

## Construction of Otherwise Isogenic Serotype 6B, 7F, 14, and 19F Capsular Variants of *Streptococcus pneumoniae* Strain TIGR4

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The polysaccharide capsule is the primary virulence factor in *Streptococcus pneumoniae*. There are at least 90 serotypes of *S. pneumoniae*, identified based on the immunogenicity of different capsular sugars. The aim of this study was to construct pneumococcal strains that are isogenic except for capsular type. Serotype 4 strain TIGR4 was rendered unencapsulated by recombinational replacement of the capsular polysaccharide synthesis (*cps*) locus with the bicistronic Janus cassette (C. K. Sung, J. P. Claverys, and D. A. Morrison, *Appl. Environ. Microbiol.* 67:5190–5196, 2001). In subsequent transformation with chromosomal DNA, the cassette was replaced by the *cps* locus derived from a strain of a different serotype, either 6B, 7F, 14, or 19F. To minimize the risk of uncontrolled recombinational replacements in loci other than *cps*, the TIGR*cps*::Janus strain was “backcross” transformed three times with chromosomal DNA of subsequently constructed capsular type transformants. Capsular serotypes were confirmed in all new capsule variants by the Quellung reaction. Restriction fragment length polymorphism (RFLP) analysis of the *cps* locus confirmed the integrity of the *cps* region transformed into the TIGR strain, and RFLP of the flanking regions confirmed their identities with the corresponding regions of the recipient. Transformants had *in vitro* growth rates greater than or equal to that of TIGR4. All four strains were able to colonize C57BL/6 mice (female, 6 weeks old) for at least 7 days when mice were intranasally inoculated with  $6 \times 10^6$  to  $8 \times 10^6$  CFU. The constructed capsular variants of TIGR4 are suitable for use in studies on the role of *S. pneumoniae* capsular polysaccharide in immunity, colonization, and pathogenesis.

A number of defined and putative virulence factors in *Streptococcus pneumoniae* have been described. Of these, the polysaccharide capsule, which protects pneumococcal cells against phagocytosis during infection, was the first to be identified and is the best studied. There are at least 90 different capsular types in *S. pneumoniae*, classified according to polysaccharide structure and immunogenicity (13), with considerable variation across serotypes in the tendency to colonize the human nasopharynx and to cause invasive disease (3, 12). Among the internationally distributed clones of *S. pneumoniae*, there are several for which variants with different capsular serotypes are known (21). It is believed that these variants arose through “capsular switching,” mediated by recombinational replacements within the capsular biosynthesis (*cps*) operon and its flanking regions (4–6), following natural transformation by DNA from other strains. The survival of such capsular variants is likely driven by the selective pressure within *S. pneumoniae*'s natural environment, the human upper respiratory tract.

In order to perform studies on the role of capsule in immunity, colonization, and pathogenesis of *S. pneumoniae*, we constructed strains that were isogenic except for the capsular type. A laboratory procedure for capsule replacement has been known since Griffith's landmark 1928 paper on transformation (11). However, as Griffith's procedure involves selection of capsular transformants in a mouse, there is a potential for introducing additional, uncontrolled changes in the strains selected. More recent attempts to obtain capsular transformants *in vitro* have involved replacements of a part of the *cps* operon,

and the type and number of possible constructs are limited to those of high homology between donor and recipient *cps* loci (22). The main limitations for controlled *in vitro* capsule transformation in *S. pneumoniae* are the large size of the DNA that had to be replaced and the lack of a suitable selective marker to identify successful transformants. The exception was serotype 3, distinguished by perhaps the smallest of all *cps* operons and by a colonial morphology distinctive enough to be used alone as a selective marker (1, 6, 15).

In an attempt to circumvent these limitations, we made use of the bicistronic, positively and negatively selectable Janus cassette recently constructed for *S. pneumoniae* by Sung et al. (27). We used Janus to replace the *cps* operon in encapsulated *S. pneumoniae*, leading to the unencapsulated, rough phenotype. In subsequent transformations, Janus was replaced by either the *cps6B*, *cps7F*, *cps14*, or *cps19F* capsule operon, leading to the construction of new encapsulated strains. The *S. pneumoniae* *cps* operon is a region of great variability, but it is flanked, in all pneumococcal isolates analyzed so far, by regions of high homology (9, 15). We selected these regions as targets for recombinational crossover in a controlled capsule transformation. The virulent TIGR4 *S. pneumoniae* strain was chosen as the host for the capsule replacements, since its complete genome sequence has been published (29) and susceptibility to a wide array of antimicrobial agents makes construction of strains with selectable resistant phenotypes relatively easy.

### MATERIALS AND METHODS

**Bacterial strains, DNA, and growth conditions.** *S. pneumoniae* strains used in this study are described in Table 1. Serotype 19F strain GA71 was kindly donated by Brian Spratt (6). Strains NY00216, GA03294, and GA03306 (all serotype 6B), GA02224 (serotype 7F), and GA02190 (serotype 14) were all clinical isolates collected by the Centers for Disease Control and Prevention's Active Bacterial

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TABLE 1. Bacterial strains and primers used in this study

Strain or primer	Description	Source or reference
<i>S. pneumoniae</i> strains		
R6		14
CP1296	R6 derivative, <i>cbp3::kan-rpsL</i> <sup>+</sup> ; Km <sup>r</sup> Sm <sup>s</sup>	27
R6S	R6 but Sm <sup>r</sup> by selection of mutants	This study
R6J	R6S but <i>cps::kan-rpsL</i> <sup>+</sup>	This study
TIGR4		29
TIGR4S	TIGR4 but Sm <sup>r</sup> by selection of mutants	This study
TIGR4J	TIGR4S but <i>cps::kan-rpsL</i> <sup>+</sup> , by transformation with TTM01-04 PCR product from R6J; Km <sup>r</sup> Sm <sup>s</sup>	This study
NY00216	Donor of type 6B capsule	26
GA02224	Donor of type 7F capsule	26
GA02190	Donor of type 14 capsule	26
GA71	Donor of type 19F capsule	6
TIGR4:6B <sup>1</sup>	TIGR4J derivative <i>kan-rpsL</i> <sup>+</sup> :: <i>cps6B</i> , by transformation with NY00216 chromosomal DNA; Km <sup>s</sup> Sm <sup>r</sup>	This study
TIGR4:6B <sup>2</sup>	TIGR4J derivative <i>kan-rpsL</i> <sup>+</sup> :: <i>cps6B</i> , by transformation with TIGR4:6B <sup>1</sup> chromosomal DNA; Km <sup>s</sup> Sm <sup>r</sup> ; 1× backcross transformant	This study
TIGR4:6B <sup>3</sup>	TIGR4J derivative <i>kan-rpsL</i> <sup>+</sup> :: <i>cps6B</i> , by transformation with TIGR4:6B <sup>2</sup> chromosomal DNA; Km <sup>s</sup> Sm <sup>r</sup> ; 2× backcross transformant	This study
TIGR4:6B <sup>4</sup>	TIGR4J derivative <i>kan-rpsL</i> <sup>+</sup> :: <i>cps6B</i> , by transformation with TIGR4:6B <sup>3</sup> chromosomal DNA; Km <sup>s</sup> Sm <sup>r</sup> ; 3× backcross transformant	This study
TIGR4:7F <sup>4</sup>	TIGR4J derivative <i>kan-rpsL</i> <sup>+</sup> :: <i>cps7F</i> , serotype 7F 3× backcross capsule transformant of TIGR4 constructed as TIGR4:6B <sup>4</sup> ; Km <sup>s</sup> Sm <sup>r</sup>	This study
TIGR4:14 <sup>4</sup>	TIGR4J derivative <i>kan-rpsL</i> <sup>+</sup> :: <i>cps14</i> , serotype 14 3× backcross capsule transformant of TIGR4 constructed as TIGR4:6B <sup>4</sup> ; Km <sup>s</sup> Sm <sup>r</sup>	This study
TIGR4:19F <sup>4</sup>	TIGR4J derivative <i>kan-rpsL</i> <sup>+</sup> :: <i>cps19F</i> , serotype 19F 3× backcross capsule transformant of TIGR4 constructed as TIGR4:6B <sup>4</sup> ; Km <sup>s</sup> Sm <sup>r</sup>	This study
GA03294	6B serotype penicillin-resistant isolate, donor of <i>pbp2x</i> for 607S transformation	26
GA03306	6B serotype cefotaxime-susceptible isolate	26
607S	GA03306 but Sm <sup>r</sup> by selection of mutants	This study
621	607S but cefotaxime resistant by transformation with <i>pbp2xf-pbp2xr2</i> PCR product of GA03306	This study
622	621 but Sm <sup>s</sup> Km <sup>r</sup> by transformation with DAM313-DAM316 PCR product of CP1296	This study
Primers		
DAM406	TCTATGCCTATTCCAGAGGAAATGGAT	D. A. Morrison
DAM351	CTAGGGCCCCCTTTCCTTATGCTTTTGGAC	27
DAM313	AGCTTTCCTCGTGGTGTAGAACAAC	27
DAM316	CTCTCAAGGTCGCCAGCTATG	27
TTM01	ATCATGACCTCCCTCGTATTGT within <i>dexB</i> in R6; 932–953	This study
TTM02	CGCGGATCCTTAATAGTGGGAATTTG; positions 7–26 correspond to positions 131–150 downstream of the <i>dexB</i> 3' end in R6	This study
TTM03	TTTGGGCCCGCCGCGTCAGTCAGTTTT; positions 10–28 correspond to positions 618–600 upstream of <i>aliA</i> 5' end in R6	This study
TTM04	AGCTTTGACTGCCGCTATTCTTC; within <i>aliA</i> in R6; 331–354	This study
TTM05	AAGGTGAGGAGATTGGGATGA; within <i>dexB</i> in R6; 1049–1069	This study
TTM06	TGTCGCAGCCTTAGCAGTTG; within <i>aliA</i> in R6; 155–174	This study
1430	TGTCCAATGAAGAGCAAGACTTGACAGTAG	15
TTM07	CTACTGTCAAGTCTTGCTCTTCATTGGACA; sequence reverse to 1430	This study
1402	CAATAATGCTACGCCCGCAAGGGCAAGT	15
TTM08	ACTTGCCCTTGCGGGCGTGACATTATTG; sequence reverse to 1402	This study
TTM09	CTAAAACAGGGGAAATTCTGGCAACAACGC; within <i>pbp2x</i> in TIGR4; 887–916	This study
TTM10	AATCGCGAAACGTCCCAGCCGTGGAAACTC; within <i>pbp1a</i> in TIGR4; 1280–1309	This study
pbp2xf	CGTGGGACTATTTATGACCGAAATGGAG	10
pbp2xr2	GGCGAATTCAGCACTGATGGAAATAA	10

Core Surveillance and kindly provided by Richard Facklam and Chris van Beneden (26). R6 (14) and TIGR4 (29) were obtained from the American Type Culture Collection. The genomic DNA of CP1296 was kindly donated by Don Morrison (27). All strains were maintained in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) or on blood agar base no. 2 medium (Becton Dickinson, Sparks, Md.) supplemented with 5% defibrinated sheep blood (Colorado Serum Company, Denver, Colo.) (SBA). Antibiotic concentrations in SBA selective media were 0.5 mg/liter for cefotaxime, 2.5 mg/liter for gentamicin, and 200 mg/liter for kanamycin and streptomycin, except for the SBA used to select streptomycin-resistant (Sm<sup>r</sup>) mutants, which contained 300 mg of streptomycin per liter. DNA templates were prepared with a genomic DNA system (Qiagen, Valencia, Calif.). Growth media and culture methods for genetic transformations have been described by Pozzi et al. (23). The transformation of TIGR4 and its derivatives was induced by competence-stimulating peptide variant 2, while vari-

ant 1 was used for strains R6 and GA03306 and their derivatives. The concentrations of donor DNA used in transformation steps were 1 µg/ml for chromosomal DNA and 100 ng/ml for PCR products.

**Serotyping of isolates.** Polysaccharide capsule types were determined based on the Quellung test with factor-specific typing sera (Statens Serum Institute, Copenhagen, Denmark) (13, 20).

**PCR amplification.** All products with sizes below 5 kb were amplified using 50 ng of genomic DNA in a final volume of 50 µl of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM MgCl<sub>2</sub>, and 0.25 mM deoxynucleoside triphosphates with 0.3 pmol of each primer, 4.5 U of *Taq* DNA polymerase (Invitrogen, Chicago, Ill.), and 0.5 U of cloned *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.). The PCR consisted of 30 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min per kb of estimated product length at 72°C, followed by a final incubation at 72°C for 5 min. All products with sizes of 8.6 kb and above were amplified using a TripleMaster PCR

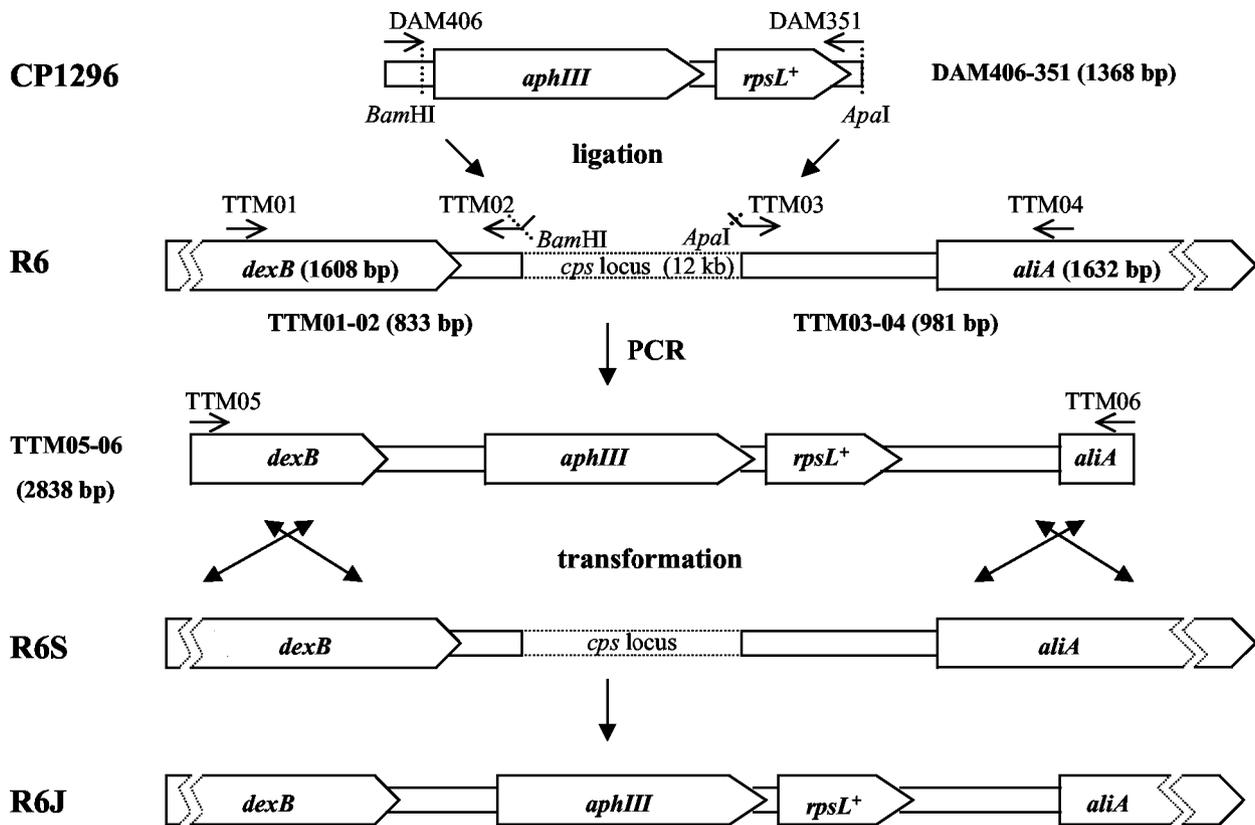


FIG. 1. Construction of the Janus cassette in the R6 *S. pneumoniae cps* locus. Pentagons represent *dexB* and *aliA* genes of R6 and *aphIII* ( $Km^r$ ) and *rpsL<sup>+</sup>* ( $Sm^s$ ) genes of Janus (size of the open reading frame is in parentheses). Oligonucleotides used to amplify cassette elements are in capital letters (see Table 1 for detailed description of the primers and annealing sites). Restriction sites of enzymes used to create sticky ends prior to the ligation step are shown by dotted lines. The two top lines show elements of CP1296 and R6 used to construct the *dexB*-Janus-*aliA* cassette by the two-step ligation procedure. The ligation product was used as a template to amplify the TTM05-06 fragment (third line) used to transform R6S (fourth line) to create R6J (fifth line) with selection for resistance to kanamycin. Sizes of the PCR products generated in this work are given in parentheses next to the product names.

system (Eppendorf, Hamburg, Germany) using cycling parameters recommended by the manufacturer.

**Construction of the *dexB-aphIII-rpsL<sup>+</sup>-aliA* cassette.** To construct a Janus-type cassette with kanamycin resistance and streptomycin sensitivity alleles embedded within DNA sequences corresponding to the genes that flank the *cps* locus, a 1,368-bp fragment containing *aphIII-rpsL<sup>+</sup>* was first amplified from chromosomal DNA of CP1296 using the primer pair DAM 406 and DAM 351. Two DNA fragments, TTM01-02 (833 bp), which contains a *Bam*HI 3' terminus, and TTM03-04 (981 bp), which contains an *Apa*I 5' terminus (both flanking the *cps* cassette), were prepared by PCR using chromosomal DNA of R6 as a template (Fig. 1). The *aphIII-rpsL<sup>+</sup>* and TTM01-02 PCR products were digested with *Bam*HI restriction nuclease, ligated by using T4 DNA ligase (New England BioLabs, Beverly, Mass.), and purified with a QIAquick PCR purification kit (Qiagen). The product of the ligation and the TTM03-04 fragment were digested with *Apa*I, ligated, and used as the template for a PCR with primers TTM05 and TTM06. Products of the PCR were run on a 1% agarose gel, and the fragment of 2,838 bp, corresponding to the expected size of *kan-rpsL<sup>+</sup>* cassette with TTM01-02 and TTM03-04 flanking regions, was purified by using a gel extraction kit (Qiagen) and used to transform R6S with selection for resistance to kanamycin ( $Km^r$ ) to create R6J. The structure of the insertion in R6J was verified by restriction fragment length polymorphism (RFLP) analysis of the TTM01-04 PCR product using *Bam*HI and *Apa*I (25).

**Construction of *cps* transformants.** A 3,195-bp PCR product containing the *kan-rpsL<sup>+</sup>* cassette amplified from chromosomal DNA of R6J with primer pair TTM01 and TTM04 was used to transform TIGR4S (encapsulated, type 4) with selection for  $Km^r$  to make the unencapsulated (rough) TIGR4J strain, which was used as a recipient in all subsequent capsule transformation steps. DNA template from each of the following strains was used to transform TIGR4J with selection for  $Sm^s$ : type 6B strain NY00216, type 7F strain GA02224, type 14 strain

GA02190 and type 19F strain GA71 (all listed in Table 1) to create encapsulated strains. For each transformation, up to five smooth colonies were picked from overnight growth on streptomycin-supplemented SBA and cultured separately. The effect of each capsule on the TIGR strain growth rate was evaluated by measuring optical density of growing broth cultures as described below. Once an isolate was identified with the appropriate capsule type and with no reduction in growth rate compared to that of TIGR4S, DNA was purified from that isolate and used to retransform TIGR4J into an encapsulated strain again ("backcross" transformation). The procedure was repeated three times to construct triple-backcross ( $3\times$  backcross) transformants (16, 24). The structure of the *cps* locus and surrounding regions was confirmed in all four  $3\times$  backcross transformants by RFLP analysis of the following PCR products: 1430-1402 (*dexB-cps-aliA* locus; size in the TIGR4 strain, 22,332 bp) digested with *Rsa*I, TTM07-09 (*pbp2x-dexB* flanking *cps* upstream region, 9,076 bp), and TTM08-10 (*aliA-pbp1a* flanking *cps* downstream region, 8,618 bp), both digested with *Tsp*509I. Restriction patterns of the  $3\times$  backcross transformants were compared with those of TIGR4 and capsule donor strains after electrophoresis in 2% OmniPur PCR plus agarose (Merck, Darmstadt, Germany).

**Effect of capsule transformation on the growth rate.** The cost of capsule replacement was evaluated by comparison of capsule transformants and TIGR4S strain growth curves. Cells harvested from overnight cultures on SBA were suspended in THY to an optical density (OD) at 600 nm of 0.1, diluted 50 times, and incubated at 37°C. The OD of broth cultures was measured every 30 min until early stationary phase was reached. For  $3\times$  backcross transformants, measurements were taken for three independent cultures and compared with those for TIGR4S. Based on OD values observed during the exponential phase of growth, the slopes were calculated as an increase in the natural logarithm per hour. The null hypothesis that the slopes of intertype transformants were identical to that of TIGR4S was tested by linear regression analysis, and *t* tests were

TABLE 2. Capsule type identification results for selected strains described in this study

Strain	Result of Quellung test for serotype <sup>a</sup> :				
	4	6B	7F	14	19F
TIGR4	Pos	Neg	Neg	Neg	Neg
TIGR4J	Neg	Neg	Neg	Neg	Neg
NY00216	Neg	Pos	—	—	—
GA02224	Neg	—	Pos	—	—
GA02190	Neg	—	—	Pos	—
GA71	Neg	—	—	—	Pos
TIGR4:6B <sup>4</sup>	Neg	Pos	—	—	—
TIGR4:7F <sup>4</sup>	Neg	—	Pos	—	—
TIGR4:14 <sup>4</sup>	Neg	—	—	Pos	—
TIGR4:19F <sup>4</sup>	Neg	—	—	—	Pos

<sup>a</sup> Pos, positive identification of particular serotype; Neg, negative result of the Quellung test; —, not determined.

performed for pairwise comparisons of each new capsule variant against TIGR4S using Prism 3.0 software (GraphPad Software, San Diego, Calif.). A *P* value of less than 0.05 was considered significant.

**Assessment of the ratio of cotransformation.** To evaluate the probability that a single transformant takes up two unlinked marker loci, we transformed TIGR4S with chromosomal DNA from a laboratory-constructed strain, 622, carrying the original Janus cassette of Sung et al. (27) inserted into *cbp3*, along with a cefotaxime-resistant (Ctx<sup>r</sup>) allele of the gene encoding penicillin binding protein 2x, allowing selection for unlinked markers encoding Ctx<sup>r</sup> and Km<sup>r</sup>. After transformation, the numbers of TIGR4 colonies resistant to cefotaxime, to kanamycin, and to both antibiotics simultaneously were counted and compared.

**Mouse colonization.** To evaluate the ability of newly constructed strains to colonize mice, four groups of four C57BL/6 experimental mice (female, 6 weeks old; Jackson Laboratory) were inoculated intranasally (19, 30) with  $6 \times 10^6$  to  $8 \times 10^6$  CFU of the 3× backcross strains, TIGR4 and TIGR4J. After 7 days, nasal washes were carried out to determine the presence and the intensity (as defined by CFU per nasal wash) of pneumococcal colonization in the animals' upper respiratory tracts.

## RESULTS

**Isolation of encapsulated transformants.** In the course of the study, all successful capsule transformants were selected based on the smooth morphology of colonies that were selected for resistance to streptomycin but susceptibility to kanamycin. The frequency of Janus replacement by an intact (functional) *cps* locus was calculated only for serotype 19F transformants. Colonies of serotype 19F cells (true for both GA71 and TIGR4:19F<sup>4</sup>) have very distinctive smooth morphology and were relatively easy to identify among rough colonies of unencapsulated cells. The frequency of *cps19F* locus transformation was estimated to be  $5.4 \times 10^{-6}$  based on the number of smooth Sm<sup>r</sup> Km<sup>s</sup> colonies growing out of TIGR4J culture transformed with chromosomal DNA of GA71. The genuine capsule transformants constituted 6.3% of all Sm<sup>r</sup> colonies isolated following transformation. The remainder may have been some combination of recombinants that did not result in transformation at the capsule locus, resistant mutants, or spontaneous revertants (as observed previously with the use of Janus [27]).

The presence of capsule was confirmed in intertype transformants of the TIGR4 strain by the Quellung reaction, and the capsule type was congruent with that expected from the donor DNA. Only the original parent strain TIGR4S was positive for serotype 4. Results with backcrosses were similar to those obtained in primary transformations (Table 2).

**Confirmation of intact capsule locus transformation.** When *RsaI* restriction fragment patterns of the *dexB-cps-aliA* (1402-1430 PCR product) were compared among capsule donors, TIGR4S, and the 3× backcross transformants, the transformants in all cases matched the donor and not the recipient fingerprints (Fig. 2, center). The largest 1430-1402 PCR product was observed for TIGR4 (22.3 kb according to the published genome sequence [29]), followed by 21.5-kb products for TIGR4:7F<sup>4</sup> and the *cps7F* donor and 18-, 16.8-, and 16.6-kb products for serotype 14, 19F, and 6B transformants and capsule donors, respectively. *RsaI* generated similar number of fragments for all five fingerprints (49 bands with sizes from 6 to 2,197 bp in TIGR4 [29] with no single band common to all five RFLP patterns [Fig. 2]).

**Confirmation that flanking regions of transformants matched the recipient strains.** RFLP analysis of regions flanking the *cps* locus also revealed polymorphisms among analyzed strains; however, *Tsp509I* fingerprints of the *pbp2x-dexB* and *aliA-pbp1a* loci (TTM09-07 and TTM08-10 PCR products, respectively) showed homology between donors and recipients not observed for the *dexB-cps-aliA* locus. The size of the TTM09-07 PCR product in TIGR4 was ~9 kb, as expected, and similar fragments were generated for NY00216 and all four 3× backcross capsule transformants. Products for GA02190, GA02224, and GA71 had sizes of 9.4, 9.7, and 2.8 kb, respectively. The *Tsp509I* patterns of TTM09-07 products generated for TIGR4 and all 3× backcross transformants except serotype 14 were identical. The RFLP fingerprints indicate that the capsule replacement crossover point in TIGR4:14<sup>4</sup> was located between enzyme restriction sites in TIGR4 at positions 700 bp upstream and 140 bp downstream of the *dexB* 5' end. Analysis of the same region in four consecutive serotype 14 transformants of TIGR4 revealed an identical fingerprint for all of them. It indicated that the evidence of the crossover point of the first capsule replacement was retained through all three backcross transformation events. All consecutive crossover points were located at or upstream of the site of the first one. RFLP analysis of the smallest of the TTM09-07 PCR products, the 2.8-kb fragment amplified for GA71, revealed its homology with the 3' end of the TTM09-07 PCR product of TIGR4.

High homology was also observed among fingerprints generated for the second flanking region, the *aliA-pbp1a* locus. The size of the TTM08-10 PCR product in TIGR4 was ~8.6 kb. Similarly sized fragments were generated for all other isolates except NY00216, for which an ~10-kb product was amplified, and GA71, which yielded no product. The TTM08-10 PCR product fingerprints for all 3× backcross transformants were identical to that of TIGR4 and different from those of all capsule donor strains.

**Calculation of backcross frequencies needed to ensure isogenic backgrounds.** Since chromosomal DNA was used to construct the original transformants, there was a risk that capsular transformants had acquired other, unlinked genetic material from the donor strains. To calculate the probability that a transformant with a change at a selected locus (the *cps* region) also picked up a gene at an unlinked region, we transformed our recipient, TIGR4S, with chromosomal DNA from a strain carrying two unlinked markers, Km<sup>r</sup> and Ctx<sup>r</sup>. The *pbp2x* gene is located 345 kb downstream of the *cbp3* gene in TIGR4

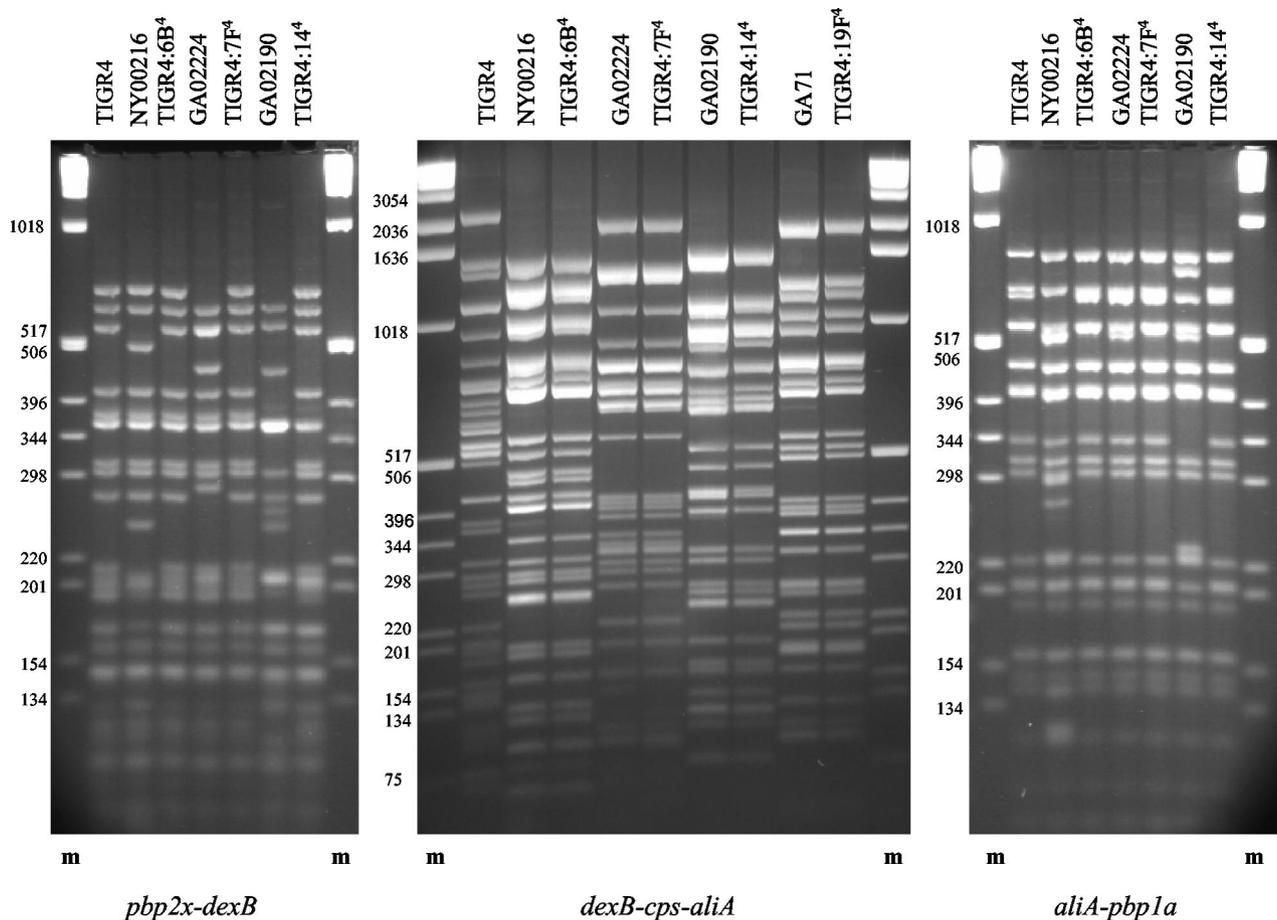


FIG. 2. RFLP patterns of the *cps* locus and its flanking regions in TIGR4, capsule transformants, and capsule donors. Lanes correspond to the *Tsp509I* fingerprints of *pbp2x-dexB* and *pbp1a-aliA* and the *RsaI* fingerprint of *dexB-cps-aliA* loci of indicated isolates. The order of fingerprints is repeated in all three modules of the picture with the exception of serotype 19F isolates GA71 and TIGR4:19F<sup>4</sup>, for which fingerprints of the *dexB-cps-aliA* locus are the only ones presented. RFLP patterns of the *cps* locus of isolates of the same serotype were identical for all four sets of strains (capsule donor and transformant), and the same was observed for flanking regions of the host strain (TIGR4) and all four capsule transformants. The exception was the TIGR4:14<sup>4</sup> fingerprint of the *pbp2x-dexB* fragment, for which a pattern different from that for TIGR4 was observed (see Results). Lanes m, 1-kb DNA ladder molecular size marker (sizes of fragments, in base pairs, are on the left of each gel).

genome (approximately one-sixth of the genome away) (29). Under identical transformation conditions, with approximately  $10^7$  CFU of the recipient cells, we obtained 77 transformants with Ctx<sup>r</sup> and 303 transformants that were Km<sup>r</sup>. With five times as many recipient cells, we obtained zero transformants that were simultaneously Km<sup>r</sup> and Ctx<sup>r</sup>. This provided an approximate 95% confidence interval for the ratio of single to double transformants of 0 to 0.008. We reasoned that among our capsular transformants, the probability that any given locus was cotransformed was therefore less than 1/100, and that if each backcross independently reduced the probability of cotransformation by a factor of at least 100, then the 3× backcross transformants should have a probability of less than  $10^{-8}$  of carrying any given gene from the donors apart from the capsule region.

**Effect of capsule transformation on the growth rate.** When the growth rates for TIGR4S and 3× backcross capsule transformants were compared, no significant differences were observed between TIGR4S and the serotype 7F and 19F transformants. However, growth rates were significantly higher for

serotype 6B and 14 transformants of the TIGR strain (*P* values of 0.002 and 0.04, respectively), indicating that capsular transformants actually grew faster than the recipient strain (Table 3).

**Ability of TIGR4 capsule transformants to colonize mice.** The presence of TIGR4 capsular transformants was demon-

TABLE 3. Results of linear regression analysis of TIGR4S and its intertype transformants' growth rate slopes

Strain	Slope ( $\Delta N/\Delta t$ ) <sup>a</sup>	95% confidence interval	<i>P</i> value versus TIGR4S
TIGR4S	0.56	0.51–0.61	
TIGR4:6B <sup>4</sup>	0.69	0.65–0.72	0.002
TIGR4:7F <sup>4</sup>	0.53	0.47–0.6	0.45
TIGR4:14 <sup>4</sup>	0.63	0.59–0.67	0.04
TIGR4:19F <sup>4</sup>	0.57	0.48–0.67	0.83

<sup>a</sup>  $\Delta N/\Delta t$ , exponential growth rate slope value calculated as change in lnOD at 600 nm per hour, where *N* is the OD and *t* is the time.

strated 7 days after mouse inoculation in nasal washes of all four animals colonized with serotype 7F or 14 (medians, 3,130 and 807 CFU/nasal wash, respectively) and in three of four mice colonized with serotype 6B or 19F strains (medians, 1,146 and 966 CFU/nasal wash, respectively). None of the four mice was colonized with TIGR4, and only one was colonized with TIGR4J.

## DISCUSSION

Transformational recombination is a known mechanism by which *S. pneumoniae* obtains genetic variation that permits adaptation to changes in the environment. Natural transformation of the capsule locus leading to changes in polysaccharide structure and immunogenicity is a phenomenon that has been observed in pneumococci (5) and in other pathogens colonizing the human respiratory tract, i.e., *Neisseria meningitidis* and *Haemophilus influenzae* (17, 28). Intertype transformation allows these microorganisms to avoid opsonization and neutralization by antibodies against serotypes to which the host was previously exposed. Uncovering the mechanisms that drive capsule switching is crucial for a better understanding of the interactions between the host and the pathogen and among different strains of pathogens competing for the host (18). Such interactions are difficult to trace in the naturally diverse population of *S. pneumoniae* due to the complexity of antigenic patterns observed. Isolates classified as closely related (members of the same epidemic clone) can be very different in their antigenic profiles (2, 4–6) and in their abilities to colonize (K. Trzcinski, C. M. Thompson, R. Malley, and M. Lipsitch, Abstr. 3rd Int. Symp. Pneumococci Pneumococcal Dis., p. 71, 2002) and probably to cause invasive disease.

For this reason, the availability of laboratory-constructed strains that are identical except for one feature (a single antigenic component) will be critical for testing these interactions in artificial or natural host models (16, 24). In the case of transformation into unrelated capsular types, such isolates can be constructed only by recombinational replacements of large fragments of chromosomal DNA, some over 20 kb in length, and crossover sites should be located within regions of high homology among pneumococcal strains. Suitable targets for recombinational crossovers seem to be *cpsA*, *cpsB*, *cpsC*, and *cpsD*, which are present at the 5' end of *cps* operons, *cpsN* and *cpsO*, which are present at the 3' end in most serotypes, regions of homology within intergenic regions surrounding the *cps* locus, and *dexB* and *aliA* genes, which flank the *cps* operon in all pneumococcal strains analyzed (15). To construct the *dexB*-Janus-*aliA* cassette suitable for capsule transformation, we chose the most external regions to eliminate any possible interactions between the *cps* operon of the recipient and newly introduced capsular genes. RFLP analysis of the fragments flanking the *dexB*-*cps*-*aliA* region in intertype transformants indicates that recombinational crossover points were located downstream of *pbp2x* and upstream of *pbp1a* but outside the *cps* operon in all of them.

Prior to this study, the construction of isogenic strains with variant polysaccharide capsule types was limited to intertype transformants of highly homologous *cps* regions (e.g., serogroup 19 variants) in which replacement of a smaller *cps* operon fragment was sufficient for serotype change (22). The

exceptions were type 3 transformants, in which the presence of a relatively small operon of three genes determines the expression of that particular capsular type in any genetic background. However, such encapsulated-to-encapsulated transformations can result in isolates expressing both capsule donor and recipient serotypes (binary types) (7, 8). Our strategy for the replacement of the entire operon overcomes these limitations.

A major obstacle in capsule transformation was the lack of selective markers to allow the identification of extremely low-frequency transformants. We solved this problem by using the Janus cassette (27). Using this cassette, we constructed four different capsular variants of the TIGR4 strain. As has been documented for serotype 3 and 37 *S. pneumoniae*, genes located outside the *cps* locus can determine the expression of particular capsular types in some strains (9). Quellung test results confirmed the donor serotype in all constructed transformants, indicating that the generated replacements included all genes necessary for functional polysaccharide capsule biosynthesis in a TIGR4 background.

Natural capsule transformation through recombinational replacements of large fragments of chromosomal DNA in *S. pneumoniae* (covering not only the whole *cps* operon but also large fragments of its flanking regions) has been described by Coffey et al. in the studies on Spain<sup>23F</sup>-1 and Spain<sup>9V</sup>-3 pandemic clone capsular variants (4, 6). Our results show that similar transformation through recombinational replacement of DNA fragments of 20 kb and above (~1% of the whole bacterial genome) can also be successfully conducted in vitro.

There were no detectable costs of capsule transformation in TIGR4S in terms of growth in vitro. Growth rates of serotype 7F and 19F transformants were not significantly different from that of TIGR4, whereas enhanced rates were observed for serotype 6B and 14 transformants. Since the *cps6B* and *cps14* loci of the capsule donors used in this study were smaller than *cps4* of TIGR4 and there was a significant increase in growth rate for these two transformants, one can speculate that the size of the *cps* operon might reflect lesser complexity and energetic cost of capsular polysaccharide biosynthesis in TIGR4 serotypes 6B and 14 than in serotype 4.

All four newly constructed capsule variants were able to colonize the upper respiratory tracts of laboratory mice, proving the strains to be suitable for use in a mouse colonization model. It is not surprising that the rough strain TIGR4J was not able to colonize, as there have been no reports to our knowledge of successful colonization of mice with rough strains and rough strain carriage is rare in humans. Previous experiments in two laboratories (19, 30; M. Lipsitch, unpublished data) have shown that different strains of *S. pneumoniae* differ widely in their ability to colonize mice at a standard dose, so the fact that the original TIGR4 strain did not colonize, while noteworthy, is consistent with prior experience.

The 3× backcross transformation steps ensured a very low likelihood of recombinational replacements involving exogenous (non-TIGR4) DNA taking place at any other locus than *dexB*-*cps*-*aliA*. Thus, the risk of changes in genotype and phenotype due to unwanted transformations was minimized. Overall, the unrelated and non-cross-reactive capsular variants of the virulent TIGR4 isolate described in this paper seem to be suitable for further studies on the role of *S. pneumoniae* capsule polysaccharide in immunity, colonization, and pathogen-

esis. The strategy described in this paper is in principle suitable for construction of any desired capsular variant of any *S. pneumoniae* strain.

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#### REFERENCES

- Avery, O. T., C. M. MacLeod, and M. McCarty. 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. *J. Exp. Med.* **79**:137–158.
- Beall, B., G. Gherardi, R. R. Facklam, and S. K. Hollingshead. 2000. Pneumococcal *pspA* sequence types of prevalent multiresistant pneumococcal strains in the United States and of internationally disseminated clones. *J. Clin. Microbiol.* **38**:3663–3669.
- Brueggemann, A. B., D. T. Griffiths, E. Meats, T. Peto, D. W. Crook, and B. G. Spratt. 2003. Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. *J. Infect. Dis.* **187**:1424–1432.
- Coffey, T. J., M. Daniels, M. C. Enright, and B. G. Spratt. 1999. Serotype 14 variants of the Spanish penicillin-resistant serotype 9V clone of *Streptococcus pneumoniae* arose by large recombinational replacements of the *cps-pbp1A* region. *Microbiology* **145**:2023–2031.
- Coffey, T. J., C. G. Dowson, M. Daniels, J. Zhou, C. Martin, B. G. Spratt, and J. M. Musser. 1991. Horizontal transfer of multiple penicillin-binding protein genes, and capsular biosynthetic genes, in natural populations of *Streptococcus pneumoniae*. *Mol. Microbiol.* **5**:2255–2260.
- Coffey, T. J., M. C. Enright, M. Daniels, J. K. Morona, R. Morona, W. Hryniewicz, J. C. Paton, and B. G. Spratt. 1998. Recombinational exchanges at the capsular polysaccharide biosynthetic locus lead to frequent serotype exchanges among natural isolates of *Streptococcus pneumoniae*. *Mol. Microbiol.* **27**:73–83.
- Dillard, J. P., M. W. Wandersea, 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.
- Garcia, E., C. Arrecubieta, R. Munoz, M. Mollerach, and R. Lopez. 1997. A functional analysis of the *Streptococcus pneumoniae* genes involved in the synthesis of type 1 and type 3 capsular polysaccharides. *Microb. Drug Resist.* **3**:73–88.
- Garcia, E., D. Llull, R. Munoz, M. Mollerach, and R. Lopez. 2000. Current trends in capsular polysaccharide biosynthesis of *Streptococcus pneumoniae*. *Res. Microbiol.* **151**:429–435.
- Gherardi, G., C. G. Whitney, R. R. Facklam, and B. Bell. 2000. Major related sets of antibiotic-resistant pneumococci in the United States as determined by pulsed-field gel electrophoresis and *pbp1a-pbp2b-pbp2x-dhf* restriction profiles. *J. Infect. Dis.* **181**:216–229.
- Griffith, F. 1928. The significance of pneumococcal types. *J. Hyg.* **27**:8–159.
- Hausdorff, W. P., J. Bryant, C. Kloek, P. R. Paradiso, and G. R. Siber. 2000. The contribution of specific pneumococcal serogroups to different disease manifestations: implications for conjugate vaccine formulation and use, part II. *Clin. Infect. Dis.* **30**:122–140.
- Henrichsen, J. 1995. Six newly recognized types of *Streptococcus pneumoniae*. *J. Clin. Microbiol.* **33**:2759–2762.
- Hoskins, J., W. E. Alborn, Jr., J. Arnold, L. C. Blaszczyk, S. Burgett, B. S. DeHoff, S. T. Estrem, L. Fritz, D. J. Fu, W. Fuller, C. Geringer, R. Gilmour, J. S. Glass, H. Khoja, A. R. Kraft, R. E. Lagace, D. J. LeBlanc, L. N. Lee, E. J. Lefkowitz, J. Lu, P. Matsushima, S. M. McAhren, M. McHenney, K. McLeaster, C. W. Mundy, T. I. Nicas, F. H. Norris, M. O'Gara, R. B. Peery, G. T. Robertson, P. Rockey, P. M. Sun, M. E. Winkler, Y. Yang, M. Young-Bellido, G. Zhao, C. A. Zook, R. H. Baltz, S. R. Jaskunas, P. R. Rostek, Jr., P. L. Skatrud, and J. I. Glass. 2001. Genome of the bacterium *Streptococcus pneumoniae* strain R6. *J. Bacteriol.* **183**:5709–5717.
- Jiang, S.-M., L. Wang, and P. R. Reeves. 2001. Molecular characterization of *Streptococcus pneumoniae* type 4, 6B, 8, and 18C capsular polysaccharide gene cluster. *Infect. Immun.* **69**:1244–1255.
- Kelly, T., J. P. Dillard, and J. Yother. 1994. Effect of genetic switching of capsular type on virulence of *Streptococcus pneumoniae*. *Infect. Immun.* **62**:1813–1819.
- Kroll, J. S., and E. R. Moxon. 1990. Capsulation in distantly related strains of *Haemophilus influenzae* type b: genetic drift and gene transfer at the capsulation locus. *J. Bacteriol.* **172**:1374–1379.
- Lipsitch, M. 1999. Bacterial vaccines and serotype replacement: lessons from *Haemophilus influenzae* and prospects for *Streptococcus pneumoniae*. *Emerg. Infect. Dis.* **5**:336–345.
- Lipsitch, M., J. K. Dykes, S. E. Johnson, E. W. Ades, J. King, D. E. Briles, and G. M. Carlone. 2000. Competition among *Streptococcus pneumoniae* for intranasal colonization in a mouse model. *Vaccine* **15**:2895–2901.
- Lund, E., and J. Henrichsen. 1978. Laboratory diagnosis, serology and epidemiology of *Streptococcus pneumoniae*. *Methods Microbiol.* **12**:241–262.
- McGee, L., L. McDougal, J. Zhou, B. G. Spratt, F. C. Tenover, R. George, R. Hakenbeck, W. Hryniewicz, J. C. Lefevre, A. Tomasz, and K. P. Klugman. 2001. Nomenclature of major antimicrobial-resistant clones of *Streptococcus pneumoniae* defined by the pneumococcal molecular epidemiology network. *J. Clin. Microbiol.* **39**:2565–2571.
- Morona, J. K., R. Morona, and J. C. Paton. 1999. Comparative genetics of capsular polysaccharide biosynthesis in *Streptococcus pneumoniae* types belonging to serogroup 19. *J. Bacteriol.* **181**:5355–5364.
- Pozzi, G., L. Masala, F. Iannelli, R. Mangenelli, L. S. Hävarstein, L. Piccoli, D. Simon, and D. A. Morrison. 1996. Competence for genetic transformation in encapsulated strains of *Streptococcus pneumoniae*: two allelic variants of the peptide pheromone. *J. Bacteriol.* **178**:6087–6090.
- Ren, B., A. J. Szalai, O. Thomas, S. K. Hollingshead, and D. E. Briles. 2003. Both family 1 and 2 PspA proteins can inhibit complement deposition and confer virulence to a capsular serotype 3 strain of *Streptococcus pneumoniae*. *Infect. Immun.* **71**:75–85.
- Sambrook, J., and D. W. Russell. 2001. Molecular cloning, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Schuchat, A., T. Hilger, E. Zell, M. M. Farley, A. Reingold, L. Harrison, L. Lefkowitz, R. Danila, K. Stefonek, N. Barrett, D. Morse, and R. Pinner. 2001. Active bacterial core surveillance of the emerging infections program network. *Emerg. Infect. Dis.* **7**:92–99.
- Sung, C. K., H. Li, J. P. Claverys, and D. A. Morrison. 2001. An *rpsL* cassette, Janus, for gene replacement through negative selection in *Streptococcus pneumoniae*. *Appl. Environ. Microbiol.* **67**:5190–5196.
- Swartley, J. S., A. A. Marfin, S. Edupuganti, L. J. Liu, P. Cieslak, B. Perkins, J. D. Wenger, and D. S. Stephens. 1997. Capsule switching of *Neisseria meningitidis*. *Proc. Natl. Acad. Sci. USA* **94**:271–276.
- Tettelin, H., K. E. Nelson, I. T. Paulsen, J. A. Eisen, T. D. Read, S. Peterson, J. Heidelberg, R. T. DeBoy, D. H. Haft, R. J. Dodson, A. S. Durkin, M. Gwinn, J. F. Kolonay, W. C. Nelson, J. D. Peterson, L. A. Umayam, O. White, S. L. Salzberg, M. R. Lewis, D. Radune, E. Holtzapple, H. Khouri, A. M. Wolf, T. R. Utterback, C. L. Hansen, L. A. McDonald, T. V. Feldblyum, S. Angiuoli, T. Dickinson, E. K. Hickey, I. E. Holt, B. J. Loftus, F. Yang, H. O. Smith, J. C. Venter, B. A. Dougherty, D. A. Morrison, S. K. Hollingshead, and C. M. Fraser. 2001. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. *Science* **293**:498–506.
- Wu, H. Y., 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.