

Requirement for Capsule in Colonization by *Streptococcus pneumoniae*

ASHALLA D. MAGEE AND JANET YOTHER*

Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294

Received 2 February 2001/Returned for modification 2 March 2001/Accepted 20 March 2001

Nasopharyngeal colonization is a necessary first step in the pathogenesis of *Streptococcus pneumoniae*. Using isolates containing defined mutations in the *S. pneumoniae* capsule locus, we found that expression of the capsular polysaccharide is essential for colonization by the type 2 strain D39 and the type 3 strains A66 and WU2. Nonencapsulated derivatives of each of these strains were unable to colonize BALB/cByJ mice. Similarly, type 3 mutants that produced <6% of the parental amounts of capsule could not colonize. Capsule production equivalent to that of the parent strain was not required for efficient colonization, however, as type 3 mutants producing approximately 20% of the parental amounts of capsule colonized as effectively as the parent. This 80% reduction in capsule level had only a minimal effect on intraperitoneal virulence but caused a significant reduction in virulence via the intravenous route. In the X-linked immunodeficient CBA/N mouse, the type 3 mutant producing ~20% of the parental amount of capsule (AM188) was diminished in its ability to cause invasive disease and death following intranasal inoculation. Following intravenous or intraperitoneal challenge, however, only extended survival times were observed. Our results demonstrate an additional role for capsule in the pathogenesis of *S. pneumoniae* and show that isolates producing reduced levels of capsule can remain highly virulent.

Streptococcus pneumoniae is an important human pathogen that causes an array of diseases including otitis media, pneumonia, meningitis, and bacteremia. The pneumococcus is a component of the normal microflora in the nasopharynx, with colonization beginning shortly after birth (2). Colonization usually results in asymptomatic carriage within the nasopharynx, which subsequently serves as the main reservoir for pneumococci causing infections in children, the elderly, the immunocompromised, and individuals suffering from chronic disease (2). Because this nasopharyngeal reservoir of bacteria is so important to the dissemination and initiation of infection, colonization is an important target for the prevention of pneumococcal disease.

A number of *S. pneumoniae* components have been implicated in the colonization process. Among these are neuraminidase, SpxB (pyruvate oxidase), and the choline binding proteins CbpA (also referred to as PspC and SpsA [11, 25]), CbpD, CbpE, CbpG, LytB, and LytC. Mutants altered in the expression of each of these proteins show decreased colonization in animal models (24, 37, 43, 46). An increase in teichoic acid expression, along with a concomitant decrease in capsule expression, is correlated with an enhanced ability of transparent-phase variants to colonize (32, 50). Additional factors that may be involved in colonization have been suggested on the basis of in vitro adherence assays (AmiA, PlpA, PsaA, and cell wall components) and protection studies (PsaA and PspA) (5, 7, 8, 15, 16, 22, 55, 56).

In systemic infections, there is an absolute requirement for the polysaccharide capsule, which functions to inhibit complement-mediated opsonophagocytosis (3, 12, 27, 54). Virulence

levels have been previously reported to correlate directly with the amount of capsule produced (32, 35), although these results have not been confirmed with isolates containing defined mutations. A role for the capsule in colonization has not been described, and in vitro studies have suggested that it may interfere with this process (1, 17, 32, 42, 44, 48). The ability of capsule-specific antibodies to reduce carriage, however, suggests that the capsule is expressed during nasopharyngeal colonization (18, 34, 36). Moreover, the varied ability of encapsulated strains of different serotypes to colonize the nasopharynx suggests some influence of the capsule on colonization (2, 14, 49, 50, 56). Here, we describe the requirement for capsule during nasopharyngeal colonization of mice and the effects of reduced capsule levels on both colonization and systemic infections.

MATERIALS AND METHODS

Bacteria and growth conditions. The strains and plasmids used in these studies are shown in Table 1. *S. pneumoniae* strains were grown in Todd-Hewitt broth (Difco) supplemented with 0.5% yeast extract (Difco) (THY) at 37°C or on blood agar base no. 2 (Difco) supplemented with 3% sheep erythrocytes (Colorado Serum Company, Denver, Colo.) at 37°C in 5% CO₂. Erythromycin was used at 0.3 µg/ml, and streptomycin was used at 100 µg/ml. For opacity determinations, strains were grown at 37°C in a candle jar on tryptic soy medium (Difco) plates containing 1% Bacto Agar (Difco) onto which 100 µl of catalase (5,000 U) was spread (50, 51).

DNA techniques. *S. pneumoniae* was transformed by induction with competence factor CSP-1 (28), as previously described (26). Plasmid DNA used for transformations was isolated using the alkaline lysis method (6). Chromosomal DNA was purified using Qiagen Genomic Tips (Qiagen, Inc., Valencia, Calif.). Insertion-duplication and deletion mutations were generated as previously described (26, 27, 58). The presence of the mutations was confirmed by Southern blot analyses for insertions and by PCR for deletions, as previously described (27). Using the digoxigenin labeling and detection system (Boehringer Mannheim, Indianapolis, Ind.), probes were generated by incorporation of digoxigenin-11-dUTP-labeled nucleotides during PCR amplification with *Taq* polymerase (Sigma). The deletion plasmid pCV182 was generated by cloning restriction fragments flanking the desired deletion into pJY4164 (Table 1). The

* Corresponding author. Mailing address: Department of Microbiology, BBRB 661/12, 845 19th St. South, Birmingham, AL 35294. Phone: (205) 934-9531. Fax: (205) 975-6715. E-mail: jyother@uab.edu.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Derivation and properties ^a	Reference(s)
Strains		
A66	Type 3 parent strain; Cps ⁺	4
AM161	pJD330 × A66, insertion-duplication mutation downstream of <i>cps3S</i> ; Em ^r Cps ⁺	This study
AM179	pCV182 × A66, Δ <i>cps3UMtnpA1</i> ; Cps ⁺	This study
AM188	A66 <i>cps3D5</i> ^(V283E) ; Cps ^r (20%)	This study
AM199	pJD369 × A66, insertion-duplication mutation downstream of <i>cps3D</i> (polar on <i>cps3S</i>); Em ^r Cps ⁻	This study
AM201	AM188 with repaired <i>cps3D5</i> mutation; Cps ⁺	This study
AM1000	D39 Δ <i>cps2ABCDETFGHI</i> ; Cps ⁻	This study
D39	Type 2 parent strain; Cps ⁺	4
JD600	WU2; Str ^r Cps ⁺	20
JD614	JD600 <i>cps3D3</i> (mutation located between bp 885 and 1802); Str ^r Cps ^r (<6%)	19, 20
JD692	JD600 <i>cps3D4</i> (mutation located between bp 1027 and 1802); Str ^r Cps ^r (<0.6%)	19, 20
JD770	pJD330 × WU2, insertion-duplication mutation downstream of <i>cps3S</i> ; Em ^r Cps ⁺	19, 20
JD908	pJD369 × WU2, insertion-duplication mutation downstream of <i>cps3D</i> (polar on <i>cps3S</i>); Em ^r Cps ⁻	19, 20
JY1060	WU2 <i>pgm-1</i> ^(K381T) ; Cps ^r (20%) PGM ^r	26
R36A	D39 serially passaged 36 times in rabbit anti-type 2 serum, Δ <i>cps2ABCDETFGHI</i> ; Cps ⁻	4, 31
WU2	Type 3 parent strain; Cps ⁺	10
Plasmids		
pJY4163 and pJY4164	Cloning vectors for insertion-duplication mutagenesis; lack origin of replication for <i>S. pneumoniae</i> ; multiple cloning site in opposite orientations in pJY4163 and pJY4164; Em ^r	58
pCV182	pJY4164::3.14-kb [<i>Sau3AI-PstI</i> (<i>'cps3DSU'</i>), <i>SacI-HindIII</i> (<i>'tnpA-plpA'</i>)];	27; this study
pJD330	pJY4163::2.38-kb <i>Sau3AI</i> (<i>'cps3DSU'</i>); Em ^r	20
pJD369	pJY4164::0.575-kb <i>PvuII-RsaI</i> (<i>'cps3DS'</i>); Em ^r	20
pJD380	pJY4164::0.356-kb <i>Sau3AI-SspI</i> fragment from pJD330 (<i>'cps3D'</i>); Em ^r	20

^a Em^r, erythromycin resistant; Str^r, streptomycin resistant; Cps⁺, encapsulated; Cps⁻, nonencapsulated; Cps^r (percentage of parental level), reduced capsule production; PGM^r, decreased PGM activity.

cps3UMtnpA deletion mutant AM179 was obtained following transformation of pCV182 into *S. pneumoniae* without selection for the Em^r marker. Deletion mutants occurring as the result of allelic replacement were identified by PCR of pooled isolates (27) and confirmed by Southern blot analysis. The AM188 mutation was repaired by transformation with pJD380 without selection for integration of the plasmid (20). Repair of the mutation in isolates exhibiting the mucoid parental colony morphology was confirmed by sequence analysis. Sequencing was performed by the University of Alabama at Birmingham Automated Sequencing Facility. Primers used for sequence analysis were Cps3D-4 (5'-ATCGCGTGTATAGAGTTTCTTG-3'; bp 2170 to 2193), Cps3D-8 (5'-GCTTTGGTTACGGAGGGTATTGC-3'; bp 1781 to 1803), and Cps3D-11 (5'-GTATACATAAAATTTATTTCCCC-3'; bp 2212 to 2234). Base pair numbers correspond to the published *cps3D* sequence (19). The 7.5-kb deletion mutation in AM1000 was made by transformation of D39 with a PCR fragment containing the deletion generated from R36A chromosomal DNA using primers C2ups-3 (5'-GTCTATCTCTATCAACTTTTC-3'; bp 1019 to 1039) and Cps2I-1 (5'-CTGAATTTGTCCCAATAAC-3'; bp 11885 to 11897). Base pair numbers correspond to the published sequence of the type 2 capsule locus (31). All primers were obtained from Oligos Etc. (Wilsonville, Oreg.).

Capsule and teichoic acid measurements. Quellung (agglutination) reactions were performed using capsule-type-specific antisera (Statens Serum Institut, Copenhagen, Denmark). For capsule measurements, cultures were grown to a density of 3×10^8 CFU/ml in THY at 37°C. Cell-associated capsule production by type 3 strains was quantified using the Stains-All assay for detecting acidic polysaccharides (41) or by an indirect enzyme-linked immunosorbent assay (ELISA). For ELISAs, duplicate samples of heat-killed cells (65°C, 30 min) were serially diluted on a microtiter plate and incubated overnight at 4°C. Capsule was detected using the anti-type 3 capsule monoclonal antibody 16.3 (10) at a 1:10,000 dilution, as described previously (27). The amount of capsule was calculated from a standard curve generated using purified type 3 polysaccharide (American Type Culture Collection). The lower limit of detection of purified type 3 capsule was 0.01 μ g/ml. Capsule production by type 2 strains was quantified in an indirect ELISA as described above, using type 2 polysaccharide-specific antiserum (Statens Serum Institut) at a 1:20,000 dilution. C-polysaccharide (teichoic acid) was measured in an indirect ELISA as described above, using polyclonal anti-C-polysaccharide antisera (Statens Serum Institut) at a 1:10,000 dilution.

Colonization and mouse virulence. Female 8- to 12-week-old BALB/cByJ and CBA/N (CBA/CaHN-*Bu^xid*) mice were used (Jackson Laboratories, Bar Harbor,

Maine). Intranasal (i.n.) inoculations were performed as previously described (56), with minor changes. Briefly, a 10- to 100-ml bacterial culture was grown to a density of 3×10^8 CFU/ml in THY at 37°C. The culture was centrifuged at $12,000 \times g$ for 20 min at 4°C. The pellet was suspended in lactated Ringer's solution, and 10 μ l of suspension containing 10^7 to 10^9 CFU of bacteria was introduced into the nares of mice. Seven days postinoculation, mice were sacrificed by asphyxiation in a CO₂ chamber. The trachea was cut at the top of the larynx, and 200 μ l of lactated Ringer's solution was washed through the nares with a tuberculin syringe fitted with Intramedic Polyethylene PE20 tubing (Becton Dickinson, Sparks, Md.). A second wash with 2 ml of Ringer's solution did not yield significant numbers of additional bacteria. Serial dilutions of the nasal washes were plated on blood agar plates containing either no antibiotic, 1 μ g of gentamicin/ml, 1 μ g of gentamicin/ml and 10 μ g of optochin (ethylhydrocupreine-HCl)/ml, or 0.3 μ g of erythromycin/ml (where applicable). From these plates, the numbers of pneumococci present in the nasal washes were determined; pneumococci are capable of growth on gentamicin but not optochin. All bacteria isolated in these washes were encapsulated, as evidenced by colony morphology and confirmed by the Quellung reaction, which produces a smaller zone of reactivity with less encapsulated strains. PCR using primers specific for pneumococcal surface protein A (*pspA*) was used to further confirm pneumococcal identity. The primer pairs used were PspA-18 (5'-CCCAAGCTTAATA TAAGTATAG-3') and PspA-11 (5'-AGGCGCGTCGA/CTCATTAACCTGCTT TCTT-3') for bp 76 to 1083 or PspA-16 (5'-GTCTCAGCCTACTGTTGT-3') and PspA-11 for bp 196 to 1083. Base pair numbers correspond to the published *pspA* sequence (58). In some cases, lungs of i.n.-infected mice were homogenized and plated on blood agar plates to test for the presence of bacteria.

For infections by either the intravenous (i.v.) or intraperitoneal (i.p.) routes, bacterial cultures were grown as described above and diluted in lactated Ringer's solution to the desired concentration, and 0.2 ml was injected. Mice were observed for 21 days. Hearts of dead mice were homogenized and plated on blood agar plates to assess phenotypes of bacteria, which did not differ from those of the infecting strains.

Statistics. The numbers of bacteria recovered from nasal washes were compared using an unpaired, two-tailed Student *t* test. The numbers of mice colonized and the numbers of mice that survived infection were each compared to those for the parent strain using a two-tailed Fisher exact test. Median times to death were compared using an unpaired, two-tailed Mann-Whitney test. Capsule production by mutant strains and that by parent strains were compared using an unpaired, two-tailed Student *t* test.

TABLE 2. Nasopharyngeal colonization of BALB/cByJ mice by A66 derivatives^a

Strain (phenotype)	Capsule production (\pm SEM) ^b	Colonization ^c	Nasal wash ^d log CFU (\pm SEM)
A66 (type 3 parent)	100 (11.2)	8/15	3.68 (0.25)
AM161 (insertion control)	82 (1.8)	3/4	3.32 (0.34)
AM179 (Δ Cps3UM)	87.7 (4.5)	4/5	3.41 (0.30)
AM188 (Cps3D ^r)	19.2 (3.3)	17/25	3.19 (0.14)
AM199 (Cps3S ⁻)	0	0/10 ^e	—
A66 + AM188	Cps ⁺ /Cps ^r	3/10 (both) ^f	4.32 (0.20) (A66) 3.85 (0.29) (AM188)
AM161 + AM199	Cps ⁺ /Cps ⁻	2/10 (A66) 0/10 ^e (both) 6/10 (AM161)	402 (0.20) — 3.60 (0.31)

^a Mice were inoculated i.n. with 10^7 bacteria.

^b Cell-associated capsule levels were determined using the Strains-All assay. Values are the percentages of capsule produced \pm standard errors of the means relative to the parent A66 and represent the means of at least four independent determinations. A66 produced 41.5 ± 4.7 μ g of capsule/ml of culture/unit of optical density at 600 nm. Capsule production by AM188 was significantly different from that by A66 ($P = 0.0004$). AM199 was nonreactive with antibody to type 3 polysaccharide, and its Strains-All value was subtracted as background from the other samples. Cps⁺, parental levels of capsule; Cps^r, reduced capsule production; Cps⁻, nonencapsulated.

^c Values are the numbers of mice colonized per total numbers of mice. Except as noted, none were significantly different from values for A66.

^d Values are the log CFU per milliliter (\pm standard errors of the means) recovered from nasal washes. The total volume of the wash was \sim 200 μ l, and the minimum detectable number of bacteria was 10 CFU per wash, equivalent to 50 CFU/ml. Em dashes indicate that no bacteria were recovered from any of the mice. Where bacteria were recovered, none of the values were significantly different from those for A66. The colony morphologies of bacteria recovered from the nasal washes were identical to those of the bacteria in the inocula.

^e Significantly different from value for A66 ($P < 0.05$).

^f The presence of both strains was distinguished by colony morphology.

RESULTS

Capsule production. The strains used in these studies produce various amounts of capsule as a result of specific mutations in either the capsule locus or other genes known to affect capsule synthesis. Their construction and properties are described in detail in Materials and Methods and in Table 1. The amount of capsule produced by each of the strains is shown in Tables 2 and 3. Synthesis of the type 3 capsule requires a UDP-Glc dehydrogenase (UDP-Glc \rightarrow UDP-glucuronic acid [GlcUA]) and the type 3 polysaccharide synthase (UDP-Glc + UDP-GlcUA \rightarrow [Glc-GlcUA]_n). Both enzymes are encoded by genes (*cps3D* and *cps3S*, respectively) in the type 3 capsule

locus, which is transcribed as a single operon (*cps3DSUM-tnpA-plpA*) (13, 19, 20). Mutants AM188, JD614, and JD692 contain different point mutations in *cps3D* that result in decreased capsule synthesis. Repair of each of these mutations results in restoration of parental levels of capsule (references 19 and 20 and this study). Mutants AM199 and JD908 contain insertions that result in loss of Cps3S and capsule expression. Resolution of the insertions restores parental levels of capsule production (reference 27 and data not shown). AM161 and JD770 contain insertions downstream of *cps3S* that do not affect capsule production (27). These strains were used as controls to ascertain any polar effects of the insertions or of the antibiotic marker (erythromycin) contained on the insertion plasmid. Cps3U, a Glc-1-P uridylyltransferase

TABLE 3. Nasopharyngeal colonization of BALB/cByJ mice by WU2 and D39 derivatives^a

Strain	Capsule production ^b (%)	Colonization ^c	Nasal wash ^d log CFU/ml (\pm SEM)
WU2 (type 3 parent)	100	9/13 ^e	3.49 (0.18)
JD770 (insertion control)	100	12/20	3.43 (0.25)
JY1060 (PGM ^r)	20	6/10	3.47 (0.41)
JD614 (Cps3D ^r)	<6	0/10 ^{**f}	—
JD692 (Cps3D ^r)	<0.6	0/10 ^{**f}	—
JD908 (Cps3S ⁻)	0	0/10 ^{**f}	—
JD770 + JD908	Cps ⁺ /Cps ⁻	0/5 ^{*f} (both) ^g	—
D39 (type 2 parent)	100	2/5 (JD770)	3.60 (0.30)
AM1000(Δ Cps2ABCDETFGH)	0	7/10	3.12 (0.28)
		0/10 ^{**f}	—

^a Mice were inoculated i.n. with 1×10^7 (WU2 and derivatives), 4×10^8 (D39), or 9×10^8 (AM1000) CFU.

^b Values are relative to the appropriate parent strain. WU2 produces approximately 46 μ g of cell-associated capsule/ml of culture/unit of optical density at 600 nm (26, 27). Capsule production by its derivatives has been reported previously (20, 26, 27), except for JD614 and JD692, which were reactive with the type 3-specific monoclonal antibody in indirect ELISAs, as indicated. JD908 was nonreactive with antibody to type 3 polysaccharide. Repair or resolution of each of the mutations restores parental levels of capsule and, for JY1060 and JD908, has been shown previously to restore parental virulence following i.p. and i.v. infections (26, 27). The polyclonal antiserum used to detect type 2 capsule reacts with other surface antigens. In the ELISA, the optical density at 405 nm of AM1000 was reduced sixfold from that of D39 and was not significantly different from that of the nonencapsulated type 2 strain R36A (data not shown). Cps⁺, parental levels of capsule; Cps⁻, nonencapsulated.

^c Values are the numbers of mice colonized per total numbers of mice. Except as noted, none were significantly different from the values for the parent.

^d See footnote *d* in Table 2. Where bacteria were recovered, none of the values were significantly different from the values for the parent.

^e Values include colonization by JD600, the WU2 (Str^r) derivative used to construct JD614 and JD692.

^f Significantly different from the value for the parent (*, $P < 0.05$; **, $P < 0.005$).

^g The presence of both strains was distinguished by colony morphology.

(Glc-1-P → UDP-Glc) and Cps3M, a phosphoglucomutase (PGM) homologue, are encoded within the type 3 locus but are not essential for capsule production or systemic infections with type 3 strains (13, 19, 26, 27). Strain AM179 contains a deletion of *cps3UMtnpA* and is not altered in capsule production (Table 2). The PGM activity necessary for conversion of Glc-6-P to Glc-1-P and synthesis of the type 3 capsule is encoded by *pgm*, which is found in strains of all capsule types and is unlinked to the capsule locus (26). PGM is involved in a number of other cellular pathways, including those leading to the teichoic acids. JY1060 contains a point mutation in *pgm* that results in decreased capsule synthesis (Table 3) and either modestly reduced virulence (in CBA/N mice) or avirulence (in BALB/cByJ mice) following systemic infection (27). Repair of the JY1060 point mutation restores parental levels of capsule and virulence (26, 27). AM1000, the capsule-negative derivative of the type 2 strain D39, was constructed by deleting the first nine genes of the D39 capsule locus (Table 3). This is the same deletion contained in R36A, which is the spontaneous, highly passaged, nonencapsulated derivative of D39 (4, 31). For all of the mutant and parent strains, no differences were detected in the teichoic acid levels, and all appeared opaque.

Nasopharyngeal colonization. The abilities of strains producing reduced levels of capsule to colonize were assessed using BALB/cByJ mice in a previously described model (56). In this model, nonanesthetized adult mice are inoculated i.n. with low-volume inocula. Under these conditions, stable colonization occurs for at least 2 weeks. The pneumococci do not cause invasive disease, and the bacteria recovered from the nasopharyngeal cavity are not the result of infections in the blood or lungs (56). In the present studies, we also did not recover bacteria from the lungs of infected mice.

As shown in Table 2, the parent type 3 strain A66 and its isogenic derivatives AM161 and AM179, which produce parental amounts of capsule, colonized at equivalent levels. These results further indicate the lack of a role for Cps3U and Cps3M (deleted in AM179) and show that insertions downstream of *cps3S* (AM161) have no effect on colonization. Reduction of capsule expression to ~20% of the parental level had no effect on the ability to colonize, as seen with AM188 (Table 2). Further, mice coinoculated with equal numbers of A66 and AM188 bacteria were colonized with similar levels of the two strains, indicating that the fully encapsulated strain did not have a competitive advantage. In contrast to these results, the nonencapsulated mutant AM199 was unable to colonize and, when coinoculated with AM161, did not impede colonization by the encapsulated strain (Table 2).

Using a second type 3 strain (WU2) and its derivatives producing various amounts of capsule, we confirmed the results obtained in the A66 background. As with A66, the nonencapsulated WU2 derivative (JD908) was unable to colonize, and the derivative producing ~20% of parental levels of capsule (JY1060) colonized as well as did WU2 (Table 3). However, mutants that produced <6% of the parental levels of capsule (JD614 and JD692) were unable to colonize the nasopharynx. A requirement for capsule production during colonization was also demonstrated using the capsule type 2 strain D39 and its nonencapsulated derivative AM1000 (Table 3).

Ability of the capsule-reduced mutant AM188 to cause invasive disease. Previous studies showed that, upon i.n. inocu-

TABLE 4. Virulence of A66 derivatives following i.n. inoculation of CBA/N mice^a

Strain	Capsule phenotype ^b	No. dead/ no. total	No. colonized/ no. alive ^c	Nasal wash ^d log CFU/ml (± SEM)
A66	Cps ⁺	8/9	0/1	—
AM188	Cps ^r	3/10** ^e	3/7	3.16 (0.23)
AM199	Cps ⁻	0/5*** ^e	0/5	—

^a Mice were inoculated i.n. with 1×10^7 (A66), 3×10^7 (AM188), or 5×10^6 (AM199) CFU.

^b Cps⁺, parental levels of capsule; Cps^r, reduced capsule production; Cps⁻, nonencapsulated.

^c Values are the number of mice colonized/number of mice surviving the infection.

^d See footnote d in Table 2.

^e Significantly different from value for A66 (*, $P < 0.05$; **, $P = 0.003$).

lation of CBA/N mice, A66 invades the host, resulting in systemic infection and death (56). These mice express the X-linked immunodeficient (XID) phenotype and respond poorly to polysaccharide antigens, including the *S. pneumoniae* capsule and the phosphocholine component of the cell wall (45, 53). Due in part to this deficiency, they are highly susceptible to pneumococcal infections (9, 10). Following i.n. inoculation of CBA/N mice, AM188 showed reduced lethality compared to that of the parent A66, and the capsule-negative AM199 was completely avirulent (Table 4). The times to death for mice that succumbed to infection with AM188 were not different from those observed with mice infected with A66 (data not shown). Mice that did not die following infection with AM188 were colonized at a frequency similar to that observed for BALB/cByJ mice (Tables 2 and 4).

Because the decreased ability of AM188 to cause lethal invasive infections following i.n. inoculation could result from a reduced ability to survive in the bloodstream, we next examined its virulence in systemic infections. When CBA/N mice were infected with AM188 via either the i.v. or the i.p. route, an extended time to death was observed compared to that with A66 (Table 5). In contrast to the i.n. infection result, however, the overall lethality of AM188 was not significantly different from the parent via these routes. In immunologically normal (BALB/cByJ) mice, AM188 was significantly reduced in its ability to kill following i.v. infection (Table 5). Following i.p. infection, however, it was attenuated only in the time required to kill. The attenuation in virulence of AM188 was due to the reduced capsule production, as repair of the *cps3D* mutation restored both capsule and virulence to parental levels (AM201 [Table 5]).

DISCUSSION

A number of surface components have been shown to be important in colonization by *S. pneumoniae*. Using an established model of nasopharyngeal colonization in mice, we have shown that capsule also has an important role in this step of the infectious process. The requirement for capsule may reflect its ability to prevent clearance of the organism by innate defenses. Alternatively, or in addition, the capsule itself may be an adhesin. Either possibility is consistent with previous observations demonstrating variability among strains of different capsular serotypes in the ability to colonize, activate complement, and bind antibody to surface antigens (2, 14, 21, 29, 49, 50, 56;

TABLE 5. Virulence of A66 derivatives following i.v. and i.p. infection of XID (CBA/N) and normal (BALB/cByJ) mice

Mouse strain	Route	Infecting strain	Dose (log CFU)	No. dead /no. total ^a	Median time to death (h)
CBA/N ^b	i.v.	A66	1.9	9/10	36
		AM188	2.0	8/10	88 ^{*c}
		AM199	6.7	0/5 ^{**c}	
	i.p.	A66	1.9	10/10	35
		AM188	2.0	8/10	51 ^{*c}
		AM199	2.7	0/5 ^{***c}	
BALB/c ByJ ^d	i.v.	A66	7.0	7/7	19
		AM188	6.9	1/7 ^{**c}	
		AM199	6.9	0/7 ^{***c}	
		AM201 ^e	6.8	5/5	13
	i.p.	A66	2.9	7/7	35
		AM188	2.2	5/7	49 ^{**c}
		AM199	2.2	0/7 ^{***c}	
		AM201	2.6	5/5	38

^a The colony morphologies of bacteria recovered postmortem were identical to those of the bacteria in the inocula.

^b The i.v. and i.p. LD₅₀s of A66 in XID mice are <10 CFU (references 9 and 57 and unpublished data).

^c Significantly different from the value for A66 (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0007$).

^d The LD₅₀s of A66 in BALB/cByJ mice are 10⁵ CFU (i.v.) and <10² CFU (i.p.) (D. E. Briles, personal communication and unpublished data).

^e Capsule production by AM201 was equivalent to that of its parent A66 (41.9 ± 4.4 and 41.5 ± 4.7 μg of capsule/ml of culture/unit of optical density at 600 nm, respectively).

M. R. Abeyta and J. Yother, unpublished data). The fact that strains producing substantially reduced levels of capsule colonize as effectively as do their parent strains indicates either that the amount of capsule produced in vitro does not reflect that produced in vivo or that there is no advantage in producing excessive amounts of capsule. Indeed, we anticipate that a reduction in the amount of capsule may be a necessary step for efficient colonization, as it would allow greater exposure of surface molecules important in adherence. A reduced amount of capsule has previously been shown to correlate with enhanced colonization by transparent-phase variants, compared to opaque-phase variants, which produce elevated levels of capsule and have enhanced virulence in systemic infections (32, 50). In in vitro studies, fully encapsulated strains show reduced adherence and invasion compared to those of nonencapsulated isolates, suggesting that the capsule is an impediment to these processes (1, 17, 32, 42, 44, 48). Reduced expression of capsule by defined mutants clearly results in greater access of antibodies and complement to the pneumococcal surface (27). In the nasopharyngeal environment, appropriate signals may result in a reduction of capsule expression and an increase in expression of factors necessary for adherence. Indeed, we would expect that transmission of *S. pneumoniae* between carriers involves strains that are already reduced in capsule production and optimized for carriage. Hence, our type 3 mutants may remain efficient colonizers because a reduction in capsule levels is the normal scenario. Little is known about the mechanisms involved in the regulation of capsule expression. If, however, the parental mechanisms of regulation remain operative in our type 3 mutants with reduced levels of capsule production, the amount of capsule necessary for effi-

cient colonization may well be less than that produced by AM188 and JY1060.

Previous studies demonstrated a correlation between i.p. infection virulence in mice and the level of capsule produced in vitro (32, 35). MacLeod and Krauss observed significant differences in the 50% lethal doses (LD₅₀s) of type 3 strains in which the amount of capsule varied by 2.5-fold (35). In contrast, we observed only modest differences in the times to death for A66 and AM188, which differ by fivefold in type 3 capsule production. Unlike the spontaneous isolates of MacLeod and Krauss and the phase variants of Kim and Weiser (32), our strains are the result of defined mutations. AM188 is altered only in capsule production. In contrast, JY1060 produces approximately the same amount of capsule as does AM188 but is completely avirulent in BALB/cByJ mice via the i.p. route (27). Here, the reduction in capsule is due to a mutation in *pgm*, and other cellular pathways are likely also affected. In addition, suppressor mutations that enhance the virulence of JY1060 do not always result in increased levels of capsule (27). Thus, reductions in capsule alone appear to have minimal effects on i.p. virulence. This is not the case for i.v. virulence, however, as both AM188 and JY1060 were significantly reduced in the ability to cause lethal infections in BALB/cByJ mice via this route (reference 27 and this study). This result may suggest that the peritoneal cavity is a relatively safe environment where bacteria do not immediately encounter the bloodstream and where a focus of infection can be maintained. Bacteria escaping this environment may then express alternative or enhanced levels of virulence factors that promote their survival in the bloodstream.

In the immunodeficient CBA/N mouse, type 3 strains inoculated i.n. cause lethal invasive infections. In contrast, AM188 was reduced in this ability but could efficiently colonize in this mouse strain. Because AM188 was highly virulent when administered i.v. in CBA/N mice, the i.n. result suggests that the capsule is either important in invasion or sufficiently reduced in quantity in AM188 by the time invasion occurs that it no longer prevents phagocytosis.

Studies with *Staphylococcus aureus* and *Streptococcus pyogenes* have also demonstrated an important role for capsule in nasopharyngeal colonization (30, 33, 52). In *S. pyogenes*, binding of the hyaluronic acid capsule to CD44 mediates adherence in vitro and in the nasopharyngeal cavity (40). The specific role or roles of the pneumococcal capsule in colonization are now under investigation, as are the effects of factors known or suspected to influence colonization and subsequent infections. In particular, the effects of capsular serotype, administration of anesthetics or antibiotics prior to i.n. inoculation, or preceding viral infection may alter the range of isolates that can become established in the nasopharyngeal cavity (23, 33, 38, 39, 47). Clearly, high levels of capsule expression are not required or even advantageous in all in vivo environments. An important step toward understanding the infectious process will thus be the identification of mechanisms involved in regulating capsule expression.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI28457 and T32 GM08111 from the National Institutes of Health and by the

University of Alabama at Birmingham Comprehensive Minority Faculty and Student Development Program.

We thank Christy Ventura for constructing pCV182, Alexis Brooks-Walter for demonstrating the i.n. infection method, and Thomas Forsee for helpful insights regarding animal experiments.

REFERENCES

- Adamou, J. E., T. M. Wizemann, P. Barren, and S. Langermann. 1998. Adherence of *Streptococcus pneumoniae* to human bronchial epithelial cells (BEAS-2B). *Infect. Immun.* **66**:820–822.
- Austrian, R. 1986. Some aspects of the pneumococcal carrier state. *J. Antimicrob. Chemother.* **18**:35–45.
- Avery, O. T., and R. Dubos. 1931. The protective action of a specific enzyme against type III pneumococcus infection in mice. *J. Exp. Med.* **54**:73–89.
- Avery, O. T., C. M. MacLeod, and M. McCarty. 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. *J. Exp. Med.* **79**:137–158.
- Berry, A. M., and J. C. Paton. 1996. Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*. *Infect. Immun.* **64**:5255–5262.
- Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**:1513–1523.
- Briles, D. E., E. Ades, J. C. Paton, J. S. Sampson, G. M. Carlone, R. C. Huebner, A. Virolainen, E. Swiatlo, and S. K. Hollingshead. 2000. Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. *Infect. Immun.* **68**:796–800.
- Briles, D. E., S. Hollingshead, A. Brooks-Walter, G. S. Nabors, L. Ferguson, M. Scholling, S. Gracenstein, P. Braun, J. King, and A. Swift. 2000. The potential to use PspA and other pneumococcal proteins to elicit protection against pneumococcal infection. *Vaccine* **18**:1707–1711.
- Briles, D. E., J. Horowitz, L. S. McDaniel, W. H. Benjamin, Jr., J. L. Claffin, C. L. Booker, G. Scott, and C. Forman. 1986. Genetic control of the susceptibility to pneumococcal infection. *Curr. Top. Microbiol. Immunol.* **124**:103–120.
- Briles, D. E., M. Nahm, K. Schorner, J. Davie, P. Baker, J. Kearney, and R. Barletta. 1981. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 *Streptococcus pneumoniae*. *J. Exp. Med.* **153**:694–705.
- Brooks-Walter, A., D. E. Briles, and S. K. Hollingshead. 1999. The *pspC* gene of *Streptococcus pneumoniae* encodes a polymorphic protein, PspC, which elicits cross-reactive antibodies to PspA and provides immunity to pneumococcal bacteremia. *Infect. Immun.* **67**:6533–6542.
- Brown, E. J. 1985. Interaction of gram-positive organisms with complement. *Curr. Top. Microbiol. Immunol.* **121**:159–187.
- Caimano, M. J., G. G. Hardy, and J. Yother. 1998. Capsule genetics in *Streptococcus pneumoniae* and a possible role for transposition in the generation of the type 3 locus. *Microb. Drug Resist.* **4**:11–23.
- Converse, G. M., III, and H. C. Dillon, Jr. 1977. Epidemiological studies of *Streptococcus pneumoniae* in infants: methods of isolating pneumococci. *J. Clin. Microbiol.* **5**:293–296.
- Cundell, D. R., B. J. Pearce, J. Sandros, A. M. Naughton, and H. R. Masure. 1995. Peptide permeases from *Streptococcus pneumoniae* affect adherence to eucaryotic cells. *Infect. Immun.* **63**:2493–2498.
- Cundell, D. R., and E. Tuomanen. 1994. Receptor specificity of adherence of *Streptococcus pneumoniae* to human type-II pneumocytes and vascular endothelial cells *in vitro*. *Microb. Pathog.* **17**:361–374.
- Cundell, D. R., J. N. Weiser, J. Shen, A. Young, and E. I. Tuomanen. 1995. Relationship between colonial morphology and adherence of *Streptococcus pneumoniae*. *Infect. Immun.* **63**:757–761.
- Dagan, R., M. Muallem, R. Melamed, O. Leroy, and P. Yagupsky. 1997. Reduction of pneumococcal nasopharyngeal carriage in early infancy after immunization with trivalent pneumococcal vaccines conjugated to either tetanus toxoid or diphtheria toxoid. *Pediatr. Infect. Dis. J.* **16**:1060–1064.
- Dillard, J. P., M. W. Vandersea, and J. Yother. 1995. Characterization of the cassette containing genes for type 3 capsular polysaccharide biosynthesis in *Streptococcus pneumoniae*. *J. Exp. Med.* **181**:973–983.
- Dillard, J. P., and J. Yother. 1994. Genetic and molecular characterization of capsular polysaccharide biosynthesis in *Streptococcus pneumoniae* type 3. *Mol. Microbiol.* **12**:959–972.
- Fine, D. P. 1975. Pneumococcal type-associated variability in alternate complement pathway activation. *Infect. Immun.* **12**:772–778.
- Geelen, S., C. Bhattacharyya, and E. Tuomanen. 1993. The cell wall mediates pneumococcal attachment to and cytopathology in human endothelial cells. *Infect. Immun.* **61**:1538–1543.
- Giebink, G. S., I. K. Berzins, S. C. Marker, and G. Schiffman. 1980. Experimental otitis media after nasal inoculation of *Streptococcus pneumoniae* and influenza A virus in chinchillas. *Infect. Immun.* **30**:445–450.
- Gosink, K. K., E. R. Mann, C. Guglielmo, E. I. Tuomanen, and H. R. Masure. 2000. Role of novel choline binding proteins in virulence of *Streptococcus pneumoniae*. *Infect. Immun.* **68**:5690–5695.
- Hammerschmidt, S. S., R. Talay, P. Brandtzaeg, and G. A. Chhatwal. 1997. SpsA, a novel pneumococcal surface protein with specific binding to secretory immunoglobulin A and secretory component. *Mol. Microbiol.* **25**:1113–1124.
- Hardy, G. G., M. J. Caimano, and J. Yother. 2000. Capsule biosynthesis and basic metabolism in *Streptococcus pneumoniae* are linked through the cellular phosphoglucomutase. *J. Bacteriol.* **182**:1854–1863.
- Hardy, G. G., A. D. Magee, C. L. Ventura, M. J. Caimano, and J. Yother. 2001. Essential role for cellular phosphoglucomutase in virulence of type 3 *Streptococcus pneumoniae*. *Infect. Immun.* **69**:2309–2317.
- Havarstein, L. S., G. Coomaraswamy, and D. A. Morrison. 1995. An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. *Proc. Natl. Acad. Sci. USA* **92**:11140–11144.
- Hostetter, M. K. 1986. Serotypic variation among virulent pneumococci in deposition and degradation of covalently bound C3b: implication for phagocytosis and antibody production. *J. Infect. Dis.* **153**:682–693.
- Husmann, L. K., D. Yung, S. K. Hollingshead, and J. R. Scott. 1997. Role of putative virulence factors of *Streptococcus pyogenes* in mouse models of long-term throat colonization and pneumonia. *Infect. Immun.* **65**:1422–1430.
- Iannelli, F., B. J. Pearce, and G. Pozzi. 1999. The type 2 capsule locus of *Streptococcus pneumoniae*. *J. Bacteriol.* **181**:2652–2654.
- Kim, J. O., and J. N. Weiser. 1998. Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae*. *J. Infect. Dis.* **177**:368–377.
- Kiser, K. B., J. M. Cantey-Kiser, and J. C. Lee. 1999. Development and characterization of a *Staphylococcus aureus* nasal colonization model in mice. *Infect. Immun.* **67**:5001–5006.
- MacLeod, C. M., R. G. Hodges, M. Heidelberger, and W. G. Bernhard. 1945. Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides. *J. Exp. Med.* **82**:445–464.
- MacLeod, C. M., and M. R. Krauss. 1950. Relation of virulence of pneumococcal strains for mice to the quantity of capsular polysaccharide formed *in vitro*. *J. Exp. Med.* **92**:1–9.
- Malley, R., A. M. Stack, M. L. Ferretti, C. M. Thompson, and R. A. Saladino. 1998. Anticapsular polysaccharide antibodies and nasopharyngeal colonization with *Streptococcus pneumoniae* in infant rats. *J. Infect. Dis.* **178**:878–882.
- Rosenow, C., P. Ryan, J. N. Weiser, S. Johnson, P. Fontan, A. Ortuist, and H. R. Masure. 1997. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. *Mol. Microbiol.* **25**:819–829.
- Rubins, J. B., and D. Charboneau. 2000. Effect of anesthetics on pathogenesis of experimentally induced murine pneumococcal pneumonia. *Comp. Med.* **50**:292–295.
- Rubins, J. B., A. H. Paddock, D. Charboneau, A. M. Berry, J. C. Paton, and E. N. Janoff. 1998. Pneumolysin in pneumococcal adherence and colonization. *Microb. Pathog.* **25**:337–342.
- Schrager, H. M., S. Alberti, C. Cywes, G. J. Dougherty, and M. R. Wessels. 1998. Hyaluronic acid capsule modulates M protein-mediated adherence and acts as a ligand for attachment of group A *Streptococcus* to CD44 on human keratinocytes. *J. Clin. Investig.* **101**:1708–1716.
- Schrager, H. M., J. G. Rheinwald, and M. R. Wessels. 1996. Hyaluronic acid capsule and the role of streptococcal entry into keratinocytes in invasive skin infection. *J. Clin. Investig.* **98**:1954–1958.
- Selinger, D. S., and W. P. Reed. 1979. Pneumococcal adherence to human epithelial cells. *Infect. Immun.* **23**:545–548.
- Spellerberg, B., D. R. Cundell, J. Sandron, B. J. Pearce, I. Idanpaan-Heikkila, C. Rosenow, and H. R. Masure. 1996. Pyruvate oxidase as a determinant of virulence in *Streptococcus pneumoniae*. *Mol. Microbiol.* **19**:803–813.
- Talbot, U. M., A. W. Paton, and J. C. Paton. 1996. Uptake of *Streptococcus pneumoniae* by respiratory epithelial cells. *Infect. Immun.* **64**:3772–3777.
- Thomas, J. D., P. Sideras, C. I. Smith, I. Vorechovsky, V. Chapman, and W. E. Paul. 1993. Co-localization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. *Science* **261**:355–358.
- Tong, H. H., L. E. Blue, M. A. James, and T. F. DeMaria. 2000. Evaluation of the virulence of a *Streptococcus pneumoniae* neuraminidase-deficient mutant in nasopharyngeal colonization and development of otitis media in the chinchilla model. *Infect. Immun.* **68**:921–924.
- Tong, H. H., J. N. Weiser, M. A. James, and T. F. DeMaria. 2001. Effect of influenza A virus infection on nasopharyngeal colonization and otitis media induced by transparent or opaque phenotype variants of *Streptococcus pneumoniae* in the chinchilla model. *Infect. Immun.* **69**:602–606.
- van der Flier, M., N. Chhun, T. M. Wizemann, J. Min, J. B. McCarthy, and E. I. Tuomanen. 1995. Adherence of *Streptococcus pneumoniae* to immobilized fibronectin. *Infect. Immun.* **63**:4317–4322.
- Webster, L. T., and A. D. Clow. 1933. Intranasal virulence of pneumococci for mice. *J. Exp. Med.* **58**:465–483.
- Weiser, J. N., R. Austrian, P. K. Sreenivasan, and H. R. Masure. 1994. Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. *Infect. Immun.* **62**:2582–2589.

51. **Weiser, J. N., Z. Markiewicz, E. I. Toumanen, and J. H. Wani.** 1996. Relationship between phase variation in colony morphology, intrastrain variation in cell wall physiology, and nasopharyngeal colonization by *Streptococcus pneumoniae*. *Infect. Immun.* **64**:2240–2245.
52. **Wessels, M. R., and M. S. Bronze.** 1994. Critical role of the group A streptococcal capsule in pharyngeal colonization and infection in mice. *Proc. Natl. Acad. Sci. USA* **91**:12238–12242.
53. **Wicker, L. S., and I. Scher.** 1986. X-linked immune deficiency (*xid*) of CBA/N mice. *Curr. Top. Microbiol. Immunol.* **124**:87–101.
54. **Winkelstein, J.** 1984. Complement and the host's defense against the pneumococcus. *Crit. Rev. Microbiol.* **11**:187–208.
55. **Wu, H., M. H. Nahm, Y. Guo, M. W. Russell, and D. E. Briles.** 1997. Intranasal immunization of mice with PspA (pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with *Streptococcus pneumoniae*. *J. Infect. Dis.* **175**:839–846.
56. **Wu, H., A. Virolainen, B. Mathews, J. King, M. W. Russell, and D. E. Briles.** 1997. Establishment of a *Streptococcus pneumoniae* nasopharyngeal colonization model in adult mice. *Microb. Pathog.* **23**:127–137.
57. **Yother, J., C. Forman, B. M. Gray, and D. E. Briles.** 1982. Protection of mice from infection with *Streptococcus pneumoniae* by anti-phosphocholine antibody. *Infect. Immun.* **36**:184–188.
58. **Yother, J., G. L. Handsome, and D. E. Briles.** 1992. Truncated forms of PspA that are secreted from *Streptococcus pneumoniae* and their use in functional studies and cloning of the *pspA* gene. *J. Bacteriol.* **174**:610–618.

Editor: E. I. Tuomanen