



Nonpneumococcal Strains Recently Recovered from Carriage Specimens and Expressing Capsular Serotypes Highly Related or Identical to Pneumococcal Serotypes 2, 4, 9A, 13, and 23A

Robert E. Gertz, Jr.,^a Fabiana C. Pimenta,^a Sopio Chochua,^a Shanda Larson,^b Anne-Kathryn Venero,^b Godfrey Bigogo,^c Jennifer Milucky,^a Maria da Gloria Carvalho,^a  Bernard Beall^a

^aRespiratory Diseases Branch, Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

^bIHRC Incorporated, Atlanta, Georgia, USA

^cKenya Medical Research Institute, Centre for Global Health Research, Kisumu, Kenya

ABSTRACT The polysaccharide capsule is a key virulence factor of *Streptococcus pneumoniae*. There are numerous epidemiologically important pneumococcal capsular serotypes, and recent findings have demonstrated that several of them are commonly found among nonpathogenic commensal species. Here, we describe 9 nonpneumococcal strains carrying close homologs of pneumococcal capsular biosynthetic (*cps*) loci that were discovered during recent pneumococcal carriage studies of adults in the United States and Kenya. Two distinct *Streptococcus infantis* strains cross-reactive with pneumococcal serotype 4 and carrying *cps4*-like capsular biosynthetic (*cps*) loci were recovered. Opsonophagocytic killing assays employing rabbit antisera raised against *S. infantis* US67cps4 revealed serotype 4-specific killing of both pneumococcal and nonpneumococcal strains. An *S. infantis* strain and two *Streptococcus oralis* strains, all carrying *cps9A*-like loci, were cross-reactive with pneumococcal serogroup 9 strains in immunodiffusion assays. Antiserum raised against *S. infantis* US64cps9A specifically promoted killing of serotype 9A and 9V pneumococcal strains as well as *S. oralis* serotype 9A strains. Serotype-specific PCR of oropharyngeal specimens from a recent adult carriage study in the United States indicated that such nonpneumococcal strains were much more common in this population than serotype 4 and serogroup 9 pneumococci. We also describe *S. oralis* and *S. infantis* strains expressing serotypes identical or highly related to serotypes 2, 13, and 23A. This study has expanded the known overlap of pneumococcal capsular serotypes with related commensal species. The frequent occurrence of nonpneumococcal strains in the upper respiratory tract that share vaccine and nonvaccine capsular serotypes with pneumococci could affect population immunity to circulating pneumococcal strains.

IMPORTANCE The distributions and frequencies of individual pneumococcal capsular serotypes among nonpneumococcal strains in the upper respiratory tract are unknown and potentially affect pneumococcal serotype distributions among the population and immunity to circulating pneumococcal strains. Repeated demonstration that these nonpneumococcal strains expressing so-called pneumococcal serotypes are readily recovered from current carriage specimens is likely to be relevant to pneumococcal epidemiology, niche biology, and even to potential strategies of employing commensal live vaccines. Here, we describe multiple distinct nonpneumococcal counterparts for each of the pneumococcal conjugate vaccine (PCV) serotypes 4 and 9V. Additional data from contemporary commensal isolates expressing serotypes 2, 13, and 23A further demonstrate the ubiquity of such strains. Increased focus upon this serological overlap between *S. pneumoniae* and its close relatives may eventually prove that most, or possibly all, pneumococcal serotypes have

Citation Gertz RE, Jr, Pimenta FC, Chochua S, Larson S, Venero A-K, Bigogo G, Milucky J, Carvalho MDG, Beall B. 2021.

Nonpneumococcal strains recently recovered from carriage specimens and expressing capsular serotypes highly related or identical to pneumococcal serotypes 2, 4, 9A, 13, and 23A. mBio 12:e01037-21. <https://doi.org/10.1128/mBio.01037-21>.

Editor Larry S. McDaniel, University of Mississippi Medical Center

Copyright © 2021 Gertz et al. This is an open-access article distributed under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/).

Address correspondence to Bernard Beall, bbeall@cdc.gov.

This article is a direct contribution from Bernard Beall, a Fellow of the American Academy of Microbiology, who arranged for and secured reviews by James Paton, University of Adelaide; Jeffrey Weiser, NYU Lagone Health; and David Stephens, Emory University.

Received 8 April 2021

Accepted 10 April 2021

Published 18 May 2021

counterparts expressed by the common upper respiratory tract commensal species *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus infantis*.

KEYWORDS capsular polysaccharide, capsular serotypes, commensal species, pneumococcal

Streptococcus pneumoniae is an opportunistic pathogen restricted primarily to humans. Its principal virulence factor is its polysaccharide capsule, currently known to have at least 100 different serotypes (1). Ninety-eight of these capsular polysaccharides are synthesized via the so-called Wzx/Wzy-dependent pathway, dependent upon a specific repeat unit membrane transporter (Wzx), specific oligosaccharide polymerase (Wzy), and glycosidic linkage to peptidoglycan (2–5). Many serotypes are grouped into structurally related serogroups. For example, serogroup 9 consists of two pairs of highly related serotypes, 9A/9V and 9L/9N. Other capsular polysaccharides, such as types 1, 2, 4, and 5, are structurally and serologically distinct from other known capsules. The pneumococcal capsular biosynthetic (*cps*) loci form 8 clusters based upon gene homology groups and sequence similarities (3).

Of the 5 serotypes that were first identified in pneumococci (6–8), 3 of them (serotypes 1, 2, and 5) have recently been characterized from nonpneumococcal commensal species (9–12). Further, in recent adult upper respiratory tract (URT) carriage assays carried out in the United States and Kenya, nonpneumococcal species expressing these and several other serotypes appear to be more common than pneumococci of the same serotypes (9, 10, 13–15). The observed directionality of horizontal transfer events, where pneumococci are most often observed as recipients of related *mitis* group DNA (16), and the abundance of *cps* loci shared between pneumococci and its close relative commensal species that reside in the URT in higher density (9, 10, 13), is consistent with pneumococcal serotype diversity originating in non-pneumococcal species. That said, species origins for shared serotypes, such as serotypes 1 and 5, have not been formally demonstrated (9, 10).

Here, we describe strains of *S. oralis* and *S. infantis* from adult carriage expressing counterparts of serotypes commonly expressed by important disease-causing strains of pneumococci, including PCV serotypes 4 and 9V, the 23-valent purified polysaccharide vaccine serotype 2, and nonvaccine serotypes 23A and 13. To our knowledge, with the exception of serotype 2 (11, 12), these serotypes have not been previously observed in nonpneumococcal species, and it is not known how common such strains are in their natural reservoir in the human URT.

RESULTS

Nonpneumococcal strains PCR positive for pneumococcal serotypes 4, 9A/V, 18A/B/C/F, and 23A recovered from U.S. adult carriage study. In a recent study of paired nasopharyngeal/oropharyngeal (NP/OP) specimens from 460 adults, 450 individuals (908 of 920 specimens) were culture negative for pneumococci (10). Isolation and characterization of nonpneumococcal strains of PCV serotypes 1 and 5 associated with the pneumococcal surveillance project (15) have been described (9, 10), as have nonpneumococcal counterparts of *cps12F* and *cps33F* (9).

Despite comprehensive attempts to recover pneumococci from all 460 paired specimens (10), there were no pneumococci recovered of serotype 4 or of serogroups 9 and 18. Only 10 individuals yielded pneumococcal isolates, including 3 that were serotype 23A (10). Fifty (47 OP and 3 NP) specimens from the 450 individuals negative for pneumococcal culture were PCR positive for serotype 4 (PCR4⁺) (Table 1). Two independent PCR4⁺ OP specimens (of the eight screened) yielded PCR4⁺, alpha-hemolytic colonies that were not pneumococci, since they were bile insoluble and negative for the CDC pneumococcal *lytA* quantitative PCR (qPCR) assay (17) (strains US302cps4 and US67cps4). Two nonpneumococcal PCV9A/9V⁺ OP isolates were recovered from this carriage survey (strains US64cps9A and US3cps9A) from two different OP specimens among the 41 total PCR9A/V⁺ specimens (Table 1). Numerous specimens were also

TABLE 1 Summary of PCR serotype and culture-positive results for serotypes 4, 9A/9V, 18C, and 23A from paired NP and OP specimens^c

PCR serotype	No. of individuals PCR positive for indicated serotype (specimens)	No. of swabs screened for nonpneumococcal strains positive for indicated PCR-serotype ^{a/} no. of colonies screened/no. of culture-positive swabs for non-Spn strain of indicated PCR serotype	PCR serotype-positive strain(s) isolated (species) ^{a,b}
4	50 (47 OP, 3 NP)	8/122/2	US302cps4 and US67cps4 (both <i>S. infantis</i>)
9A/9V	41 (all OP)	3/50/2	US3cps9A (<i>S. oralis</i>), US64cps9A (<i>S. infantis</i>)
18A/B/C/F	36 (all OP)	2/68/1	US164cps18 (<i>S. infantis</i>)
23A	9 (6 OP, 3 NP)	1/84/1	US1133ps23A (<i>S. infantis</i>)
Total	136 (130 OP, 6 NP)		

^aAttempts were made to isolate pneumococci from all specimens (both negative and positive for the CDC pneumococcal qPCR *lytA* assay).

^bAll nonpneumococcal strains were negative for the CDC pneumococcal qPCR *lytA* assay and were recovered from OP specimens.

^cSpecimens were taken from 450 pneumococcal culture-negative individuals.

PCR serogroup 18 positive (PCR18⁺ or PCR23A⁺), and, subsequently, single nonpneumococcal PCR18⁺ and PCR23A⁺ strains were recovered (US164cps18 and US1133cps23A, respectively). All 6 nonpneumococcal strains (optochin resistant and bile insoluble) were recovered from OP specimens (Table 1). Of the 136 specimens PCR positive for serotypes 4, 9A/V, 23A, and 18A/B/C/F, 130 were OP specimens, consistent with the oropharynx providing the primary reservoir for nonpneumococcal strains that carry homologs of pneumococcal *cps* loci (13). In contrast, NP specimens are relatively enriched for pneumococcal strains (18).

PCR4⁺ nonpneumococcal strains have capsules serologically cross-reactive with serotype 4. Although weak compared to serotype 4 pneumococci, positive Quellung reactions were seen for nonpneumococcal strains US302cps4 and US67cps4 employing pneumococcal type 4 antiserum (not shown), which corresponded to specific positivity of US302cps4 and US67cps4 extracts in immunodiffusion experiments employing this same antiserum (Fig. 1A). Antiserum prepared against strain US67cps4 also strongly and specifically reacted with these same extracts (Fig. 1A). Nonpneumococcal and pneumococcal strains of various serotypes other than type 4 were unreactive in immunodiffusion assays employing these two antisera, as shown for serotype 1 *S. mitis* (10) and *S. pneumoniae*.

Cross-species serotype 4-specific OPK. There was reproducible opsonophagocytic killing (OPK) activity against nonpneumococcal strains US302cps4 and US67cps4 employing serotype 4-specific pneumococcal typing antiserum but none detected employing control antiserum against strain US64cps9A (Fig. 1B). Antiserum prepared against US67cps4 also exhibited serotype-specific OPK against serotype 4 pneumococci and the two nonpneumococcal PCR4⁺ strains, while antiserum against US64cps9A lacked OPK activity against US67cps4, US302cps4, and serotype 4 pneumococci.

Nonpneumococcal *cps* loci. The pneumococcal *cps4* locus is one of 7 cluster 1 *cps* loci (3) that shares features with *cps5* and *cps12* loci from nonpneumococcal species (9). Serotype 4 pneumococci contain *N*-acetyl-L-fucosamine in their capsules, corresponding to the synthetic genes *fnIA* to *fnIC* (*fnIA*-*fnIC*) (3, 5), which were also observed in the two nonpneumococcal counterparts (Fig. 1C). These two strains were tentatively assigned as *S. infantis* due to the characteristic downstream location of the cell division gene *ftsA* and upstream location of two *aliB*-like genes (9, 11). The *cps4* loci between the species also share highly related *wciI* (initial sugar transferase) and *wciJ* (glucosyl transferase) genes. The shared seroreactivity between serotype 4 pneumococci and these two nonpneumococcal strains, observed in Quellung reactions (not shown), immunodiffusion experiments (Fig. 1A), and OPK assays (Fig. 1B), is consistent with highly conserved serotype 4-specific genes.

Also consistent with shared ancestry and close structural relatedness between serotype 4 pneumococcal capsules and capsules from the two nonpneumococcal PCR4⁺ strains US67cps4 and US302cps4 was the gene order shared between the three 16,368- to 16,429-bp loci and very close relatedness (93.3 to 96.4% sequence identity) between alleles of the 5 central genes that encode serotype-specific synthetic functions (Fig. 1C). The 5 genes include the repeat unit oligosaccharide synthase gene

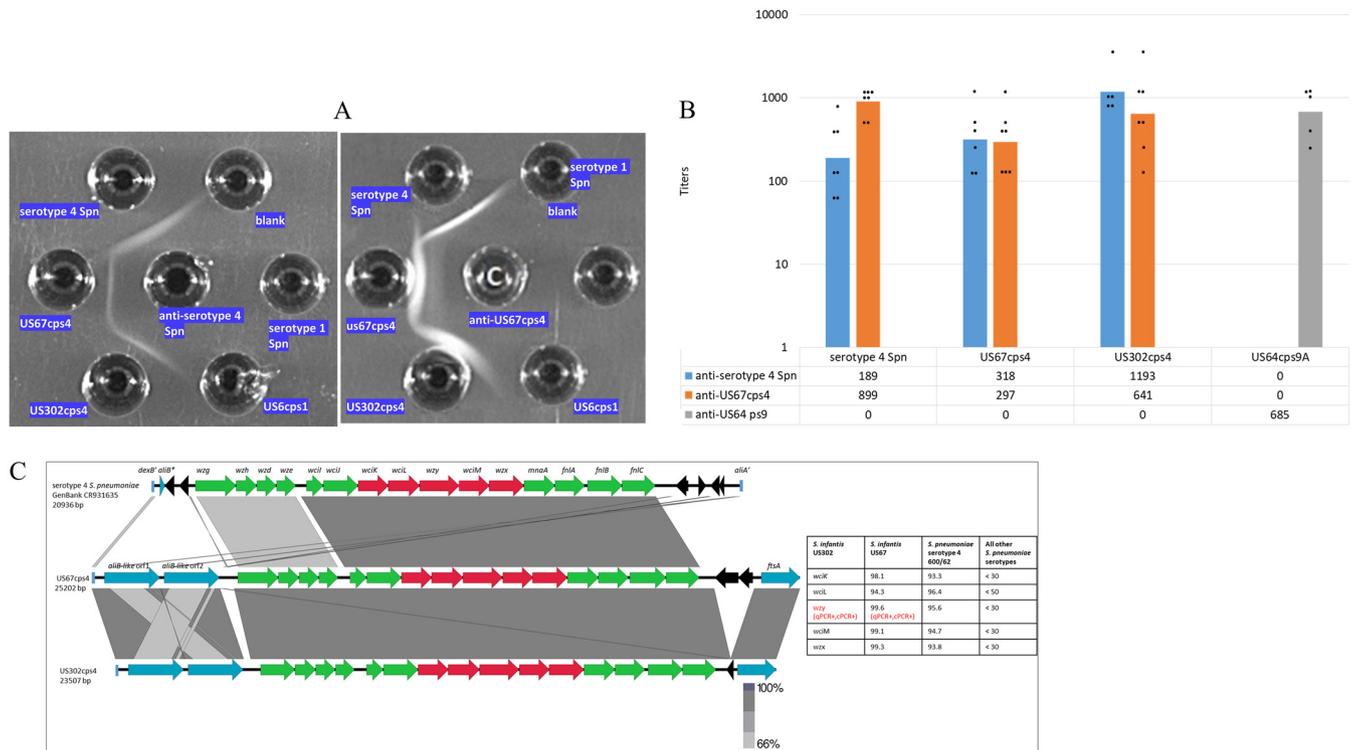


FIG 1 (A) Reactivity of pneumococcal (Spn) typing serum (left) and antiserum raised against US67cps4 (right) against serotype 4 pneumococcal strain and two nonpneumococcal strains carrying *cps4*-like operons (US67cps4 and US302cps4). Negative-control strains include a serotype 1 pneumococcal strain and serotype 1 *Streptococcus mitis* strain US6cps1 (10). (B) Cross-species and serotype 4-specific opsonophagocytic killing activity of antisera prepared against serotype 4 *S. pneumoniae* and nonpneumococcal strain US67cps4. Geometric mean titer (GMT) values are shown with >50% killing compared with growth in the complement control wells. Titers used to calculate GMTs are indicated with black circles. (C) Alignment of *cps4* loci from serotype 4 pneumococci and nonpneumococcal strains US6cps4 and US302cps4 recovered from adult OP specimens. The pneumococcal sequence between the 3' end of *dexB* (*dexB'*) and the 5' end of *aliA* (*aliA'*) is aligned with corresponding sequences from the two nonpneumococcal strains that lie downstream of *dexB*. The *cps4* operon genes indicated in green are relatively highly conserved with counterparts from *cps* operons other than *cps4*, while genes in red indicate genes that share >94% sequence identity only within *cps4* counterparts (indicated in the accompanying table as well as indication of positivity of the 2 strains with qPCR and cPCR assays for serotype 4). Also indicated in the table is the tentative assignment of species *S. infantis* due to characteristic gene arrangement at this genomic region. Genes indicated in blue are unrelated to capsule biosynthesis. A remnant of an *aliB* gene is indicated (*aliB**) in the pneumococcal strain. Remnants of transposase genes are indicated in black. Black lines are indicative of small localized homologous segments. The different gray shades shared between the *aliB*-like genes are indicative of high homology shared between all 4 genes.

(wzy), which shared ~96% sequence identity between species and allowed PCR detection employing conventional PCR (cPCR) (19) and qPCRs (20) specific for serotype 4.

PCR9A/9V⁺ nonpneumococcal strains have capsules serologically related to serogroup 9 and specifically react with typing factor serum 9d. Three PCR9A⁺ nonpneumococcal specimens and strains retrospectively isolated from pneumococcal carriage specimens (10, 14) were positive for the qPCR-serotyping reaction targeting *wzx9A-wzx9V* (20). One of the three PCR9A⁺ strains (US64cps9A) was also positive for the cPCR assay targeting *wzx9A-wzx9V* (21). Positive Quellung reactions were observed for the three nonpneumococcal PCR9A⁺ strains when employing pooled serogroup 9 antisera containing all 4 factors for resolution of serotypes 9A, 9V, 9L, and 9N pneumococci (not shown) but not when the 3 strains were reacted with 3 of the individual factors used for resolving serogroup 9 (factors 9e, 9g, and 9f). US64cps9A displayed an obviously stronger Quellung result, with pooled serogroup 9 antisera compared to the other two PCR9A⁺ strains, that was slightly more noticeable than the reactivity with the serotype 9A pneumococcal reference strain. Barely detectable positive Quellung reactions were observed for the 3 PCR9A⁺ nonpneumococcal strains when reacted with factor 9d (reacts with pneumococcal serotypes 9A and 9V only). According to this result, these 3 strains are tentatively serotype 9A, since they did not quell with the 9V-specific factor 9g. Strains of all four pneumococcal serogroup 9 serotypes, and all 3 PCR9A⁺ nonpneumococcal strains, displayed strongly positive Quellung reactions

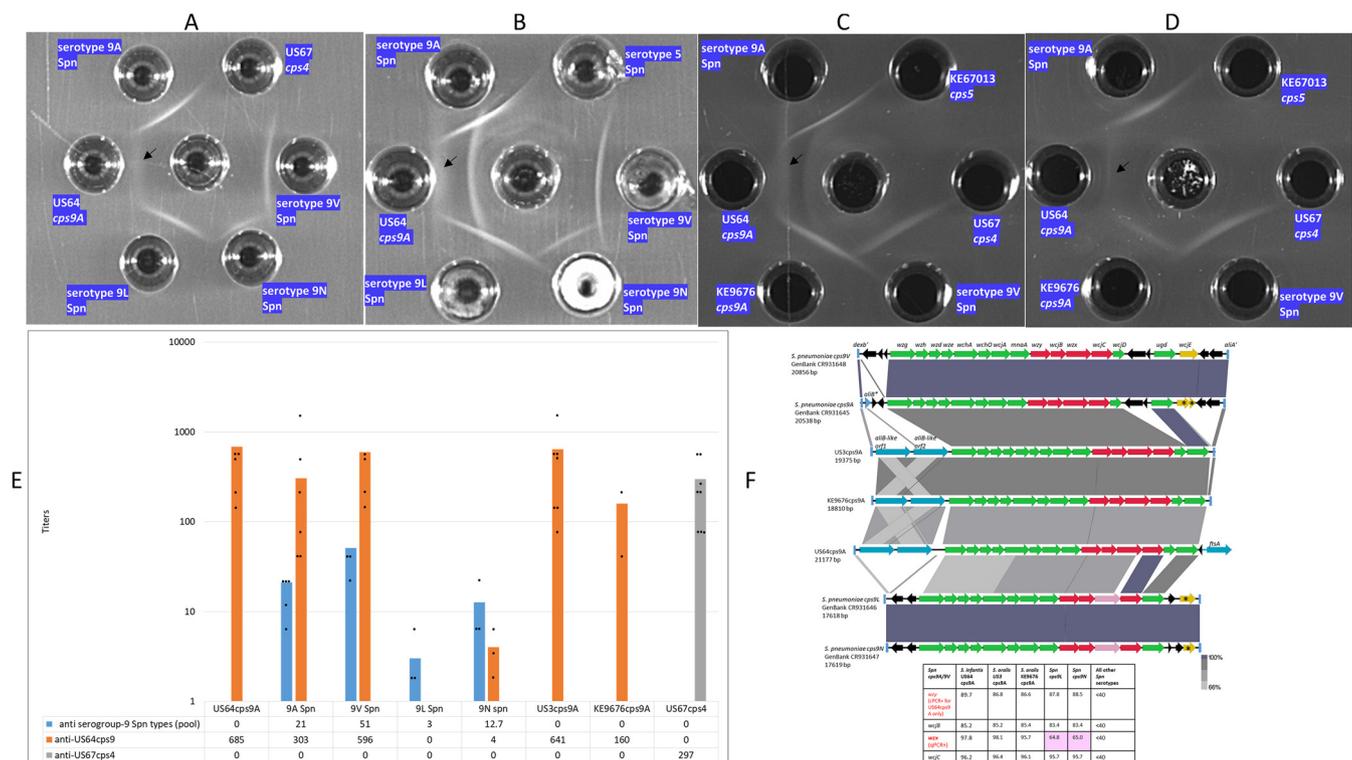


FIG 2 (A) Cross-reactivity of nonpneumococcal strains with pneumococcal typing sera against serogroup 9. Pooled pneumococcal serogroup 9 typing factors (center) are reacted against extracts of pneumococcal serotypes 9A, 9L, 9N, and 9V. A negative-control extract of nonpneumococcal strain US67cps4 is included. A faint serogroup 9 precipitate (arrow) is visible between US64cps9A extract and the centrally placed pooled pneumococcal serogroup 9 typing antisera. (B) Anti-US64cps9A sera (center) reacted against extracts of pneumococcal serotypes 9A, 9L, 9N, 9V, and US64cps9A. A negative-control extract of control serotype 5 *S. pneumoniae* is included. The antiserum-distal precipitate corresponds to the precipitate showing identity with the corresponding precipitate from KE9676cps9A in panel C. (C) Anti-US64cps9A sera (center) reacted against extracts of serotype 9A Spn, US64cps9A, KE9676cps9A, and serotype 9V Spn. Negative controls of nonpneumococcal strain US67cps4 and KE67013cps5 are included. The faint precipitate from US64cps9A (arrow) shows apparent serologic identity to the corresponding precipitate from KE9676cps9A. (D) Pooled serogroup 9 typing factors (center) reacted against extracts of serotype 9A Spn, US64cps9A, KE9676cps9A, and serotype 9V Spn. Negative-control extracts of nonpneumococcal strains US67cps4 and KE67013cps5 are included. A faint serogroup 9 precipitate (arrow) is visible between US64cps9A extract and the centrally placed pooled Spn serogroup 9 typing antisera and appears to share serologic identity with the corresponding precipitate from KE9676cps9A. (E) Cross-species and serotype 9A/9V-specific opsonophagocytic killing activity of antisera prepared against serotype 9A nonpneumococcal strain US64cps9A and pooled antisera against *S. pneumoniae* serogroup 9 serotypes. Geometric mean titer (GMT) values are shown with >50% killing compared with growth in the complement control wells. Titers used to calculate GMTs are indicated with black circles. (F) Alignment of *cps* loci from serogroup 9 pneumococci and nonpneumococcal strains US3cps9A, US64cps9A, and KE9676cps9A recovered from adult OP specimens. The pneumococcal sequence between the 3' end of *dexB* (*dexB'*) and the 5' end of *aliA* (*aliA'*) is aligned with corresponding sequences from the three nonpneumococcal strains that lie downstream of *dexB*. The *cps* operon genes indicated in green are relatively highly conserved with counterparts from *cps* operons other than from serogroup 9, while genes in red share >83% sequence identity among the 4 pneumococcal serogroup 9 operons, with the exception of *wzx*, which distinguishes 9A/9V (indicated in pink and in the accompanying table), and indicates positivity of the 3 nonpneumococcal strains with *wzx*-specific qPCR. The active *wcjE* acetylase gene in *cps9V* is indicated in gold, and inactive forms in the other 3 pneumococcal types are indicated with an asterisk. The nonpneumococcal *cps9A*-like operons lack *wcjE* genes. Genes indicated in blue are not involved in capsule biosynthesis. A remnant of an *aliB* gene is indicated (*aliB**) in one pneumococcal strain. Remnants of transposase genes are indicated in black. The different gray shades shared between the *aliB*-like genes are indicative of the high homology shared between all 4 genes.

when mixed with antiserum prepared against US64cps9A (data not shown). Since serotype 9A capsule contains glucuronic acid, these strains were subjected to an assay measuring uronic acids (22–24). All three nonpneumococcal PCR9AV⁺ strains yielded uronic acid assay values corresponding to more than 100 μg/ml, as predicted by the glucuronic acid standard, with strain US64cps9A giving higher readings than both pneumococcal and nonpneumococcal serotype 9A strains (see Fig. S1 in the supplemental material).

Immunodiffusion assay results employing serogroup 9 typing sera and anti-US64cps9A (Fig. 2A to D) were in agreement with Quellung testing. A faint precipitate is shown for US64cps9A extract reacted with pooled serogroup 9 sera in Fig. 2A, with clear precipitate bands shown for each of the 4 serogroup 9 pneumococcal strains. Despite positive factor 9d Quellung results with serotypes 9A and 9V pneumococci as well as the three nonpneumococcal PCR9AV⁺ strains (not shown), reproducible

immunoprecipitates were not apparent when reacting factor 9d with any pneumococcal or nonpneumococcal serogroup 9 strains (data not shown).

Antiserum prepared against US64cps9A reacted with each of the 4 serogroup 9 pneumococcal serotypes (Fig. 2B and C). This antiserum produced a diffuse immunoprecipitate close to the US64cps9A extract (compare to same extract reacting with pooled serogroup 9 antiserum in Fig. 2A) and a sharper band close to the central antiserum well (Fig. 2C). Neither of these two bands was evident by employing various extracts from PCR9AV-negative nonpneumococcal strains. Both US3cps9A (not shown) and KE9676cps9A showed the same reactivity to these antisera as US64cps9A (Fig. 2C and D), with the exception of the absence of the apparently US64cps9A-specific precipitate (antiserum-proximal) shown in Fig. 2B and, to a lesser extent, in Fig. 2C. Presumably this relatively strong precipitate line is due to a distinct surface antigen unrelated to the 9A polysaccharide that is expressed by US64cps9A and not expressed by the other strains shown.

Cross species-specific opsonophagocytic activities against serotypes 9A and 9V. Consistent with observed reactivity in Quellung reactions with pooled serogroup 9 pneumococcal typing antisera and with factor 9d, antiserum against US64cps9A was highly active in OPK assays against pneumococci of serotypes 9A and 9V with low OPK activity against pneumococci of serotypes 9L and 9N (Fig. 2E). Employing anti-US64cps9A, high OPK activity was observed against serotypes 9A and 9V pneumococci and against strains US3cps9A, US64cps9A, and KE9676cps9A. No OPK activity was observed against US67cps4. Pooled serogroup 9 pneumococcal typing antisera showed modest OPK activity against pneumococci of serotypes 9A, 9V, and 9N but low activity against serotype 9L pneumococci and no observable OPK activity against the three *cps9A*-positive nonpneumococcal strains. Antisera raised against serotype 4 strains had no activity against serotype 9A pneumococci or putative serotype 9A nonpneumococcal strains.

US64cps9A, US3cps9A, and KE9676cps9A *cps* operons. The four pneumococcal serogroup 9 capsular polysaccharide structures are very similar, and their *cps* loci comprise cluster 6 (3, 5). The 5-unit repeating structures of serotypes 9A/9V, 9N, and 9L are very similar but distinct from each other. The pneumococcal serotype 9A/9V *wzx* (flippase) gene is more discriminatory than *wzy* (repeat unit polymerase gene) when distinguishing between the two syntenic pneumococcal *cps* pairs (9A/9V and 9L/9N), and the pneumococcal *wzx* is much more homologous than *wzy* to their counterparts in the three nonpneumococcal PCR9A⁺ strains (Fig. 2F). It follows that the *wzx9V-9A*-specific qPCR assay (19) was positive for these 3 strains while *wzy*-directed cPCR (21) was negative for 2 of the 3 strains (Fig. 2F).

Serotypes 9V and 9A share the same repeating oligosaccharide subunit structure that differs between them only by lack of WcjE-mediated O-acetylation in relatively rare serotype 9A strains that arise from mutation of the *cps9V* O-acetyltransferase gene, *wcjE* (3, 5, 25). The serotype 9A/9V-specific OPK activity (Fig. 2E), the specific Quellung positivity with serotyping factor 9d (not shown), the high interspecies similarity between the *cps9A-cps9V* flippase gene (*wzx*) alleles, the presence of the *wcjD* O-acetyltransferase gene (absent in serotypes 9L and 9N), and the lack of a *wcjE* homolog (Fig. 2F) are all features consistent with these three serogroup 9 nonpneumococcal strains expressing the serotype 9A polysaccharide.

Judging from previous observations (9, 11), the *dexB* and *ftsA* genes flanking the putative *cps9A* locus within US64cps9A together with the upstream two full-length *aliB*-like genes was suggestive of *S. infantis*. The *cps* region organization in strains US3cps9A and KE9676cps9A (flanking *dexB* and *aliA*, two full-length *aliB*-like genes) was suggestive of *S. oralis* (Fig. 2F).

Cross-reactivity of US1133cps23A with pooled serogroup 23 sera and reactivity of anti-US1133cps23A with pneumococcal serogroup 23 serotypes. Nonpneumococcal strain US1133cps23A was recovered through screening for *wzy23A*-positive (PCR23A⁺) isolates from an OP specimen (Table 1) using qPCR (20). Pooled antiserum against the three serogroup 23 serotypes 23A, 23B, and 23F produced weak positive Quellung reactions with US1133cps23A (not shown); however, individual

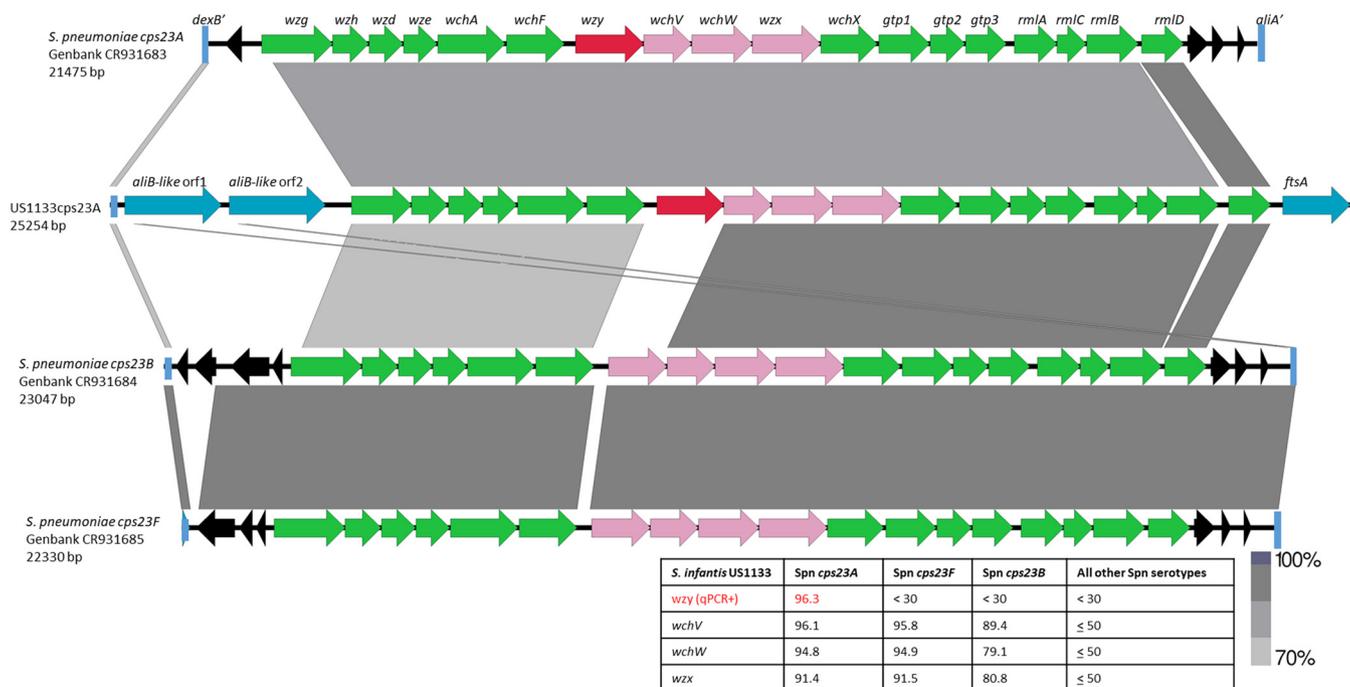


FIG 3 Alignment of *cps* loci from serogroup 23 pneumococci and nonpneumococcal strains US1133cps23A recovered from adult OP specimens. The pneumococcal sequence between the 3' end of *dexB* (*dexB'*) and the 5' end of *aliA* (*aliA'*) is aligned with corresponding sequence from US1133cps23. The *cps* operon genes indicated in green are relatively highly conserved with counterparts from non-serogroup 23 *cps* operons, while the *wzy* gene alleles in red depict dissimilarity to all other known pneumococcal *cps* genes. Genes indicated in pink share ≥83% sequence identity between 2 and 3 corresponding serogroup 23 *cps* genes. Genes indicated in blue are not involved in capsule biosynthesis. Remnants of transposase genes are indicated in black.

serogroup 23 factor sera did not produce detectable results. Antisera raised against nonpneumococcal strain US1133cps23A quelled pneumococcal strains of serotypes 23A, 23B, and, more weakly, 23F (not shown). The strongest Quellung reactivity of anti-US1133cps23A was observed with serotype 23A pneumococci. It is notable that antiserum prepared against serotype 23F pneumococci cross-reacts very little with serotype 23A and not at all with 23B, which correlates to their known polysaccharide structures (26, 27). The high sequence identity between the US1133cps23A and pneumococcal *cps23A* *wzy* alleles is consistent with the possibility of an identical capsular chemical structure shared between US1133cps23A and serotype 23A pneumococci (Fig. 3).

Despite the positive Quellung results employing pooled serogroup 23 pneumococcal typing serum and antiserum prepared against US1133cps23A, we did not see US1133cps23A extract reactivity in immunodiffusion experiments with pooled serogroup 23 typing antisera. Antisera raised against US1133cps23A did not visibly react with the three serogroup 23 pneumococcal serotypes and generated only a weak band against US1133cps23A extract (not shown).

Nonpneumococcal serotype 2 Kenya carriage OP strain. Due to the extreme abundance and allelic diversity of serotype 2-specific amplicons from apparent pneumococcus-negative URT specimens recovered during a pneumococcal carriage survey performed in Kenya during 2009 (13), we attempted to recover a nonpneumococcal PCR2⁺ strain from recent (2013) carriage specimens recovered from adults in the same area (14). Although there were no serotype 2 pneumococci recovered among 2,590 adults, a nonpneumococcal strain (KE9746cps2) that was *wzy2* positive by cPCR (21) and tested strongly Quellung positive for serotype 2 was recovered from an adult OP specimen. This result correlated with a pronounced serotype 2-specific immunoprecipitate in immunodiffusion (Fig. 4A) and higher uronic acid assay values than from the corresponding serotype 2 reference strain (Fig. S1). The KE9746cps2 *cps2* operon closely resembled the pneumococcal cluster 2 *cps2* operon (3) and *cps2* from recently described serotype 2 *S. oralis* strains (11, 12) (Fig. 4B).

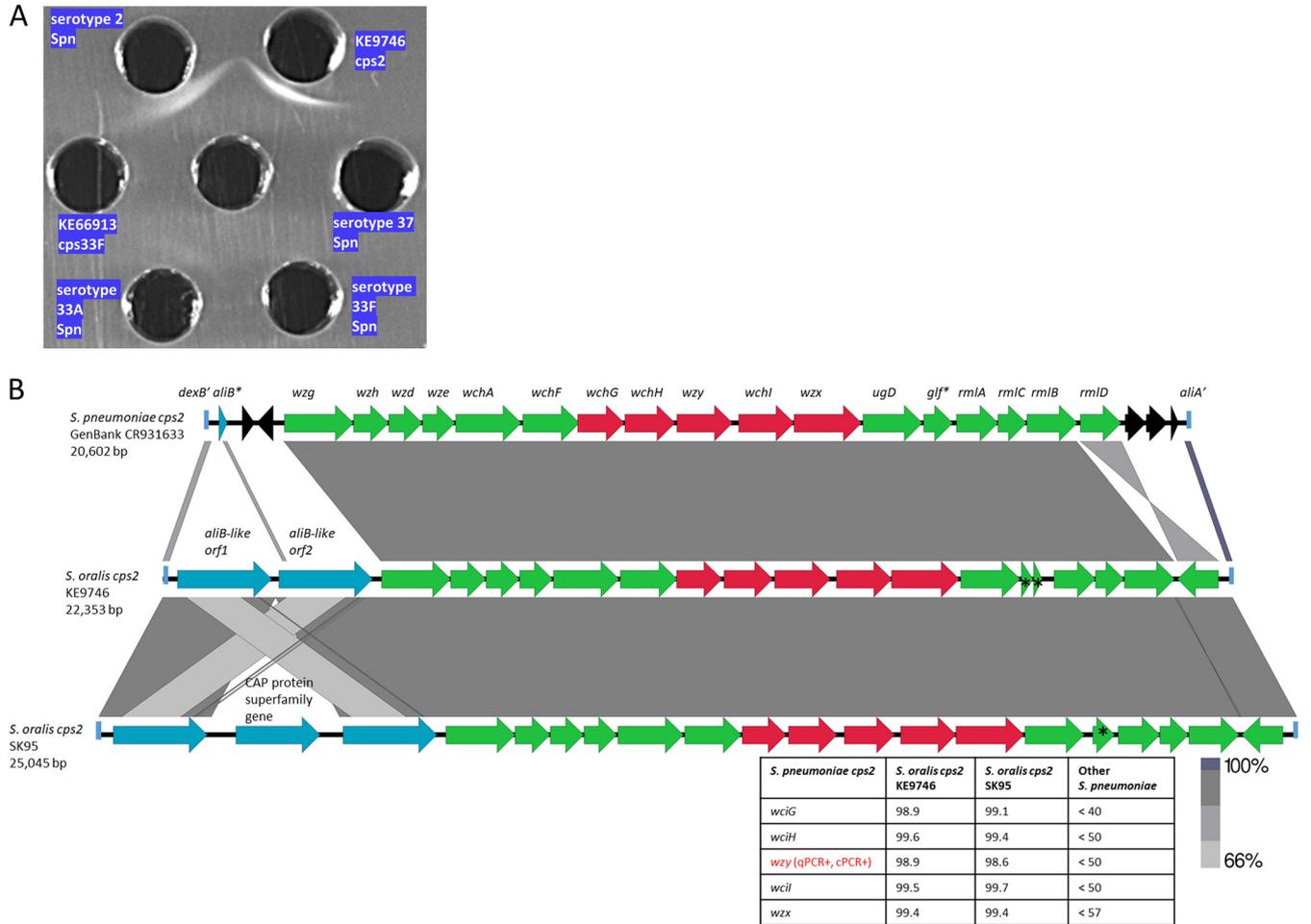


FIG 4 (A) Typing antiserum against pneumococcal serotype 2 reacted against serotype 2 *S. pneumoniae* and *S. oralis* strain KE9746cps2. A control *S. oralis* strain carrying a cps33F-like locus and pneumococcal serotypes 33A, 33F, and 37 are also included. (B) Alignment of *cps2* loci from serotype 2 pneumococcal reference and nonpneumococcal strains: KE9746cps2 recovered from adult OP specimens and previously described serotype 2 *S. oralis* strain SK95 (11, 12). The pneumococcal sequence between the 3' end of *dexB* (*dexB'*) and the 5' end of *aliA* (*aliA'*) is aligned with corresponding sequences from the two nonpneumococcal strains that lie downstream of *dexB*. The *cps2* operon genes indicated in green are relatively highly conserved with counterparts from *cps* operons other than *cps2*, while genes in red indicate genes that share >99% sequence identity only within *cps2* counterparts (indicated in the accompanying table as well as the indication of positivity the 2 strains with qPCR and cPCR assays for serotype 2). Genes indicated in blue are unrelated to capsule biosynthesis. A remnant of an *aliB* gene is indicated (*aliB**) in the pneumococcal strain. Remnants of transposase genes are indicated in black. The different gray shades shared between the *aliB*-like genes are indicative of the high homology shared between all 4 genes. Truncated and presumably inactive *glf* gene segments (required for galactopyranose synthesis) are indicated with asterisks.

U.S. serotype 13 NP strain. US3019cps13 was fortuitously sent to the CDC *Streptococcus* laboratory from an adult prison inmate among one carriage isolate and three invasive isolates recovered during a serotype 12F invasive pneumococcal disease (IPD) outbreak and was the sole isolate in this study identified initially employing the Quellung reaction rather than through PCR serotyping. Among the 4 isolates sent to the CDC, the 3 invasive isolates were serotype 12F pneumococcal strains (bile soluble) and single NP isolate was serotype 13 and not bile soluble. Extracts of US3019cps13 provided serotype 13-specific immunoprecipitates when reacted against pneumococcal typing serum (Fig. 5A).

The *cps13* locus represents one of 23 different cluster 4 pneumococcal serotypes (3). The apparent serologic identity between US3019cps13 and serotype 13 pneumococci corresponded to the high homology (68 to 94% sequence identity) shared between each gene conserved of the 16 gene *cps* operons (Fig. 5B). The *wzy13* gene, which shares 91% sequence identity with its counterpart in US3019cps13, is the most divergent and unique

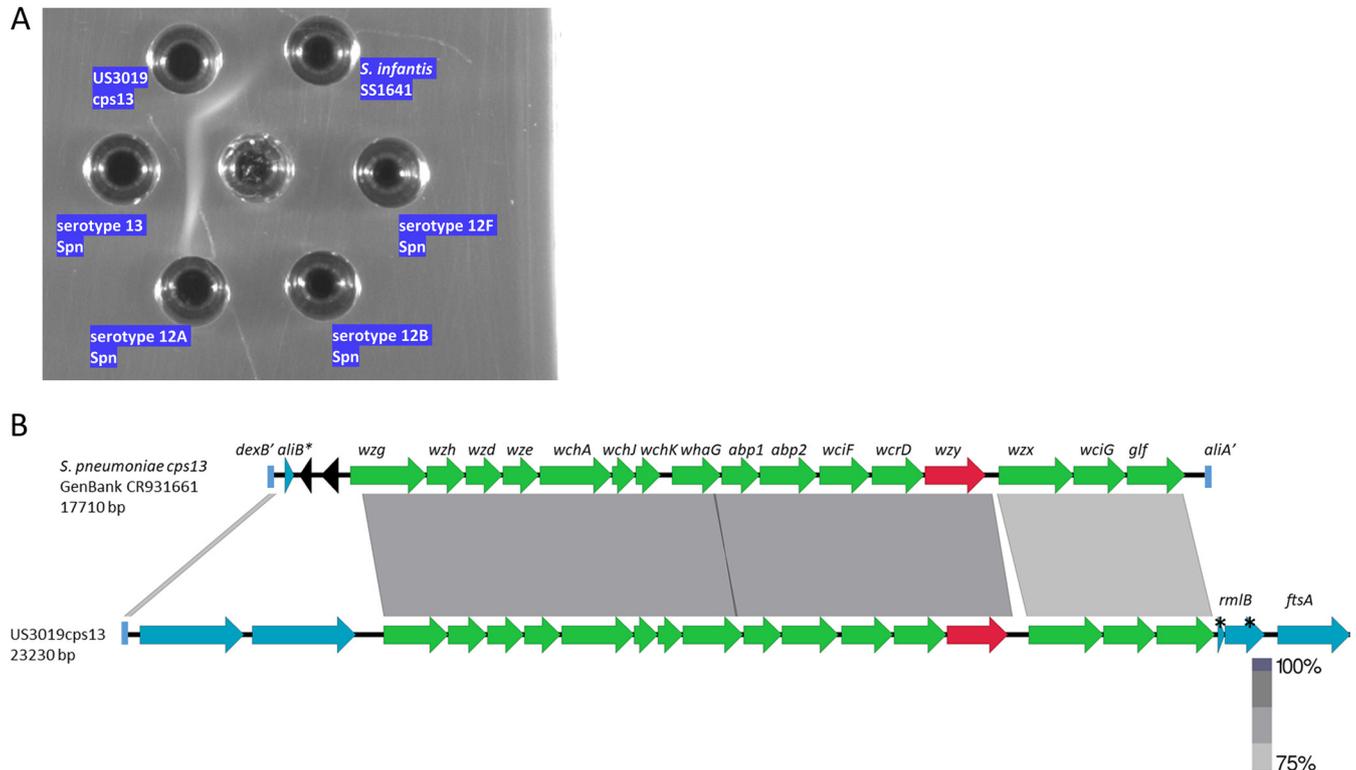


FIG 5 (A) Pneumococcal typing antiserum against serotype 13 (center well) reacted against extracts of nonpneumococcal strain US3019cps13 and the serotype 13 pneumococcal reference strain. Also included are negative-control extracts of *S. infantis* strain SS1641 and serogroup 12 pneumococcal extracts. (B) Alignment of *cps13* loci from the serotype 13 pneumococcal reference and nonpneumococcal strain US3019. The pneumococcal sequence between the 3' end of *dexB* (*dexB'*) and the 5' end of *aliA* (*aliA'*) is aligned with corresponding sequence from US3019cps13 that lies downstream of *dexB*. The *cps13* operon genes indicated in green are relatively highly conserved with counterparts from *cps* operons other than *cps13* (up to 87% identity), while *wzy13* shares >99% sequence identity only within *cps13* counterparts and shares 91% sequence identity with US3019cps13. Genes indicated in blue are not associated with capsule biosynthesis. A remnant of an *alIB* gene is indicated (*alIB**) in the pneumococcal strain. Remnants of transposase genes are indicated in black. Inactive remnants of an *rmlB* rhamnose biosynthetic gene are indicated by asterisks.

of the pneumococcal *cps13* operon genes in that it shares <50% identity with all other known *cps* genes from other serotypes.

US164cps18 nonpneumococcal strain from U.S. carriage survey. Despite PCR assays positive for serogroup 18 *wzy* alleles, we were unable to detect positive Quellung or immunodiffusion results for US164cps18 when employing serogroup 18 pooled or individual pneumococcal serotyping factors. Serogroup 18 (18A, 18B, 18C, and 18F) *cps* loci are representative of the cluster 2 group (40 serotypes that include rhamnose in their capsules) and include the above-described *cps23A* and *cps2* loci, all of which contain rhamnose biosynthetic and transferase genes (*rmlA-D* and *wchF*, respectively, shown in Fig. 3, 4B, and 6).

Core genome phylogeny of recent PCR serotype-positive nonpneumococcal strains. The 9 PCR serotype-positive nonpneumococcal strains described in this work were subjected to genomic sequencing followed by phylogenetic clustering with previously described strains (Fig. 7, Table 2), including nonpneumococcal strains described from other studies (Table S1). To extend the included diversity, 24 invasive nonpneumococcal strains submitted to the CDC *Streptococcus* laboratory were added to the phylogenetic analysis. These include strains of *S. mitis*, *S. oralis*, and *S. pseudopneumoniae*.

Six of the 9 PCR serotype-positive strains from this study, including US67cps4, US302cps4, US64cps9A, US164cps18, US1133cps23A, and US3019cps13, formed a cluster that included 4 previously described cluster 1 strains of *S. infantis* (28) and a previously described strain (US24) of serotype 5 *S. infantis* (9). Three strains of *S. oralis* were apparent, including KE9746cps2 and US3cps9A, that clustered with strains categorized as *S. oralis* subsp. *oralis* (28). This cluster included 7 additional strains previously

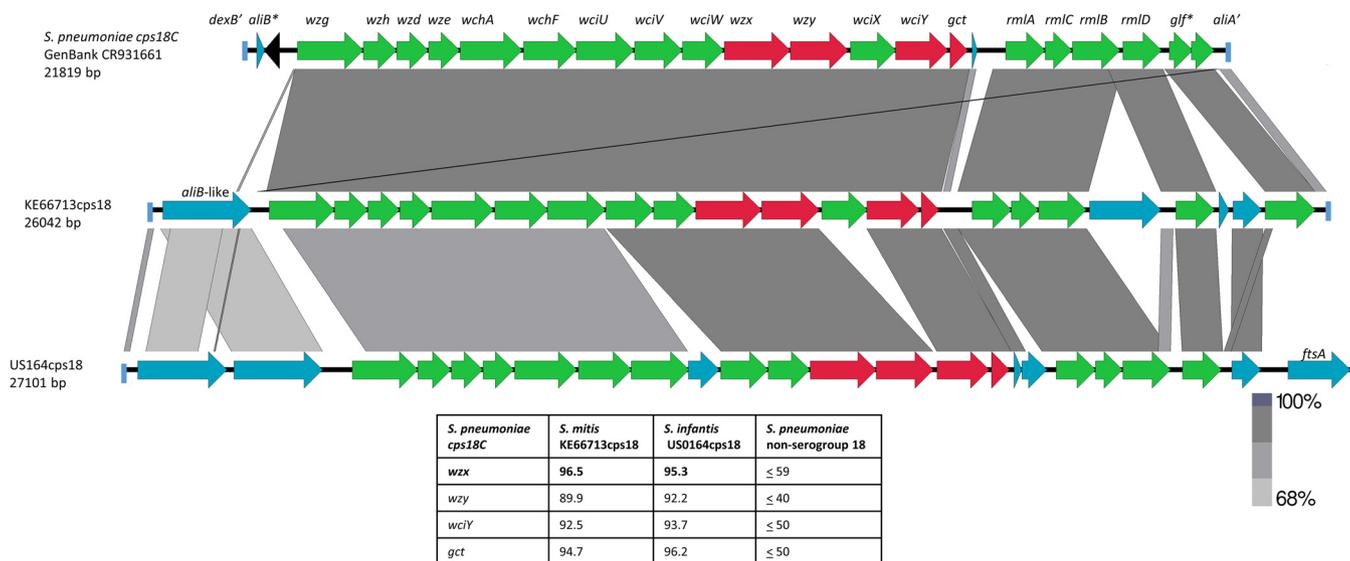


FIG 6 Alignment of *cps* locus from serotype 18C pneumococcus and nonpneumococcal strains KE66713cps18, from a previous study (9), and US164cps18, both recovered from adult OP specimens. The pneumococcal sequence between the 3' end of *dexB* (*dexB'*) and the 5' end of *aliA* (*aliA'*) is aligned with corresponding sequences from the nonpneumococcal strains. The *cps* operon genes indicated in green are relatively highly conserved with counterparts from *cps* operons other than from serogroup 18, while the gene alleles in red depict dissimilarity to all other known pneumococcal *cps* genes. Genes indicated in blue are not involved in capsule biosynthesis. Remnants of transposase genes are indicated in black.

identified to carry homologs of *S. pneumoniae* *cps2*, *cps5*, *cps15A*, *cps47F*, and *cps12* loci (9, 11). Strain KE9676cps9A clustered with previously identified *S. oralis* subsp. *tigurinus* strains and included strains carrying homologs of pneumococcal *cps16A* (11) and *cps33F* (9).

Figure 7 depicts much of the known diversity of nonpneumococcal strains that carry close homologs of pneumococcal *cps* loci and includes strains that have been previously characterized at genomic and serologic levels (11, 28). Importantly, Fig. 7 depicts 22 nonpneumococcal isolates that we have retrospectively recovered from recent pneumococcal carriage surveys (9, 10, 13–15) that represent 10 different serotypes or serogroups. These include 5 strains within the *S. oralis* subsp. *oralis* cluster (28) carrying representatives of *cps* homology clusters 1, 4, 6, and 8 (3), including *cps5*, *cps15A*, *cps9A*, *cps12*, and *cps33A-F* recovered in U.S. or Kenya pneumococcal carriage surveys. Five distinct *cps2*⁺ strains (described in references 11 and 12 in addition to KE9746cps2 of this study) shown to express serotype 2 are shown within two of the three *S. oralis* subspecies shown.

Cluster 1 *S. infantis* (28) also represents broad *cps* diversity with 7 distinct strains carrying homologs of *cps18*, *cps4*, *cps13*, *cps5*, *cps23A*, and *cps9A* of *cps* homology clusters 1, 2, 4, and 6 (3). The presence of two genetically distinct *cps4*⁺ strains within this species is consistent with previous observations that individual *cps* loci are representative of much greater strain diversity within nonpneumococcal species than within pneumococci (9–11, 13).

Evident in this phylogram (Fig. 7) are numerous, diverse strains that are described here and elsewhere (28) as *Streptococcus mitis* simply by virtue of their close relatedness to, as well as genetic separation from, the two phylogenetically distinct branches that define *S. pneumoniae* and *S. pseudopneumoniae*. These 3 highly related entities are well demarcated phylogenetically from *S. oralis* and *S. infantis*. Seven independent *S. mitis* strains from recent carriage surveys that carry homologs of pneumococcal *cps* operons are depicted, including 5 independent serotype 1 isolates recovered within the United States (10) as well as *cps5*⁺ and *cps18*⁺ strains recovered in Kenya (9). Several additional *S. mitis* strains with pneumococcal serologic overlap shown in Fig. 7 and Table S1 have been described by Sørensen and colleagues (11). Strains designated *S. mitis* also included several invasive isolates erroneously submitted to active bacterial

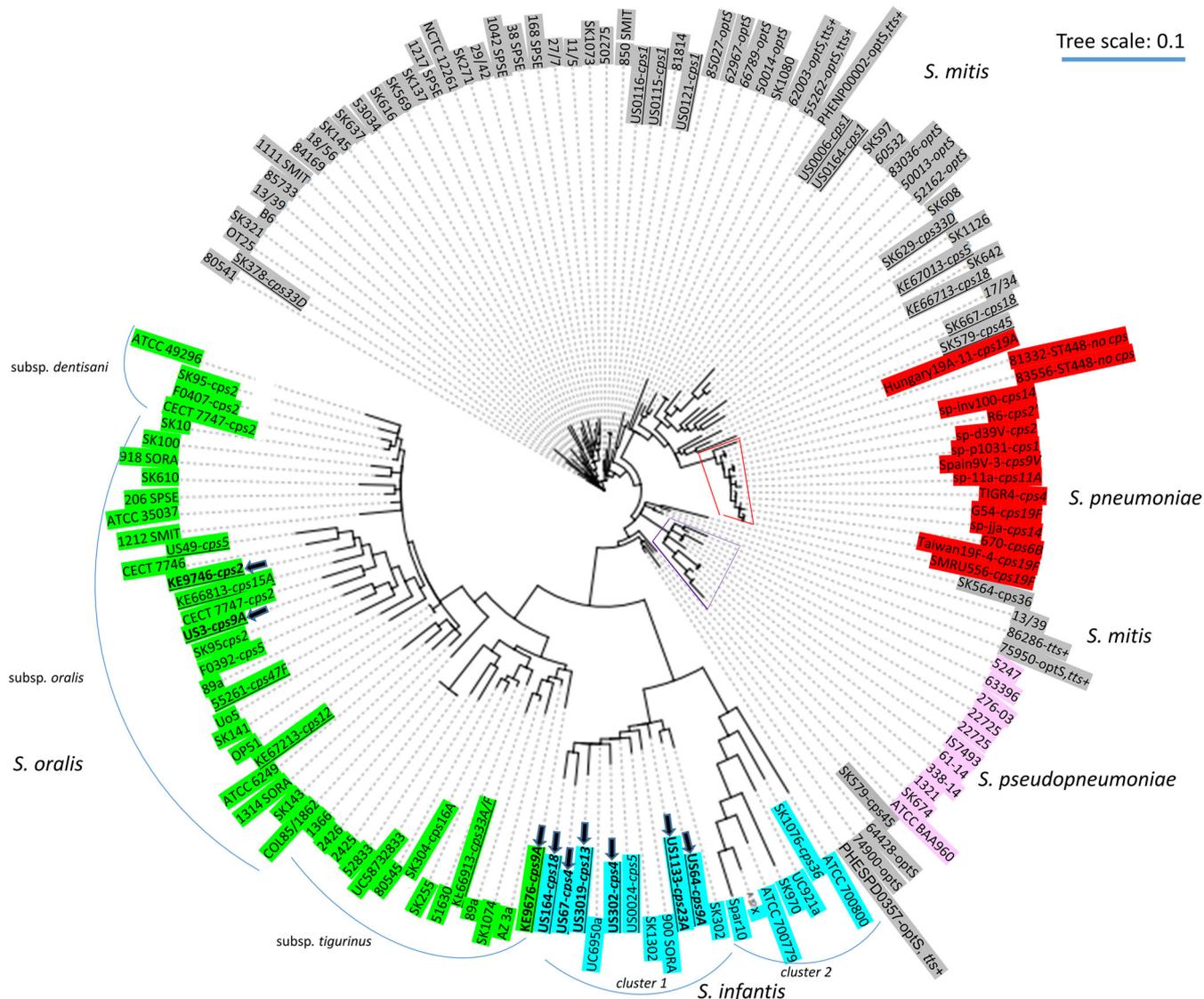


FIG 7 Maximum parsimony core genomic tree based upon kSNP3.0 analysis (33) of genomes accounting for 9 nonpneumococcal carriage isolates carrying homologs of pneumococcal *cps* loci that are the focus of this study are indicated with arrows. Strains from previous studies that carry *cps* loci highly similar to pneumococcal counterparts (9–11) are underlined. All strains are described in Table S1. The k-mer size employed was 19. The scale is based on 322 core single-nucleotide polymorphisms (SNPs). The number of core SNPs was 258. The number of noncore SNPs was 2,043,461. The number of SNPs in at least 0.5 genomes was 77,922. Clusters indicative of *S. oralis* subspecies and *S. infantis* clusters 1 and 2 are indicated by virtue of described clustering and subspecies designations (28). The species *S. pneumoniae* and *S. pseudopneumoniae* are indicated in red and purple highlights, respectively. *S. mitis*, *S. infantis*, and *S. oralis* are indicated in gray, blue, and green highlights, respectively.

core surveillance (ABCs) as pneumococci (unpublished data), some of which carry the *tts* gene, known to confer the Wzy-independent serotype 37 (29), and two similar *tts*⁺ clinical isolates recovered in the United Kingdom (30). It is interesting that the majority of these invasive *S. mitis* strains also shared the typical pneumococcal phenotype of optochin sensitivity (Table S1).

DISCUSSION

Here, we build upon data derived from recent pneumococcal carriage surveys confirming that many pneumococcal capsular serotypes are widely dispersed among related commensal species residing in the URT (9–11, 13). Our knowledge of this ecological niche is extremely limited, and here we modestly expand the known shared capsular serology and strain diversity within the subset of the Mitis group that consists of the five species *S. pneumoniae*, *S. pseudopneumoniae*, *S. mitis*, *S. oralis*, and *S. infantis*.

TABLE 2 Nonpneumococcal strains carrying *cps* locus homologs of pneumococcal strains of known serotypes described in the manuscript

Strain	Yr and country of origin ^a	Species/phylogenetic cluster ^b	Source of isolate	Capsular serology employing pneumococcal typing sera	Seroreactivity of rabbit antiserum prepared against strain
US302cps4	2015–2016 United States	<i>S. infantis</i> /1	Adult OP	Serotype 4	Not tested
US67cps4	2015–2016 United States	<i>S. infantis</i> /1	Adult OP	Serotype 4	Specific quelling and killing of serotype 4 pneumococci and of <i>S. infantis</i> strain US302cps4
US3cps9A	2015–2016 United States	<i>S. oralis</i> /subsp. <i>oralis</i>	Adult OP	Serotype 9A	Not tested
KE9676ps9A	2013 Kenya	<i>S. oralis</i> /subsp. <i>tigurinis</i>	Adult OP	Serotype 9A	Not tested
US64cps9A	2015–2016 United States	<i>S. infantis</i> /1	Adult OP	Serotype 9A	Specific quelling of pneumococci of all 4 serogroup 9 serotypes; also specific quelling of the 3 <i>cps</i> 9A-positive nonpneumococcal strains; high killing activity of serotypes 9A and 9V pneumococci and of the 3 nonpneumococcal serotype 9A strains
KE9746ps2	2013 Kenya	<i>S. oralis</i> /subsp. <i>oralis</i>	Adult OP	Serotype 2	Not tested
US3019ps13	2019 United States	<i>S. infantis</i> /1	Adult NP	Serotype 13	Not tested
US1133ps23A	2015–2016 United States	<i>S. infantis</i> /1	Adult OP	Serogroup 23	Specific quelling activity against pneumococci of serogroup 23 serotypes; highest quelling against serotype 23A, followed by 23B and more weakly against 23F; OPK activity
US164cps18	2015–2016 United States	<i>S. infantis</i> /1	Adult OP	No reactivity	Not tested

^aIsolates recovered during 2015 to 2016 were retrospectively collected from stored specimens from a previously described U.S. adult pneumococcal carriage survey (10, 15). Isolates recovered during 2013 were retrospectively collected from stored specimens from a previously described Kenya pneumococcal carriage survey (14). The isolate recovered during 2019 was originally mistaken for a pneumococcal carriage isolate during a pneumococcal outbreak and was sent to the CDC *Streptococcus* laboratory for characterization.

^bAssignments are based on core genomic phylogeny as shown in Fig. 7.

This work gives a glimpse of the vast genomic diversity within the individual species of *S. mitis*, *S. oralis*, and *S. infantis*, among which expression of pneumococcal capsular serotypes is broadly dispersed. We note that the strains assigned here as *S. mitis* appear to lack a single major branch point that segregates them as a single species in our phylogenetic analysis.

It is relevant here to provide a summary of recent surveillance-based findings pertaining to the capsular serologic overlap found between pneumococci and close relative species. From relatively limited specimen testing in the United States, we have recovered isolates of commensal Mitis group species carrying homologs of pneumococcal *cps* loci (9 and 10 and this work) that predict vaccine serotypes or serogroups (1, 4, 5, 9V/9A, 18C/18A/18B/18F) and nonvaccine types (23A, 13). Extensive testing of URT specimens from individuals in the United States in the post-PCV era has demonstrated that pneumococcal carriage of PCV serotypes 1, 4, 5, and 9A/9V is rare in both young children and individuals 65 years or older (14, 15, 28, 29). While nonvaccine serotype 13 pneumococci have not been observed in these pneumococcal carriage studies, strains of the nonvaccine serotype 23A have been increasingly prevalent in pediatric carriage (31, 32) and among the top carried strains in older adults (10, 15), although carriage of any pneumococci is uncommon in older adults in the United States. All 5 of these vaccine serotypes or serogroups are also currently rare causes of IPD within the United States, although there has been resurgence of serotype 4 IPD in western states (33). Within ABCs in the year 2018, there were 8 IPD cases due to the nonvaccine serotype 13 and nonvaccine serotype 23A continued to be a major cause of IPD in the post-PCV vaccine era with 125 case isolates (34).

Studies in Kenya that have combined PCR serotype deduction and pneumococcal strain isolation together with limited nonpneumococcal strain isolation have been very informative. From relatively limited URT specimen testing in HIV-positive and HIV-negative adults, we have recovered isolates of commensal species carrying close homologs of pneumococcal *cps* loci (9 and 13 and this work) that predict vaccine serotypes or serogroups (types 2, 5, 9V/9A, 12F/12A/12B, 18C/18A/18B/18F, and 33F/33A) or nonvaccine types (15A/15F). Within certain adult high pneumococcal carriage populations in Kenya, so-called pneumococcal serotype-specific PCR signals from oropharyngeal specimens have been observed to be manyfold higher for numerous serotypes from nonpneumococcal strains than from pneumococcal strains (13). For example, 30 to 40% of adult URT specimens from both HIV-positive ($n = 118$) and HIV-negative ($n = 40$)

individuals in Kenya collected during 2009 were PCR²⁺ and PCR⁵⁺; however, comprehensive attempts to recover serotype 2 or 5 pneumococcal isolates from these specimens as well as from additional specimens collected from adults and young children during 2009 and 2013 have yielded only a few serotype 5 pneumococcal isolates and no serotype 2 pneumococcal isolates (13, 14). In accord with the abundance of their high serotype-specific PCR signal, culture-based screening of a limited number of specimens from this same area (9 and this work) has resulted in recovery of serotype 2 and serotype 5 nonpneumococcal strains (*S. oralis* strain KE9746cps2 described here and previously described *S. mitis* strain KE67013cps5 [9]). Although only one specimen collected from 158 adults in this previous study was PCR^{9A/9V+} and several serotype 9V isolates have been recovered from individuals in this area, a serotype 9A *S. oralis* strain (KE9676cps9A described here) has subsequently been recovered from an adult residing in this region during 2013.

The abundance of these capsular serotypes within nonpneumococcal species appears to coincide with great genomic diversity compared to pneumococci of the same or related serotypes. The known clonal diversity of serotypes 1, 4, 5, and 9A/9V is relatively limited within pneumococci (35, 36) (<https://pubmlst.org/>). In contrast, the nonpneumococcal isolates of the same serotypes described to date most often are distantly genetically related, with serotypes 5 and 9A even expressed from multiple nonpneumococcal species (this study for serotype 9A; reference 9 describes serotype 5 strains of *S. mitis*, *S. oralis*, and *S. infantis*). In a prior study, each of 4 randomly selected 230-bp serotype 2-specific amplicons from individual pneumococcus-negative specimens had a unique sequence that shared 98 to 99% sequence identity to each other and 97 to 98% identity to the corresponding pneumococcal reference *cps2* sequence, suggestive of broad strain diversity (13). Nonpneumococcal serotype 5-specific amplicons from that study revealed even more striking abundance (~40% of adult specimens) and diversity, with 6 unique 295-bp alleles sequenced that shared 96 to 99% sequence identity to the corresponding highly conserved pneumococcal serotype 5 *cps5* sequence.

There are many questions pertaining to what impact, if any, that the extensive interspecies capsular serotype overlap in the URT has on pneumococcal biology and carriage distributions. Studies to date have only scratched the surface of the extent of this overlap. In the carriage studies discussed (9, 10, 13–15), available URT specimens were screened comprehensively for pneumococcal isolates, while nonpneumococcal isolate screening has only been performed in limited URT specimens. Currently, this area of study lacks nonpneumococcal strain carriage data from younger individuals that more commonly carry pneumococcal strains. Pneumococci of serotypes 1, 2, and 5 were among the most deadly pathogens in the early 1900s (7) yet are currently rare causes of disease in the United States. PCR²⁺ signals have been observed within recent adult carriage specimens in the United States (data not shown); however, at the CDC we have not recovered serotype 2 pneumococci from specimens of any kind during the past 25 years (unpublished data). In view of the absence of serotypes 1 and 5 pneumococci in U.S. carriage specimens from all ages (10, 15, 31, 32), rarity in disease even before PCV implementation (35), and relatively frequent occurrence within nonpneumococcal species, it is tempting to speculate that immune responses to these commensal strains expressing these capsules serve to limit carriage distributions of their pneumococcal counterparts. At this point, such observations are only speculative and are subjective. For example, serotype 23A pneumococci were increasingly carried by children in the post-PCV era (32, 33), and serotype 23A strains of pneumococci were found within adult carriage specimens (10, 15). The nonpneumococcal strain from adult carriage described in this study that was cross-reactive with serotype 23A is likely to be representative of significant carriage of such strains judging from PCR-serotyping assays performed upon pneumococcal-negative carriage specimens. The scenario for serotype 9A carriage among nonpneumococcal strains in the United States is different from that seen for serotypes 1 and 5 in that the nearly identical PCV serotype 9V was

commonly carried and a common cause of IPD before PCV implementation (35). Currently, however, it is evident that PCR9A/9V⁺ specimens are found fairly frequently among adults in the United States, and our isolation data strongly suggest that these PCR signals correspond in part or in totality to different commensal nonpneumococcal species expressing serotype 9A.

The increasingly evident capsular serologic overlap between pneumococci and commensal species further highlights that the capsule is not a sole virulence determinant that distinguishes pneumococci from closely related species. Most members of *S. mitis* carry *cps* loci that are organizationally similar to those within *S. pneumoniae*. Besides capsule, pneumococci express an array of surface and secreted virulence factors that mediate evasion or exploitation of host immune responses, tissue damage, and invasion (37).

Whether commensal nonpneumococcal strains confer any protective immunity to pneumococci of the same serotypes is a question that is relevant for global pneumococcal strain distributions, past and present. It is conceivable that such naturally available commensal strains could serve as live vaccines. Recently, it has been demonstrated that mucosal immunization of mice with previously described *S. mitis* serotype 5 (9) induces Th17 and serotype-specific IgG/IgA responses against pneumococcal infection (38). It is not clear what impact current pneumococcal vaccines have on nonpneumococcal strains of vaccine serotypes, although a limited analysis suggested that such strains carrying *cps* loci corresponding to PCV serotypes were less common among vaccinated individuals (10). Key to understanding the underlying seroepidemiology of the Mitis group will be systematic surveillance for commensal strains expressing recognized pneumococcal serotypes in different age groups and populations and an analysis of their carriage-mediated effects upon individual and population immunity to pneumococcal carriage.

MATERIALS AND METHODS

Strains and genomic sequencing. All 9 isolates described in this study, carrying homologs of pneumococcal *cps* operons, were recovered from PCR serotype-positive URT specimens that were negative for the pneumococcal *lytA* qPCR assay (9, 10). These strains were classified as nonpneumococcal based upon optochin resistance, bile insolubility, and the absence of the *piaA* gene, which is highly conserved and ubiquitous among encapsulated pneumococcal strains (39). Eight of the nine isolates were recovered from OP specimens and 1 from an NP specimen. These 9 strains and 28 additional isolates recovered through ABC's IPD surveillance (26 invasive nonpneumococcal strains sent to verify as nonpneumococcal, two nontypeable pneumococci) were subjected to genomic sequencing on the Illumina MiSeq platform as previously described (33, 34, 36). Accession numbers for the 9 isolates that are the focus of this study, and 28 additional strains employed for phylogenetic analysis are provided in Table S1 in the supplemental material.

U.S. nonpneumococcal carriage strains. Four hundred sixty paired respiratory OP and NP swabs from a pneumococcal carriage study in the United States carried out during 2015 to 2016 in healthy adults aged 65 years and older were assessed for pneumococcal carriage using culture-based methods (10, 15) and 450 of these individuals were culture negative for pneumococci. A limited *lytA* qPCR-negative subset of the corresponding 450 paired specimens was assessed for nonpneumococcal strains positive for certain PCR serotypes detected by sequential cPCR and qPCR assays (19–21) (updates at <https://www.cdc.gov/streplab/pneumococcus/resources.html>). From this U.S. carriage study, nonpneumococcal strains US67cps4, US302cps4, US64cps9A, US3cps9A, US164cps18, and US1133cps23A were obtained from stored OP swab specimens and isolated as previously described (9, 10). Strain US3019cps13 was collected from an adult NP carriage specimen during an investigation of an IPD outbreak in a U.S. prison during 2019 (our unpublished data).

Kenya nonpneumococcal carriage strains. Nonpneumococcal strain KE9746cps2 and KE9676cps9A were retrospectively recovered from stored adult OP specimens (2,028 of 2,590 [78.3%] individuals were HIV positive) collected during 2013 from a carriage study in Kenya (14). We lack information linking these isolates with HIV status.

***cps* operon alignments.** Reference pneumococcal *cps* operons used in figures are described in depth in references 3 and 5. Each of these reference loci lay between the *dexB* 3' end and the *alia* 5' ends. Corresponding genomic segments were extracted from genomic sequences of the 9 nonpneumococcal strains that were the study's focus and subjected to Prokka to identify and annotate open reading frames (40). The annotated sequences were analyzed by BLAST, and the *cps* regions were aligned into figures using EasyFig 2.2.3 (41).

Species assignments Strains were assigned species through clustering with previously speciated strains (9, 28) employing core genomic kSNP3.0 (42) analysis to generate a single-nucleotide polymorphism matrix and a parsimony phylogenetic tree that represents a consensus tree and node support

based on an extended majority rule consensus of the equally most parsimonious trees from a sample of 100 trees. A phylogenetic tree was generated employing the iTOL (43) program (<https://itol.embl.de/>).

Institutional approvals. Centers for Disease Control and Prevention (CDC) and local institutional review boards approved the studies (CDC protocol number 6725), and it was approved by ethics committees as described previously (9, 14, 15).

Preparation of streptococcal antisera. This protocol (number 3028GERRABC) was approved by the Institutional Animal Care and Use Committee (IACUC). Antisera against formalin-fixed strains US67cps4, US64cps9A, and US1133cps23A were prepared exactly as described for CDC capsular typing antisera prepared against pneumococcal strains (44).

Serology. Latex agglutination and the Quellung reaction employing CDC rabbit polyclonal typing antiserum were used as described previously (44). Double immunodiffusion assays employing pneumococcal typing sera and antisera prepared against commensal streptococci were carried out as previously described (9, 10) using Pierce standard Ouchterlony agarose gel plates (Pierce number 31111; ThermoFisher Scientific).

OPK assays. OPK assays were performed employing HL-60 cells and complement source (baby rabbit serum; Pel-Freez, Brown Deer, WI) as outlined previously (45). Bacterial strains were grown overnight in Todd-Hewitt broth supplemented with 0.5% yeast extract. A 500- μ l aliquot was incubated in 25 ml of the same growth medium and grown to an optical density at 650 nm of 0.4. Glycerol was added to each culture to a final concentration of 20% before dispensing 1-ml aliquots in cryovials and flash freezing in an ethanol-dry ice bath. Frozen cultures were kept at $\leq -70^{\circ}\text{C}$ for further use. Viable counts of aliquots were performed to determine the concentration of pneumococci before and after the freezing protocol, and 1,000 CFU/20 μ l was used in each OPK assay. For each nonpneumococcal strain (US64cps9A and US67cps4), serum samples were obtained from 2 to 3 rabbits inoculated over a period of 6 weeks with the formalin-fixed vaccine. Pooled samples from 2 to 3 animals were used for OPK experiments following verification of capsule expression by immunodiffusion and Quellung reaction against the reference serotype 4 and serogroup 9 *S. pneumoniae* strains.

Geometric mean titer (GMT) values with $>50\%$ killing compared with the growth in the complement control wells were generated across 4 to 8 assay runs. For serotype 4 pneumococcal typing serum, antiserum against US67cps4, pooled pneumococcal serogroup 9 antiserum, and antiserum against US64cps9A, 2-fold dilutions (1/8 through 1/1,024) and 3-fold dilutions (1/400 through 1/874,800) were employed. An additional 2-fold series from 1:400 (1:400 through 1:51,200) of pneumococcal type 4 antiserum was also used. Given the lack of response with types 9L and 9N, we additionally assayed high concentrations (from 1:1 through 1:128) for serogroup 9 strains.

Uronic acid assay. Glucuronic acid within serotypes 2 and 9A was assayed by a method that exploits the appearance of a chromogen when uronic acid is heated to 100°C in concentrated sulfuric acid-tetraborate followed by the addition of meta-hydroxydiphenyl (22), as used for bacterial suspensions (23, 24). Strains were grown on trypticase soy agar containing 5% sheep's blood plates overnight at 37°C , 5% CO_2 , which was used to inoculate Todd Hewitt broth plus neopeptone (THBN) to mid-log phase and then held overnight at 4°C . The next day, 500 μ l was transferred to 30 ml THBN at 37°C , 5% CO_2 and incubated to the end of log phase. Dilution was used to standardize the A_{660} readings to 0.5. Aliquots of 10 ml were then centrifuged at $8,000 \times g$ to obtain pellets. Pellets were washed $3 \times$ in 1 ml $1 \times$ phosphate-buffered saline (PBS) at 4°C at $8,000 \times g$. Pellets were resuspended in 1 ml of 150 mM Tris-HCl, pH 7, and 1 mM MgSO_4 and centrifuged again at 4°C at $8,000 \times g$. The supernatant was removed, and the pellets were resuspended in 500 μ l of 150 mM Tris-HCl, pH 7, and 1 mM MgSO_4 , and 200 μ l was transferred to a new tube; 1.2 ml of 12.5 mM sodium tetraborate in concentrated sulfuric acid was added, and the tubes were mixed and then heated in a hot bath at 100°C for 5 min and transferred to an ice bath. A blank of 200 μ l 150 mM Tris-HCl, pH 7, 1 mM MgSO_4 , and 1.2 ml 12.5 mM sodium tetraborate was prepared and heated to 100°C for 5 min. Twenty microliters of 0.15% m-hydroxybiphenyl in 0.5% NaOH was added to each sample, with 20 μ l of 0.5% NaOH substituted in the blank. The samples were immediately transferred to borosilicate cuvettes and read at 520 nm. Standards of glucuronic acid were prepared in water, including 1 mg/ml, 0.1 mg/ml, 0.02 mg/ml, 0.01 mg/ml, and 0.005 mg/ml, and, as with the samples, 200 μ l of each preparation was added to 12.5 mM sodium tetraborate in concentrated sulfuric acid, mixed well, heated to 100°C , transferred to an ice bath, had 20 μ l 0.15% m-hydroxybiphenyl in 0.5% NaOH added, and were transferred to borosilicate cuvettes for readings at 520 nm.

Data availability. The BioProject number for SAMN files from this work as well as references 7 and 8 (all numbers are provided in Table S1) is [PRJNA480039](https://doi.org/10.21968/PRJNA480039).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 0.1 MB.

TABLE S1, DOCX file, 0.04 MB.

ACKNOWLEDGMENTS

We thank Zhongya Li, Theresa Tran, and Hollis Walker for generating whole-genome sequences of the nonpneumococcal streptococcal strains that were the focus of this study. We are very grateful to So Hee Park for her assistance with the OPK technique. We thank Rex Howard and Will Thomas for their guidance with antisera preparation. We

thank the following individuals who organized and contributed to the U.S. adult pneumococcal carriage project: Fernanda C. Lessa, Nadine G. Roupael, Nancy M. Bennett, H. Keipp Talbot, Lee H. Harrison, Monica M. Farley, and Cynthia G. Whitney. We thank the following individuals who contributed to the Kenya adult pneumococcal carriage project: Laura Conklin, Geoffrey Jagero, Mothoni Junghae, Daniel Feikin, and Robert Breiman.

REFERENCES

- Ganaie F, Saad JS, McGee L, van Tonder AJ, Bentley SD, Lo SW, Gladstone RA, Turner P, Keenan JD, Breiman RF, Nahm MH. 2020. A new pneumococcal capsule type, 10D, is the 100th serotype and has a large cps fragment from an oral *Streptococcus*. mBio 11:e00937-20. <https://doi.org/10.1128/mBio.00937-20>.
- Sorensen UBS, Henrichsen J, Chen H-C, Szu SC. 1990. Covalent linkage between the capsular polysaccharide and the cell wall peptidoglycan of *Streptococcus pneumoniae* revealed by immunochemical methods. Microb Pathog 8:325–334. [https://doi.org/10.1016/0882-4010\(90\)90091-4](https://doi.org/10.1016/0882-4010(90)90091-4).
- Mavroidi A, Aanensen DM, Godoy D, Skovsted IC, Kalltoft MS, Reeves PR, Bentley SD, Spratt BG. 2007. Genetic relatedness of the *Streptococcus pneumoniae* capsular biosynthetic loci. J Bacteriol 189:7841–7855. <https://doi.org/10.1128/JB.00836-07>.
- Larson TR, Yother J. 2017. *Streptococcus pneumoniae* capsular polysaccharide is linked to peptidoglycan via a direct glycosidic bond to β -D-N-acetylglucosamine. Proc Natl Acad Sci U S A 114:5695–5700. <https://doi.org/10.1073/pnas.1620431114>.
- Aanensen DM, Mavroidi A, Bentley SD, Reeves PR, Spratt BG. 2007. Predicted functions and linkage specificities of the products of the *Streptococcus pneumoniae* capsular biosynthetic loci. J Bacteriol 189:7856–7876. <https://doi.org/10.1128/JB.00837-07>.
- Cooper G, Rosenstein C, Walter A, Peizer L. 1932. The further separation of types among the pneumococci hitherto included in group IV and the development of therapeutic antisera for these types. J Exp Med 55:531–554. <https://doi.org/10.1084/jem.55.4.531>.
- Cecil RL, Plummer N. 1932. Pneumococcus type II pneumonia, a clinical and bacteriologic study of one thousand cases with especial reference to serum therapy. JAMA 98:779–786. <https://doi.org/10.1001/jama.1932.02730360001001>.
- Kauffmann F, Mørch E, Schmith K. 1940. On the serology of the pneumococcus-group. J Immunol 39:397–426.
- Pimenta F, Gertz RE, Jr, Park SH, Kim E, Moura I, Milucky J, Roupael N, Farley MM, Harrison LH, Bennett NM, Bigogo G, Feikin DR, Breiman R, Lessa FC, Whitney CG, Rajam G, Schiffer J, Carvalho MDG, Beall B. 2018. *Streptococcus infantis*, *Streptococcus mitis*, and *Streptococcus oralis* strains with highly similar cps5 loci and antigenic relatedness to serotype 5 pneumococci. Front Microbiol 9:3199.
- Lessa FC, Milucky J, Roupael N, Bennett NM, Talbot K, Harrison LH, Farley MM, Walston J, Pimenta F, Gertz RE, Jr, Rajam G, Carvalho MDG, Beall B, Whitney CG. 2018. *Streptococcus mitis* expressing pneumococcal serotype 1 capsule. Sci Rep 8:17959. <https://doi.org/10.1038/s41598-018-35921-3>.
- Skov Sørensen UB, Yao K, Yang Y, Tettelin H, Kilian M. 2016. Capsular polysaccharide expression in commensal streptococcus species: genetic and antigenic similarities to *Streptococcus pneumoniae*. mBio 7:e01844-16. <https://doi.org/10.1128/mBio.01844-16>.
- Nahm MH, Brissac T, Kilian M, Vlach J, Orihuela CJ, Saad JS, Ganaie F. 2020. Pneumococci can become virulent by acquiring a new capsule from oral streptococci. J Infect Dis 222:372–380. <https://doi.org/10.1093/infdis/jiz456>.
- Carvalho MDA, Pimenta FC, Moura I, Roundtree A, Gertz RE, Jr, Li Z, Jagero G, Bigogo G, Junghae J, Conklin L, Feikin DR, Breiman RF, Whitney CG, Beall BW. 2013. Non-pneumococcal mitis-group streptococci confound detection of pneumococcal capsular serotype-specific loci in upper respiratory tract. PeerJ 1:e97. <https://doi.org/10.7717/peerj.97>.
- Kobayashi M, Bigogo G, Kim L, Mogeni OD, Conklin LM, Odoyo A, Odiembo H, Pimenta F, Ouma D, Harris AM, Odero K, Milucky JL, Ouma A, Aol G, Audi A, Onyango C, Cosmas L, Jagero G, Farrar JL, da Gloria Carvalho M, Whitney CG, Breiman RF, Lessa FC. 2020. Impact of 10-valent pneumococcal conjugate vaccine introduction on pneumococcal carriage and antibiotic susceptibility patterns among children aged <5 years and adults with human immunodeficiency virus infection: Kenya, 2009–2013. Clin Infect Dis 70:814–826.
- Milucky J, Carvalho MG, Roupael N, Bennett NM, Talbot HK, Harrison LH, Farley MM, Walston J, Pimenta F, Lessa FC, Bower M, McNair N, Williams S, Presmanes E, Tunali A, Thomas S, Hollick R, Langdon J, Salar Sepehri A, Sharova A. 2019. *Streptococcus pneumoniae* colonization after introduction of 13-valent pneumococcal conjugate vaccine for US adults 65 years of age and older, 2015–2016. Vaccine 37:1094–1100. <https://doi.org/10.1016/j.vaccine.2018.12.075>.
- Dowson CG, Hutchison A, Brannigan JA, George RC, Hansman D, Liñares D, Tomasz A, Smith JM, Spratt BG. 1989. Horizontal transfer of penicillin-binding protein genes in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. Proc Natl Acad Sci U S A 86:8842–8846. <https://doi.org/10.1073/pnas.86.22.8842>.
- Carvalho MGS, Tondella ML, McCaustland K, Weidlich L, McGee L, Mayer LW, Steigerwalt A, Whaley M, Facklam RR, Fields B, Carlone G, Ades EW, Dagan R, Sampson JS. 2007. Evaluation and improvement of real-time PCR assays targeting *lytA*, *ply*, and *psaA* genes for detection of pneumococcal DNA. J Clin Microbiol 45:2460–2466. <https://doi.org/10.1128/JCM.02498-06>.
- Farrar JL, Odiembo H, Odoyo A, Bigogo G, Kim L, Lessa FC, Feikin DR, Breiman RF, Whitney CG, Carvalho MG, Pimenta FC. 2020. Limited added value of oropharyngeal swabs for detecting pneumococcal carriage in adults. Open Forum Infect Dis 7:ofaa368. <https://doi.org/10.1093/ofid/ofaa368>.
- Pai R, Gertz RE, Beall B. 2006. Sequential multiplex PCR approach for determining capsular serotypes of *Streptococcus pneumoniae* isolates. J Clin Microbiol 44:124–131. <https://doi.org/10.1128/JCM.44.1.124-131.2006>.
- Pimenta FC, Roundtree A, Soysal A, Bakir M, Du Plessis M, Wolter N. 2013. Sequential triplex real-time PCR assay for detecting 21 pneumococcal capsular serotypes that account for a high global disease burden. J Clin Microbiol 51:647–652. <https://doi.org/10.1128/JCM.02927-12>.
- Da Gloria Carvalho M, Pimenta FC, Jackson D, Roundtree A, Ahmad Y, Millar EV, O'Brien KL, Whitney CG, Cohen AL, Beall BW. 2010. Revisiting pneumococcal carriage by use of broth enrichment and PCR techniques for enhanced detection of carriage and serotypes. J Clin Microbiol 48:1611–1618. <https://doi.org/10.1128/JCM.02243-09>.
- Blumenkrantz N, Asboe-Hansen G. 1973. New method for quantitative determination of uronic acids. Anal Biochem 54:484–489. [https://doi.org/10.1016/0003-2697\(73\)90377-1](https://doi.org/10.1016/0003-2697(73)90377-1).
- Morona JK, Morona R, Paton JC. 2006. Attachment of capsular polysaccharide to the cell wall of *Streptococcus pneumoniae* type 2 is required for invasive disease. Proc Natl Acad Sci U S A 103:8505–8510. <https://doi.org/10.1073/pnas.0602148103>.
- Zheng YD, Pan Y, He K, Li N, Yang D, Du GF, Ge R, He QY, Sun X. 2020. SPD_1495 contributes to capsular polysaccharide synthesis and virulence in *Streptococcus pneumoniae*. mSystems 5:e00025-20. <https://doi.org/10.1128/mSystems.00025-20>.
- Calix JJ, Oliver MB, Sherwood LK, Beall BW, Hollingshead SK, Nahm MH. 2011. *Streptococcus pneumoniae* serotype 9A isolates contain diverse mutations to *wcjE* that result in variable expression of serotype 9V-specific epitope. J Infect Dis 204:1585–1595. <https://doi.org/10.1093/infdis/jir593>.
- Robbins JB, Austrian R, Lee CJ, Rastogi SC, Schiffman G, Henrichsen J, Mäkelä PH, Broome CV, Facklam RR, Tiesjema RH. 1983. Considerations for formulating the second-generation pneumococcal capsular polysaccharide vaccine with emphasis on the cross-reactive types within groups. J Infect Dis 148:1136–1159. <https://doi.org/10.1093/infdis/148.6.1136>.
- Ravenscroft N, Omar A, Hlozek J, Edmonds-Smith C, Follador R, Serventi F, Lipowsky G, Kuttel MM, Cescutti P, Faridmoayer A. 2017. Genetic and structural elucidation of capsular polysaccharides from *Streptococcus*

- pneumoniae* serotype 23A and 23B, and comparison to serotype 23F. *Carbohydr Res* 450:19–29. <https://doi.org/10.1016/j.carres.2017.08.006>.
28. Jensen A, Scholz CF, Kilian M. 2016. Re-evaluation of the taxonomy of the Mitis group of the genus *Streptococcus* based on whole genome phylogenetic analyses, and proposed reclassification of *Streptococcus dentisani* as *Streptococcus oralis* subsp. *dentisani* comb. nov., *Streptococcus tigurinus* as *Streptococcus oralis* subsp. *tigurinus* comb. nov., and *Streptococcus oligofermentans* as a later synonym of *Streptococcus cristatus*. *Int J Syst Evol Microbiol* 66:4803–4820. <https://doi.org/10.1099/ijsem.0.001433>.
 29. Lull D, Muñoz R, López R, García E. 1999. A single gene (*tts*) located outside the cap locus directs the formation of *Streptococcus pneumoniae* type 37 capsular polysaccharide. *J Exp Med* 190:241–252. <https://doi.org/10.1084/jem.190.2.241>.
 30. Sheppard CL, Kapatai G, Broughton K, Schaefer U, Hannah M, Litt DJ, Fry NK. 2017. Clinical streptococcal isolates, distinct from *Streptococcus pneumoniae*, but containing the beta-glucosyltransferase *tts* gene and expressing serotype 37 capsular polysaccharide. *PeerJ* 5:e3571. <https://doi.org/10.7717/peerj.3571>.
 31. Sharma D, Baughman W, Holst A, Thomas S, Jackson D, da Gloria Carvalho M, Beall B, Satola S, Jerris R, Jain S, Farley MM, Nuorti JP. 2013. Pneumococcal carriage and invasive disease in children before introduction of the 13-valent conjugate vaccine: comparison with the era before 7-valent conjugate vaccine. *Pediatr Infect Dis J* 32:e45–e53. <https://doi.org/10.1097/INF.0b013e3182788fdd>.
 32. Desai AP, Sharma D, Crispell EK, Baughman W, Thomas S, Tunalı A, Sherwood L, Zmitrovich A, Jerris R, Satola SW, Beall B, Moore MR, Jain S, Farley MM. 2015. Decline in pneumococcal nasopharyngeal carriage of vaccine serotypes after the introduction of the 13-valent pneumococcal conjugate vaccine in children in Atlanta. *Georgia Pediatr Infect Dis J* 34:1168–1174. <https://doi.org/10.1097/INF.0000000000000849>.
 33. Beall B, Walker H, Tran T, Li Z, Varghese J, McGee L, Li Y, Metcalf BJ, Gierke R, Mosites E, Chochua S, Pilishvili T. 2020. Upsurge of conjugate vaccine serotype 4 invasive pneumococcal disease clusters among adults experiencing homelessness in California, Colorado, and New Mexico. *J Infect Dis* <https://doi.org/10.1093/infdis/jiaa501>.
 34. Metcalf BJ, Chochua S, Walker H, Tran T, Li Z, Varghese J, Snippes Vagnone PM, Lynfield R, McGee L, Li Y, Pilishvili T. 2020. Invasive pneumococcal strain distributions and isolate clusters associated with persons experiencing homelessness during 2018. *Clin Infect Dis Nov* 5:ciaa1680. <https://doi.org/10.1093/cid/ciaa1680>.
 35. Gertz RE, Jr, McEllistrem MC, Boxrud DJ, Li Z, Sakota V, Thompson TA, Facklam RR, Besser JM, Harrison LH, Whitney CG, Beall B. 2003. Clonal distribution of invasive pneumococcal isolates from children and selected adults in the United States prior to 7-valent conjugate vaccine introduction. *J Clin Microbiol* 41:4194–4216. <https://doi.org/10.1128/JCM.41.9.4194-4216.2003>.
 36. Beall B, Chochua S, Gertz RE, Jr, Li Y, Li Z, McGee L, Metcalf BJ, Ricaldi J, Tran T, Walker H, Pilishvili T. 2018. A population-based descriptive atlas of invasive pneumococcal strains recovered within the U.S. during 2015–2016. *Front Microbiol* 9:2670. <https://doi.org/10.3389/fmicb.2018.02670>.
 37. Weiser JN, Ferreira DM, Paton JC. 2018. *Streptococcus pneumoniae*: transmission, colonization and invasion. *Nat Rev Microbiol* 16:355–367. <https://doi.org/10.1038/s41579-018-0001-8>.
 38. Shekhar S, Åmdal HA, Petersen FC. 30 March 2021. Vaccination with the commensal *Streptococcus mitis* expressing pneumococcal serotype 5 capsule elicits IgG/IgA and Th17 responses against *Streptococcus pneumoniae*. *Front Immunol* <https://doi.org/10.3389/fimmu.2021.676488>.
 39. Whalan RH, Funnell SG, Bowler LD, Hudson MJ, Robinson A, Dowson CG. 2006. Distribution and genetic diversity of the ABC transporter lipoproteins PiuA and PiaA within *Streptococcus pneumoniae* and related streptococci. *J Bacteriol* 188:1031–1038. <https://doi.org/10.1128/JB.188.3.1031-1038.2006>.
 40. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30:2068–2069. <https://doi.org/10.1093/bioinformatics/btu153>.
 41. Sullivan MJ, Petty NK, Beatson SA. 2011. Easyfig: a genome comparison visualizer. *Bioinformatics* 27:1009–1010. <https://doi.org/10.1093/bioinformatics/btr039>.
 42. Gardner SN, Slezak T, Hall BG. 2015. kSNP3.0: SNP detection and phylogenetic analysis of genomes without genome alignment or reference genome. *Bioinformatics* 31:2877–2878. <https://doi.org/10.1093/bioinformatics/btv271>.
 43. Letunic I, Bork P. 2019. Interactive Tree of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res* 47:W256–W259. <https://doi.org/10.1093/nar/gkz239>.
 44. Lund E, Henriksen J. 1978. Laboratory diagnosis, serology and epidemiology of *Streptococcus pneumoniae*, p 1241–1262. In Bergan T, Norris JR (ed), *Methods in microbiology*. Academic Press, London, United Kingdom.
 45. Romero-Steiner S, Libutti D, Pais LB, Dykes J, Anderson P, Whitin JC, Keyserling HL, Carlone GM. 1997. Standardization of an opsonophagocytic assay for the measurement of functional antibody activity against *Streptococcus pneumoniae* using differentiated HL-60 cells. *Clin Diagn Lab Immunol* 4:415–422. <https://doi.org/10.1128/CDLI.4.4.415-422.1997>.