (Fig. 2A to F). The mean capsule radius indicated that the ΔadcAll mutant expressed a capsule 5.6 times thicker than the wild-type (WT) D39 (capsule width of 61 ± 1.8 nm versus 343 ± 8.3 nm for the D39 and ΔadcAll strains, respectively; n = 30 for each strain). Monosaccharide composition of capsule extracts for the ΔadcAll mutant and WT D39 strain extracts were assessed biochemically using gas chromatography-mass spectrometry (GC-MS). Total polysaccharide in capsule extracts demonstrated a 1.5-fold increase in the ΔadcAll mutant compared to the wild-type strain, largely due to a 2-fold increase in rhamnose content (Fig. 1C). Despite these changes in polysaccharide content, the hyperencapsulated ΔadcAll strain was still recognized by serotype-specific antisera (Fig. 2G to I). The small amount of GalNac detected was probably from teichoic acids extracted with the capsular polysaccharide. Overall, these data demonstrated that partial deletion of adcAll modified the polysaccharide content of the capsule with overexpression of rhamnose-containing polysaccharides. To assess whether the ΔadcAll mutant phenotype was serotype specific, additional ΔadcAll mutant strains were obtained in capsular serotype 4, 6A, 6B, and 17F strains. Partial deletion of adcAll in the 6A and 6B serotypes also resulted in a mucoid phenotype suggestive of increased capsule thickness but did not affect capsule thickness in the serotype 4 and 17F strains (Fig. 1E).
Consistent association of the \textit{adcAll} mutation with increased capsule expression by D39. To characterize further the relationship between partial deletion of \textit{adcAll} and increased capsule thickness, additional transformation and phenotyping experiments were performed. Increased capsule expression was also detected in \textit{ΔadcAll} strains made using the kanamycin resistance cassette \textit{kana} instead of \textit{cat} and if the deletion included the immediate downstream gene (\textit{phtD}) (Fig. 1E and Table 1). Combined deletion of \textit{adcA} and \textit{adcAll} did not result in an increased capsule thickness phenotype. When the \textit{adcAll} mutation was created in an unencapsulated D39 strain (\textit{ΔcpsD}), colony volumes were similar to the parental strain and markedly lower than with \textit{ΔadcAll} mutations in the WT D39 strain (Fig. 1E). The frequency with which deletion of \textit{adcAll} resulted in a strain with an increased capsule thickness was investigated using multiple transformants made using the \textit{adcAll} deletion constructs or by transformation with genomic DNA extracted from a \textit{ΔadcAll} strain mutant. Of the 100 transformants, 44\% (\textit{kana}) or 42\% (\textit{cat}) had increased capsule thickness when transformed with the PCR construct and 78\% (18 out of 23) when transformed with genomic DNA (Table 1). The remaining mutant clones either had a normal capsule thickness or were unencapsulated. Growth of the \textit{ΔadcAll} strain in chemically defined medium (CDM) supplemented with 33 \text{mM} cations (\text{Mn}^{2+} or \text{Zn}^{2+}), 5\% sucrose, or recombinant PhTD (50 \text{μg/ml}) or in CDM depleted of cations by treatment with 1 \text{mM EDTA} did not reduce increased capsule expression (data not shown; measured using capillary tube colony volume). The increased capsule thickness phenotype was stable, with 100\% of 100 colonies retaining a thick capsule after a single mucoid colony was cultured in THY (Todd-Hewitt broth supplemented with yeast extract) liquid medium followed by plating on blood agar plates over five generations. These data show that transformation of the \textit{S. pneumoniae} D39 strain with a deletion construct affecting \textit{adcAll} frequently results in transformants with a marked increase in capsule quantity.

The hyperencapsulated D39 \textit{ΔadcAll} strain is resistant to complement-mediated phagocytosis. The capsule is an essential virulence factor that prevents opsonophagocytosis of \textit{S. pneumoniae} but at a metabolic cost during \textit{S. pneumoniae} growth (7, 26). We therefore investigated the phenotypes of the hyperencapsulated D39 \textit{ΔadcAll} strain in vitro and in murine infection models. Growth of the \textit{ΔadcAll} strain was similar to the WT D39 in complete medium THY and in CDM (supplemented with 33 \text{μM} zinc to overcome effects of loss of \textit{adcAll} on zinc transport) (Fig. 3A and B). In contrast, in blood approximately 1 log_{10} more \textit{ΔadcAll} bacteria were recovered after 4 h of incubation compared to the D39 WT strain, with large differences in CFU persisting at 6 h (Fig. 3C). Flow cytometry demonstrated increased resistance to opsonization with complement and macrophage phagocytosis of the D39 \textit{ΔadcAll} strain compared to the D39 WT strain (Fig. 4A to C). The D39 \textit{ΔadcAll} strain also had increased resistance to killing by neutrophils compared to the WT strain; these differences were lost if bacteria were opsonized in heat-treated (i.e., complement-deficient) sera or in phosphate-buffered saline (PBS) alone, demonstrating that the differences were largely complement dependent (Fig. 4D). Adhesion assays showed there was no defect for the D39 \textit{ΔadcAll} strain in binding to the respiratory epithelium cell line Detroit 562 compared to the WT strain (Fig. 5A). Hence, increased capsule expression by the \textit{ΔadcAll} strain was

### Table 1

| DNA source for transformation | No. of clones analyzed | Capsule phenotype | Absent* | Normal | Thick |
|------------------------------|------------------------|-------------------|--------|--------|-------|
| PCR fragment \textit{adcAll}:\textit{kana} | 100 | 14 (1) | 32 | 44 |
| PCR fragment \textit{adcAll}:\textit{cat} | 100 | 45 (4) | 13 | 42 |
| Genomic DNA R6 \textit{ΔadcAll}:\textit{cat1} 1st | 4 | 0 | 0 | 4 |
| Genomic DNA R6 \textit{ΔadcAll}:\textit{cat1} 2nd | 4 | 1 | 0 | 3 |
| Genomic DNA R6 \textit{ΔadcAll}:\textit{cat2} | 15 | 1 | 3 | 11 |

*aNumbers in parentheses are numbers of absent capsule strains sequenced all of which contained the Q308 stop codon mutation in \textit{cps2E}.
The hyperencapsulated \( \Delta adcAII \) strain has increased virulence. Both colony forming units (CFU) in nasal washes at day 5 and competitive infection experiments demonstrated that the hyperencapsulated D39 \( \Delta adcAII \) strain colonized the nasopharynx to a similar degree as the WT D39 (Fig. 5B; Table 2), results which are consistent with the lack of a difference between the strains for adhesion to Detroit 562 cells. In contrast, the hyperencapsulated \( \Delta adcAII \) strain had increased virulence during systemic or pneumonic infection. In competitive infection experiments using a sepsis model (intraperitoneal [i.p.] inoculation), the D39 \( adcAII \) strain strongly outcompeted the WT strain (Table 2), and in a murine sepsis model using pure inocula of each strain, 80% of
mice infected with the D39 ΔadcAII strain progressed to fatal infection by 40 h compared to 40% of mice infected with WT D39 (Fig. 6A). Finally, in a pneumonia model higher CFU was recovered in both the lungs and blood from mice infected with the ΔadcAII mutant compared to wild-type D39 (Fig. 6B and C).

**Transcriptome analysis of wild-type and hyperencapsulated ΔadcAII strains.** To investigate mechanisms causing increased capsule production by the ΔadcAII strain, a transcriptome microarray analysis was performed on WT D39, one hyperencapsulated ΔadcAII and one ΔadcAII/phtD strain clone, and one ΔadcAII::cat unencapsulated clone (Cl44) (Table 3). Three independent RNA extracts for each strain were submitted to transcriptomic analysis. In total, 89 genes showed significant changes in expression (>1.5-fold, \(P < 0.05\)) between the wild-type D39 and hyperencapsulated ΔadcAII strain (78 with reduced and 11 with increased expression in the mutant strain including the deleted adcAII and downstream phtD genes), 96% (86/89) of which also showed comparable changes in expression in the thick-capsule ΔadcAII/phtD strain. In contrast, 11% (10/89) of these genes showed similar changes in expression in the Cl44 ΔadcAII strain without increased capsule expression, suggesting that the gene expression changes were linked to the capsule phenotype. Expression of the D39 capsule locus genes was not significantly different between the strains. Genes showing increased expression in the hyperencapsulated ΔadcAII and ΔadcAII/phtD strains included genes related to zinc uptake (adcR, adcA, phtA, and phtE), suggesting compensatory effects.

**FIG 4** The ΔadcAII mutant has increased resistance to complement and phagocytosis. (A) Mean fluorescence index (MF; measured in arbitrary units) of C3b/iC3b deposition on WT D39 or ΔadcAII mutant measured using flow cytometry in 25% human serum. Error bars represent SDs. ***, \(P < 0.001\), unpaired t test. (B) Examples of flow cytometry histograms for C3b/iC3b deposition on WT D39 or ΔadcAII mutant in 100% human serum. Gray shadowing indicates the results for bacteria incubated in PBS alone. (C) Flow cytometry quantification of macrophage (THP-1 cells) phagocytosis of isothiocyanate fluorescein-labeled WT D39, R6 (unencapsulated derivative of D39), and the ΔadcAII mutant for 1 h at 37°C (50 CFU/cell). The percentage of fluorescent macrophages was quantified by flow cytometry, and the data are expressed as means (SD) of the percentage of the results for the WT D39 strain. ***, \(P < 0.01\), unpaired Student’s \(t\) tests. (D) Mean proportions of WT D39 (white columns) and the ΔadcAII mutant (black columns) surviving incubation with fresh human neutrophils for 45 min (MOI of 500 bacteria/neutrophil). Data are given for bacteria preincubated in PBS, 25% normal human serum, or 25% heat-inactivated human serum (no complement activity). Error bars represent SDs, and \(P\) values were obtained using unpaired \(t\) tests.
due to loss of the AdcAII zinc transporter. The other genes showing increased expression in the hyperencapsulated strains encode proteins of unknown function or containing LysM domains predicted to be involved in cell wall metabolism (27). Three of the operons that showed reduced expression in the hyperencapsulated strains are predicted to be involved in pyrimidine synthesis: SPD_0608-09, encoding a predicted orotatedecarboxylase and phosphoribosyltransferase and being part of a larger operon encompassing SPD_0608 to SPD_0618 (28); SPD_0851-52, predicted to encode a dihydroorotate dehydrogenase electron transfer subunit (29); and SPD_1131, predicted to encode a carbamoylphosphate synthase large subunit required for pyrimidine synthesis from glutamine (30). Other genes showing reduced expression in the hyperencapsulated strains have roles in iron uptake (SPD_0224, -0226, and -1650), carbohydrate uptake (SPD_0279, 0362, 1050-1053, 1501, and 1987-95), and riboflavin synthesis (SPD_0166-69). Of particular interest, the hyperencapsulated strains showed reduced expression of SPD_0450, SPD_0452, and SPD_0453, from the SpnD39III (ST5556II) type I restriction-modification system, respectively; this is discussed in detail below.

**Increased capsule thickness of the ΔadcAII strains correlated closely with specific hsd alleles.** The *S. pneumoniae* SpnD39III (ST5556II) type I restriction-modification locus undergoes genetic variation due to recombination within the locus between pairs of inverted repeats, generating six allelic variants which are linked to

**TABLE 2** Competitive index data for infection models using a mixed inoculum of 50% WT D39 and 50% D39 ΔadcAII hyperencapsulated strain

| Infection model        | Inoculation route and CFU               | Sample source (time point) | CI (SD) | n   | P value |
|------------------------|----------------------------------------|-----------------------------|---------|-----|---------|
| Nasopharyngeal colonization | Intranasal, 5 × 10⁶ CFU | Nasal washes (5 days) | 1.04 (0.15) | 4   | 0.58    |
| Sepsis                  | Intraperitoneal, 5 × 10⁶ CFU            | Blood (24 h)                | 4.6 (0.62) | 7   | <0.0001 |
opaque (increased capsule expression) and transparent (reduced capsule expression) colony morphology (14–16). This suggests that the detected changes in expression of genes within the SpnD39III (ST5556II) locus could reflect differences in the proportions of the allelic variants between the WT and \(\text{/H9004 adcAII}\) strains, and these differences could underpin the hyperencapsulated phenotype of the latter. Hence, the proportion of each of the six SpnD39III (ST5556II) variants was obtained for multiple individual \(\text{/H9004 adcAII}\) strains expressing either thick or normal-size capsules using a previously described assay based on PCR followed by restriction digestion of the products (14) (Table 4). This showed a clear correlation between capsule phenotype and the dominant SpnD39III variant. The WT D39 strain contained a mixture of the SpnD39III variants, mainly SpnD39IIIC with also a significant proportion of the SpnD39IID and F variants. With one exception, SpnD39IIIC (3 strains) and F (5 strains) were the dominant variants found in the hyperencapsulated \(\text{/H9004 adcAII}\) strains, whereas SpnD39IID (7 strains) or A (1 strain) was the dominant variant found in the \(\text{/H9004 adcAII}\) strains with normal capsule thickness. To try to link increased capsule formation by some \(\text{ΔadcAll}\) mutants to changes in the dominant alleles of the SpnD39III (ST5556II) type I restriction-modification locus, the hyperencapsulated \(\text{ΔadcAll}\) strain was transformed with genomic DNA from D39 mutant strains with locked SpnD39III (ST5556II) alleles due to an inactivated \(\text{creX}\) gene. Flow cytometry analysis of complement sensitivity was used to rapidly assess capsular phenotype for 10 transformants for each allele (A to F). All transformants retained the complement-resistant phenotype of the hyperencapsulated \(\text{ΔadcAll}\) strain, even those made using the SpnD39III (ST5556II) alleles associated with a normal capsule width in \(\text{ΔadcAll}\) transformants (A and D) (Fig. 7), suggesting they all remained hyperencapsulated.

**Genome sequence data for \(\text{ΔadcAll}\) strains.** Genome sequencing of one \(\text{ΔadcAll}\) and one \(\text{ΔadcAll/pht}\) strain confirmed they contained the expected partial deletion of \(\text{adcAll}\) or \(\text{adcAll/pht}\), respectively, with insertion of the antibiotic resistance
| Gene name | Predicted/known function | ΔadcAll (AII) thick | ΔadcAll/phtD (AII+Pcl4) thick | ΔadcAll (Cl44) none |
|-----------|--------------------------|---------------------|------------------------------|---------------------|
| spd_0052  | purL                     | −2.45               | −2.36                        | 1.24                |
| spd_0053  | purF                     | −2.27               | −2.34                        | 1.25                |
| spd_0055  | purN                     | −2.13               | −2.18                        | 1.36                |
| spd_0090  | abc transporter lipoprotein | −2.02             | −2.19                        | 2.04                |
| spd_0166  | ribH                     | −2.76               | −3.44                        | 1.56                |
| spd_0167  | ribB                     | −2.51               | −3.34                        | 1.71                |
| spd_0168  | ribE                     | −2.52               | −3.30                        | 1.66                |
| spd_0169  | ribD                     | −2.40               | −3.32                        | 1.67                |
| spd_0167  | ppiD                     | −2.28               | −2.11                        | 1.46                |
| spd_0176  | adeAII                   | −2.01               | −1.37                        | 1.72                |
| spd_0265  | adhP                     | −1.80               | −1.83                        | 1.63                |
| spd_0279  | cellB                    | −2.28               | −1.79                        | 1.76                |
| spd_0300  | oligohydrolutezyme       | −2.49               | −1.60                        | 1.32                |
| spd_0362  | mtiF                     | −2.38               | −2.40                        | 1.40                |
| spd_0364  | amino acid ABC transporter ATPase | −3.00             | −2.80                        | 1.84                |
| spd_0444  | lytB                     | −1.55               | −1.69                        | 1.38                |
| spd_0450  | cexX                     | −3.39               | −4.24                        | −1.43               |
| spd_0452  | hsdS (hsdS2)             | −3.62               | −6.41                        | 1.25                |
| spd_0453  | hsdS (hsdS1)             | −2.01               | −2.36                        | 1.15                |
| spd_0466  | blpT                     | −1.79               | −2.26                        | 1.52                |
| spd_0472  | blpA                     | −2.21               | −3.47                        | 1.59                |
| spd_0473  | blpY                     | −1.50               | −2.17                        | 1.44                |
| spd_0533  | bpmA                     | −1.59               | −2.14                        | 2.26                |
| spd_0595  | bpmB                     | −1.55               | −1.72                        | −0.64               |
| spd_0608  | pyrF                     | −1.65               | −1.60                        | 1.02                |
| spd_0609  | pyrE                     | −1.77               | −1.67                        | 1.02                |
| spd_0610  |                      | −2.18               | −2.26                        | 1.45                |
| spd_0611  |                      | −1.76               | −1.94                        | 1.19                |
| spd_0612  |                      | −2.07               | −2.06                        | 1.00                |
| spd_0613  |                      | −1.70               | −1.83                        | 1.09                |
| spd_0614  |                      | −1.76               | −1.77                        | 1.11                |
| spd_0615  |                      | −1.51               | −2.25                        | 1.25                |
| spd_0616  |                      | −1.56               | −2.38                        | 1.13                |
| spd_0617  |                      | −1.76               | −2.64                        | 1.25                |
| spd_0618  |                      | −1.71               | −2.51                        | 1.20                |
| spd_0851  |                      | −1.90               | −1.90                        | 1.16                |
| spd_0852  |                      | −2.32               | −2.28                        | 1.11                |
| spd_0853  |                      | −1.71               | −1.65                        | 1.09                |
| spd_0888  | adcAII                   | −3.87               | −3.03                        | −5.95               |
| spd_0889  | phtD                     | −1.78               | −2.50                        | −4.2                |
| spd_1009  | serB                     | −1.51               | −1.24                        | 1.60                |
| spd_1011  | hlxK                     | −1.63               | −1.26                        | 1.58                |
| spd_1035  | pts system, IIA component | −4.70             | −4.83                        | −1.91               |
| spd_1036  | pts system, IIA component | −7.26             | −5.84                        | −2.93               |
| spd_1050  | lacD                     | −1.61               | −1.60                        | 1.44                |
| spd_1051  | lacC                     | −1.62               | −1.62                        | 1.49                |
| spd_1052  | lacB                     | −1.58               | −1.56                        | 1.46                |

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DISCUSSION

In this work, we have described that mutation of the zinc transporter lipoprotein gene adcAII in the S. pneumoniae D39 strain leads to an unexpected and striking increase in capsule expression in 42% of the resulting mutants. This phenotype occurred with ΔadcAII mutations made by transformation either with a PCR construct or with genomic DNA from another ΔadcAII mutant and was stable over many bacterial generations. A similar mucoid phenotype was also observed with the ΔadcAII mutation in two of the four other S. pneumoniae capsular serotypes investigated. The increased capsule quantity was very marked, with EM showing a greater-than-5-fold increase in capsule width and nuclear magnetic resonance (NMR) showing a 60% increase in the quantity of monosaccharides in purified capsule. This level of increase in capsule expression is markedly greater than that seen between opaque and transparent TIGR4
capsular switched (less than 2-fold) (31) and 6B strains (32), justifying describing the D39 ΔadcAll strain as hyperencapsulated. The phenotypic consequence of the increased capsule expression was a high degree of resistance to complement-mediated immunity and hypervirulence in mouse models of pneumonia and sepsis. These

TABLE 4 Proportions of variants (identified by PCR analysis) for the SpnD39III (ST5556II) type I restriction-modification system for selected ΔadcAll mutant strains divided into those with thick and normal capsule thicknesses

| Phenotype     | Strain | Proportion (%) of SpnD39III (ST5556II) variant: |
|---------------|--------|-----------------------------------------------|
|               |        | A    | B    | C    | D    | E    | F    |
| Wild type     | D39    | 2.2  | 0    | 67.2 | 15.6 | 0    | 15.0 |
| Thick capsule | Cl82   | 1.3  | 0    | 3.8  | 7.1  | 1.0  | 86.8 |
|               | Cl72   | 1.7  | 0    | 4.3  | 9.2  | 0    | 84.8 |
|               | Cl10   | 1.3  | 0    | 4.0  | 8.8  | 0    | 85.9 |
|               | Cl38   | 2.0  | 0    | 1.9  | 8.4  | 2.3  | 85.4 |
|               | Cl3 2P | 1.0  | 0    | 2.5  | 6.4  | 1.6  | 88.6 |
|               | Cl3 1P | 0.7  | 0    | 87.8 | 9.8  | 0    | 1.7  |
|               | Cl3 1P | 1.7  | 0    | 84.8 | 10.3 | 0    | 3.3  |
|               | Cl1 1G | 2.2  | 0    | 83.1 | 11.1 | 0    | 3.7  |
|               | Cl7 1G | 0    | 0    | 7.7  | 92.3 | 0    | 0    |
|               | All   | 3.8  | 0.6  | 74.9 | 17.8 | 0    | 2.8  |
| Normal capsule| Cl88   | 0    | 0    | 6.0  | 92.7 | 0.00 | 1.3  |
|               | Cl28   | 0    | 0    | 6.7  | 93.3 | 0    | 0    |
|               | Cl35   | 0    | 0    | 6.92 | 93.1 | 0    | 0    |
|               | Cl6    | 0    | 0.7  | 5.71 | 93.6 | 0    | 0    |
|               | Cl73   | 0    | 0    | 9.88 | 90.1 | 0    | 0    |
|               | Cl20   | 0    | 0    | 9.93 | 90.1 | 0    | 0    |
|               | Cl17   | 0    | 0    | 9.0  | 8.9  | 88.2 | 0    |
|               | Cl11   | 0.4  | 1.7  | 16.4 | 80.8 | 0    | 0.6  |
|               | Cl36   | 94.3 | 5.8  | 0    | 0    | 0    | 0    |

FIG 7 Flow cytometry analysis of complement sensitivity of the hyperencapsulated ΔadcAll strain after transformation with locked SpnD39III (ST5556II) alleles (A to F) containing an inactivated creX gene. ΔFP441, ΔFP442, ΔFP443, ΔFP444, ΔMR0559, and ΔMR0560 are all double mutant strains carrying the adcAll mutation and an extra one in allele SpnIIIB, allele SpnIIIC, allele SpnIIIA, allele SpnIIID, allele SpnIIIE, and allele SpnIIIF, respectively. (A) Fluorescence index (MFI measured in arbitrary units multiplied by proportion of bacteria positive for C3b/iC3b) of C3b/iC3b deposition on ΔadcAll mutants and ΔadcAll fixed SpnD39III allele transformants (alleles A to F) as a proportion of the fluorescence index for the wild-type normal-capsule-thickness D39 strain. The data were measured using flow cytometry after preincubation in 30% human serum. Error bars represent SDs, and 10 transformants were tested for each double mutant strain. For all mutant strains, the P value for results compared to D39 was <0.001 (unpaired t tests). (B) Examples of flow cytometry histograms for C3b/iC3b deposition on WT D39 (dark gray line) and one ΔadcAll/SpnD39IIIΔ allele (light gray line) double mutant transformant. Gray shading indicates the results for bacteria incubated in PBS alone.
TABLE 5 Mutation construction, capsule phenotype, and (where available) cps2E gene genome sequence data for S. pneumoniae strains

| Strain/clone | Gene deletion | Antibiotic resistance | Mutant construction | Capsule ratio/D39 | Capsule phenotype | Mutation in cps2E |
|--------------|---------------|-----------------------|---------------------|------------------|------------------|------------------|
| D39 800      | None          |                       |                     | 1                | Normal           | None             |
| D39 WT       | None          |                       |                     | 1                | Normal           | None             |
| ΔadcAII      | ΔadcAII       | Cm                    | New transformation  | 3.7              | Thick            | None             |
| C10          | ΔadcAII       | Kana                  | New transformation  | 3                | Thick            | None             |
| C157         | ΔadcAII       | Kana                  | New transformation  | 0.5              | Unencapsulated   | Stop codon aa 308|
| C11 1P       | ΔadcAII       | Cm                    | Back-crossing with ΔadcAII | 0.5   | Unencapsulated   | Not sequenced    |
| C11 1G       | ΔadcAII       | Cm                    | Back-crossing with ΔadcAII | 2.9   | Thick            | None             |
| C12 2P       | ΔadcAII       | Cm                    | Back-crossing with ΔadcAII | 0.9   | Normal           | Not sequenced    |
| C13 1G       | ΔadcAII       | Cm                    | Back-crossing with ΔadcAII | 3.1   | Thick            | Not sequenced    |
| C13 1P       | ΔadcAII       | Cm                    | Back-crossing with ΔadcAII | 3.2   | Thick            | Not sequenced    |
| C13 2P       | ΔadcAII       | Cm                    | Back-crossing with ΔadcAII | 3.1   | Thick            | None             |
| C15          | ΔadcAII       | Cm                    | Back-crossing with ΔadcAII | 3.8   | Thick            | None             |
| C15 1G       | ΔadcAII       | Cm                    | Back-crossing with ΔadcAII | 3.7   | Thick            | Not sequenced    |
| C16 1P       | ΔadcAII       | Cm                    | Back-crossing with ΔadcAII | 0.5   | Unencapsulated   | Not sequenced    |
| C16 2P       | ΔadcAII       | Cm                    | Back-crossing with ΔadcAII | 0.5   | Unencapsulated   | Stop codon aa 308|
| C17 1P       | ΔadcAII       | Cm                    | Back-crossing with ΔadcAII | 3.5   | Thick            | Not sequenced    |
| C17 1G       | ΔadcAII       | Cm                    | Back-crossing with ΔadcAII | 3.1   | Thick            | None             |
| Allcl 1      | ΔadcAII       | Cm                    | New transformation  | 1                | Normal           | None             |
| Allcl 17     | ΔadcAII       | Cm                    | New transformation  | 1.1              | Normal           | None             |
| Allcl 20     | ΔadcAII       | Cm                    | New transformation  | 1.1              | Normal           | None             |
| Allcl 28     | ΔadcAII       | Cm                    | New transformation  | 1.15             | Normal           | None             |
| Allcl 31     | ΔadcAII       | Cm                    | New transformation  | 0.5              | Unencapsulated   | Stop codon aa 308|
| Allcl 35     | ΔadcAII       | Cm                    | New transformation  | 0.85             | Unencapsulated   | Stop codon aa 308|
| Allcl 36     | ΔadcAII       | Cm                    | New transformation  | 1.05             | Normal           | E for K aa 322   |
| Allcl 38     | ΔadcAII       | Cm                    | New transformation  | 2.05             | Thick            | E for K aa 322   |
| Allcl 44     | ΔadcAII       | Cm                    | New transformation  | 0.5              | Unencapsulated   | Stop codon aa 308|
| Allcl 72     | ΔadcAII       | Cm                    | New transformation  | 2.8              | Thick            | E for K aa 322   |
| Allcl 73     | ΔadcAII       | Cm                    | New transformation  | 1.15             | Normal           | None             |
| Allcl 75     | ΔadcAII       | Cm                    | New transformation  | 1.2              | Normal           | None             |
| Allcl 78     | ΔadcAII       | Cm                    | New transformation  | 1.8              | Thick            | E for K aa 322   |
| Allcl 82     | ΔadcAII       | Cm                    | New transformation  | 2.6              | Thick            | E for K aa 322   |
| Allcl 88     | ΔadcAII       | Cm                    | New transformation  | 1                | Normal           | None             |
| All + Pcl4   | ΔadcAII + phtD| Cm                    | New transformation  | 2.2              | Thick            | Not sequenced    |

*a*, amino acid position.

...phenotypes are exaggerated versions of the well-described effects of the capsule on S. pneumoniae evasion of host immunity (7), demonstrating that under a normal level of expression the capsule effects on immune evasion have not reached maximal potential. Previous data have shown that capsule expression comes at a metabolic cost which inhibits growth when cultured in defined medium and that the capsule prevents adhesion by respiratory epithelium (26, 33, 34). However, surprisingly, these negative aspects of capsule expression were not identified with the hyperencapsulated ΔadcAII strain. The serotype 2 S. pneumoniae capsule repeating unit is a hexasaccharide consisting of one glucuronic acid, two glucose, and three rhamnoses (6, 35). NMR demonstrated that the relative proportion of glucose to rhamnose was altered in the ΔadcAII strain compared to WT D39, shifting from almost 1 to 1 in the latter to closer to the expected 2-to-3 ratio. This would be compatible with an increased proportion of the total S. pneumoniae glucose pool being used for capsule production. The larger comparative increase in capsule width compared to changes in monosaccharide quantity suggests the organization of the capsule may have been altered, perhaps with more loosely packed but longer capsule strands in the ΔadcAII strain compared to D39.

Why there is increased expression of the capsule in the ΔadcAII strain is not clear. The close linkage to adcAII suggests a role for disruption of zinc utilization, yet the hyperencapsulated phenotype did not occur with mutation of the other S. pneumoniae zinc uptake lipoprotein gene adcA (19) and was not affected by zinc availability. Combined deletion of adcA and ΔadcAII was also not associated with the hyperencapsulated phenotype, but the double mutation had major effects on S. pneumoniae...
physiology (19) which could have obscured or suppressed the mucoid phenotype. Overall regulation of *S. pneumoniae* capsule expression is poorly understood and is further complicated by the large number of different capsular carbohydrate structures with potentially significant differences in regulatory mechanisms. Factors affecting thickness of the capsule layer include regulation of *cps* locus gene expression by RitR (an orphan two-component signal transduction component) (36), CpsR (a GntR family regulator) (37), and RegM (38), as well as the conserved *S*. *cpsABCD* (also termed *wzg*, *wzh*, *wzd*, and *wze*) genes of the *cps* locus (39–41). Two *S. pneumoniae* quorum-sensing systems (LuxS/AI-2 and the Rgg/small hydrophobic peptide system) increase capsule thickness (42–44), which can also be regulated independently of gene transcription by the supply of capsule monosaccharide precursors (45) or by increased capsule shedding mediated by LytA (12). However, our transcriptome analysis did not identify increased *cps* locus gene expression or any effects on the abovementioned known regulators of capsule expression in the Δ*adcAII* strain.

Another potential mechanism causing the hyperencapsulated phenotype in the Δ*adcAII* mutant was identified by effects on transcription of the SPD_0450-0453 locus. This encodes the SpnD39III (ST5556II) type I restriction-modification system, allelic variants of which correlate with capsule thickness for several serotypes (14–16). We found that the hyperencapsulated phenotype of Δ*adcAII* mutants was associated with a predominance of either the SpnD39IIIIC or F allelic variant, whereas SpnD39IIIID was the dominant allele for the majority of Δ*adcAII* mutants with normal capsule thickness. This link between the hyperencapsulated phenotype of the Δ*adcAII* strain and specific alleles of the SpnD39III (ST5556II) system seems unlikely to be coincidental given the known effects of this restriction-modification system on capsule expression. However, transformation with fixed SpnD39III (ST5556II) alleles, including those associated with normal capsule thickness (A and D), did not alter the hyperencapsulated phenotype of the Δ*adcAII* mutant, showing that any effects of SpnD39III alleles on the capsule thickness of the Δ*adcAII* mutation are not readily reversed by switching alleles. This situation is further confused by the similarity in allele composition of the wild-type D39 strain and the Δ*adcAII* mutant and by differences between our data and published papers in which SpnD39III alleles are linked to thick or thin capsule phenotypes. Manso et al. found that A, E, and F allele strains were largely opaque but C strains were more transparent, Li et al. found that only E allele strains (termed *hsdSa* in their paper) were opaque, and Oliver et al. found that the A and B alleles were opaque and the others transparent (14–16). The presumed mechanism of capsule regulation by SpnD39III is differential methylation of genes or regulatory regions (14, 15), but the genes involved remain undetermined. Our transcriptome data have identified multiple additional genes showing differential expression between hyperencapsulated Δ*adcAII* strains and wild-type D39 or a normal-capsule-width Δ*adcAII* mutant, some of which could be involved in mediating increased capsule expression. These include three operons annotated as being involved in pyrimidine metabolism, suggesting a potential role for pyrimidine in controlling capsule expression. Which genes showing differential expression between the Δ*adcAII* strains and WT D39 strains are involved in the capsular phenotype and whether differential regulation is related to differences in methylation will require considerably more detailed genetic studies.

Interestingly, 50% of independently obtained hyperencapsulated Δ*adcAII* strains contained an identical nonsynonymous SNP affecting the *cps* locus gene *csp2E*. The SNP is predicted to affect the cytoplasmic tail of Csp2E, a glucose phosphate transferase that initiates the assembly of capsule components on the cell membrane and is partially conserved among most capsular serotypes (39). Point mutations of *csp2E* that affect capsule expression have been previously described (32, 39), suggesting a causative role for this SNP for the Δ*adcAII*-related capsule phenotypes. However, the same SNP was not present in one lineage of Δ*adcAII* with increased capsule thickness (the original transformant and four back-crossed derivatives) and was also identified in one out of eight normal-capsule-thickness Δ*adcAII* strains. All the unencapsulated Δ*adcAII* transformants also contained the same SNP in *csp2E* predicted to introduce a stop codon.
This high frequency of cspE2 stop codon mutations suggests that partial deletion of adcAII causes significant physiology stress to S. pneumoniae that may induce loss of capsule production as an escape mutation.

To conclude, we have identified that in the S. pneumoniae D39 strain a hyperencapsulated phenotype is an unexpected consequence of targeted mutation of adcAII, which encodes a zinc ABC transporter lipoprotein. This strain will be a useful tool for investigating how the capsule affects S. pneumoniae interactions with the host. The hyperencapsulated phenotype partially correlated with both a nonsynonymous SNP in cspE2 and changes in allelic dominance within the SnpD39II (ST5556II) restriction-modification system. Further investigation of genes showing differential expression between normal and hyperencapsulated D39 strains could help to further identify the underlying mechanism(s) controlling S. pneumoniae capsule thickness.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The ΔadcAII, ΔphtD, ΔadcA/adcAII, and ΔadcAII/phtD mutant strains were created either in the wild type or in the ΔcspD D39 strain as well as in wild-type serotype 4 (TIGR4), 6A, 6B (strains 6Aa and 6Ba, respectively, from the work of Hyams et al. [31]), and 17F (46) strains by gene replacement using genomic DNA or PCR-amplified fragments obtained from the corresponding R6 mutants and standard transformation protocols for S. pneumoniae (19). The cat and kanA genes were inserted in the reverse orientation without promoter or terminator sequences to avoid affecting expression of adjacent genes. Mutant identities were verified by PCR with primers flanking the cloned regions. S. pneumoniae was grown at 37°C with 5% CO₂ in air in THY or on Columbia agar containing 5% blood. Working stocks grown to an optical density (OD) of 0.4 (−10⁵ CFU/ml) were made using THY and stored at −80°C in 10% glycerol as single-use aliquots. CFU were confirmed by colony counting of log₉ serial dilutions of bacteria cultured overnight on 5% Columbia blood agar. Growth curves were determined by measuring OD₅₉₅ for bacteria cultured in 2.5 ml of THY or chemically defined medium (CDM) supplemented with 33 μM Zn in 24-well plates sealed with a transparent film and incubated at 37°C in a FLUOstar reader. To measure blood growth, 1 × 10⁶ CFU/ml of S. pneumoniae was inoculated into 1 ml of heparinized human blood and incubated at 37°C, with plating of serial dilutions at 0, 4, and 6 h to assess bacterial CFU.

Capsule size measurement and microscopy. An indirect method was developed to measure capsule size by determining the size of the bacterial pellet. Briefly, 12 ml of culture was centrifuged, the pellet was resuspended in 120 μl of PBS, and 35 μl was loaded in a microcapillary tube. After centrifugation for 15 min at 800 × g, the height of the pellet within the tube was measured with a ruler. Electron microscopy of mid-log-phase S. pneumoniae fixed in 3% paraformaldehyde (PAF) was performed using a ruthenium red and London resin capsule-preserving protocol as previously described (33). Capsule thickness was calculated by direct measurement of the surface layer for 30 randomly chosen S. pneumoniae bacteria/strain using ImageJ software.

Confocal microscopy on bacteria was performed using an Olympus FV1000 confocal laser scanning microscope with a 63× objective. Bacteria were fixed for 30 min with 4% PFA (Sigma) on slides (Thermo Scientific; SuperFrost Plus 10149870) and subsequently stained with anti-serotype 2 antibody (Statens Serum Institute) plus Alexa Fluor 546-conjugated anti-rabbit antibody. DNA was stained with 4,6-diamidino-2-phenylindole (DAPI).

Capsular polysaccharide extraction and quantification. Capsular polysaccharides were extracted from 1 liter of culture, and bacteria were resuspended in 10 ml of 0.15 M Tris buffer (pH 8) supplemented with 0.1% deoxycholate and incubated for 10 min at 37°C and then for 35 min at 50°C. Cell debris was removed by centrifugation under acidic condition. Proteins were eliminated from the supernatant by two successive extractions using a 5:1 ratio of chloroform and butanol, before precipitating capsular polysaccharides in 80% ethanol. Pellets were dried, resuspended in 0.1 M phosphate buffer (pH 7.2), and incubated with DNase and RNase for 1 h at 37°C, and then trypsin was added for 2 h at 37°C before purification of capsular polysaccharide by ion exchange on a column of DEAE Sepharose. Monosaccharide composition was established by GC and GC-MS as alditol acetate derivatives. Briefly, samples were hydrolyzed in 4 M trifluoroacetic acid (TFA) for 4 h at 100°C and reduced with sodium borohydride in 0.05 M NaOH for 4 h. Reduction was stopped by dropwise addition of acetic acid until pH 6 was reached, and borate salts were codistilled by repetitive evaporation in dry methanol. Peracetylation was performed in acetic anhydride at 100°C for 2 h. All monosaccharide derivatives were identified according to their specific retention times and electron ionization MS (EI-MS) fragmentation patterns (47).

Phagocytosis, neutrophil killing, complement deposition, and adhesion assays. Flow cytometry phagocytosis and complement deposition assays were performed as previously described (7, 48) using S. pneumoniae incubated for 30 min with human serum (25%), human heat-inactivated serum (25%), or just Hanks balanced salt solution (HBSS) medium. For macrophage phagocytosis, THP-1 monocytes cultured in suspension in RPMI medium supplemented with 10% fetal bovine serum (FBS) were treated for 24 h with 10 nM phorbol 12-myristate 13-acetate (PMA) to induce cell adhesion and macrophage differentiation. Flow cytometry was performed using a fluorescence-activated cell sorting (FACS)Verse machine (BD), and the data were analyzed with FlowJo software. For neutrophil killing assays, fresh human neutrophils were purified using a magnetically activated cell sorting (MACS) neutrophil isolation kit (Miltenyi Biotec) and resuspended in HBSS medium at a concentration of 1 × 10⁶ cells/ml. S.
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