

Invasive *Streptococcus oralis* Expressing Serotype 3 Pneumococcal Capsule, Japan

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We report 2 adult cases of invasive disease in Japan caused by *Streptococcus oralis* that expressed the serotype 3 pneumococcal capsule and formed mucoid colonies. Whole-genome sequencing revealed that the identical serotype 3 pneumococcal capsule locus and *hyl* fragment were recombined into the genomes of 2 distinct *S. oralis* strains.

Streptococcus oralis is a viridans streptococcus that is divided into 3 subspecies *S. oralis* subsp. *oralis*, *dentisani*, and *tigurinus* (1). Differentiation between these subspecies and other α -hemolytic streptococci, including *S. pneumoniae*, remains difficult because they share similar biochemical properties. *S. oralis* inhabits the oral cavity and can cause severe infections in persons with immunodeficiency (2). Antimicrobial drug resistance and capsule expression studies have demonstrated that gene transfer can occur from oral *Streptococcus* spp. to *S. pneumoniae* (3–5). Most oral *Streptococcus* spp. have a pneumococcus-like capsule locus and produce capsular polysaccharides (6).

We report 2 cases of invasive streptococcal disease in older adults in Japan (Table). Case 1 occurred in a 69-year-old man with gastric cancer; case 2 occurred in a 78-year-old man with bacteremic meningitis who had no underlying disease. Both patients were successfully treated with antimicrobial agents. The bacterial isolates (ASP0312-Sp from case 1 and SP2752 from case 2) contained α -hemolytic bacteria that formed characteristic mucoid colonies on blood agar (Table). Quellung reactions were strongly positive for pool R or pneumococcal serotype 3 antisera (Statens Serum Institut, <https://en.ssi.dk>), suggesting that the isolates were *S. pneumoniae* serotype 3. However, both isolates were optochin-resistant and bile-insoluble. Moreover, multilocus sequence

typing (MLST) showed that the sequences of all 7 alleles of ASP0312-Sp and 5 alleles of SP2752 differed from those registered in the MLST database (<https://pubmlst.org>) (Table). For SP2752, the allele numbers were 341 for *gdh* and 406 for *spi*. Furthermore, we observed nucleotide differences between ASP0312-Sp and SP2752 in *aroE* (31 different bp), *gdh* (34 bp), *gki* (25 bp), *recP* (25 bp), *spi* (14 bp), *xpt* (47 bp), and *ddl* (15 bp), which indicated that the strains were distinct. These results suggested that the 2 strains were non-pneumococcal *Streptococcus* spp.

For species identification, we performed phylogenetic analyses of whole-genome sequences (Appendix, <https://wwwnc.cdc.gov/EID/article/28/8/21-2176-App1.pdf>). Homologous core gene clustering showed that ASP0312-Sp and SP2752 belonged to the *S. oralis* clade (Figure); they were distant from one another, which was consistent with the MLST results.

To investigate recombination events, we compared the sequences surrounding the capsule loci of ASP0312-Sp and SP2752 with those of *S. oralis* subsp. *tigurinus* osk_001 and *S. pneumoniae* serotype 3 OXC141 (Appendix Figure). For ASP0312-Sp, the sequence corresponding to the downstream region of *nsik* up to the 5' terminus of the gene encoding the cell wall binding repeat protein in osk_001 was replaced by a fragment of ≈ 30 kb from pneumococcus. For SP2752, the sequence encoding an ATPase up to the 5' terminus of the gene encoding the cell wall binding repeat protein in osk_001 was replaced by a fragment of ≈ 16 kb from pneumococcus. The capsule sequences of ASP0312-Sp and SP2752 were 100% identical to the corresponding sequences located from 303730 to 312820 bp in HU-OH (GenBank accession no. AP018937.1), a serotype 3 pneumococcal strain that was isolated in Japan (7).

We performed homology searches of 36 known pneumococcal virulence genes because multifragment recombination has been demonstrated during the capsular transformation process in pneumococcal populations (8). In ASP0312-Sp and SP2752, the *hyl* gene, which encodes hyaluronate lyase (9), was located distantly from the capsule locus and shared 96% identity with that of *S. pneumoniae*. We did not detect homologs of the other 35 genes for either isolate.

A recent study reported that acapsular pneumococcus became virulent after transformation with the capsule gene from SK95, which is an oral *S. mitis* strain (5). This previous study demonstrated a cross-species transformation from a commensal streptococcal species to pneumococcus (5). Our results complement this report, although the direction of transformation in our study was reversed. Our analyses of 2 human

Table. Characteristics of invasive *Streptococcus oralis* expressing serotype 3 pneumococcal capsule from 2 adult patients, Japan*

| Case | Onset date | Isolate ID | Source | Positive Quellung reaction | No. different bases | | | | | | |
|------|--------------|------------|------------|----------------------------|---------------------|------------|------------|-------------|------------|------------|------------|
| | | | | | <i>aroE</i> | <i>gdh</i> | <i>gki</i> | <i>recP</i> | <i>spi</i> | <i>xpt</i> | <i>ddl</i> |
| 1 | January 2015 | ASP0312-Sp | Blood | Pool R, serotype 3 | 61 | 30 | 44 | 32 | 4 | 41 | 37 |
| 2 | April 2014 | SP2752 | Blood, CSF | Pool R, serotype 3 | 54 | -† | 40 | 33 | -† | 47 | 36 |

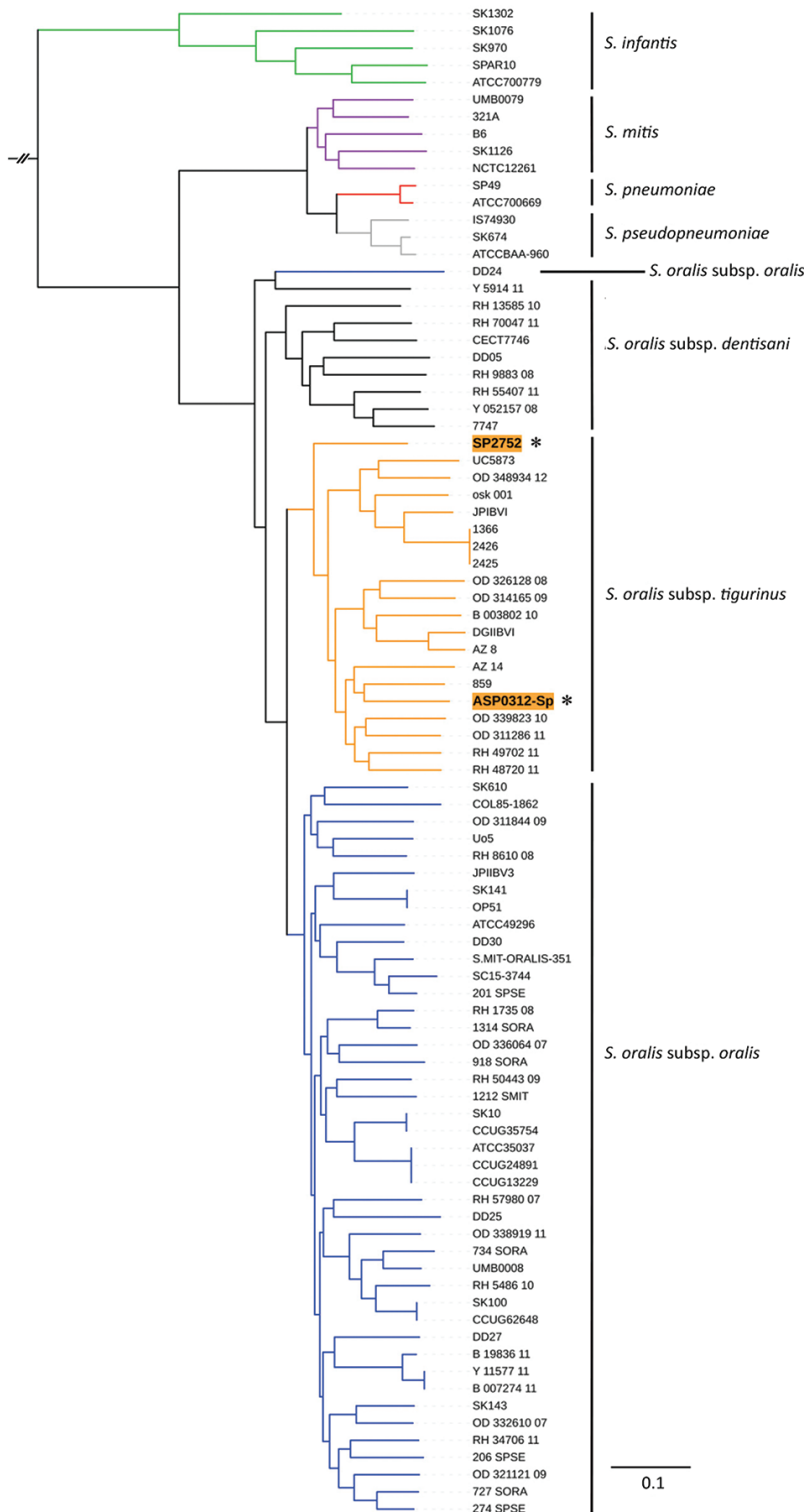


Figure. Phylogenetic analysis of invasive *Streptococcus oralis* expressing serotype 3 pneumococcal capsule from 2 adult patients, Japan. Asterisks and orange shading indicate genomes from isolates ASP0312-Sp and SP2752 identified in this study. Homologous core gene clusters of 71 strains from 3 *Streptococcus oralis* subsp., 2 *S. pneumoniae*, 5 *S. mitis*, 5 *S. infantis*, and 3 *S. pseudopneumoniae* were downloaded from the National Center for Biotechnology Information database (<https://www.ncbi.nlm.nih.gov>) and compared with the ASP0312-Sp and SP2752 genomes. Branch lengths represent the genetic distance. Scale bar indicates nucleotide substitutions per site.

patients with invasive disease caused by *S. oralis* provided evidence of cross-species gene transfer from pneumococcus to a commensal streptococcal species. Acquisition of capsule and *hyl* genes might have increased pathogenicity (9,10) and contributed to progression of invasive disease in these 2 cases.

In conclusion, because of discrepancies between phenotypic and biochemical analyses, we used MLST and whole-genome sequencing to identify streptococcal species in these 2 patients. Our study indicates a potential pitfall for identifying and serotyping pneumococci that can occur if the bacteria are not isolated. Thus, when α -hemolytic streptococci are isolated from a sterile site, clinicians should request molecular analyses to identify the causative species, regardless of the mucoid phenotype.

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Hepatitis E Virus Outbreak among Tigray War Refugees from Ethiopia, Sudan

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Appendix

Culturing Methods, Biochemical Tests, Serotyping, and Multilocus Sequence Typing (MLST)

The isolated streptococcal strains were incubated at 37°C on agar plates prepared with BD Columbia Agar with 5% Sheep Blood (Becton Dickinson, <https://www.bd.com>). Optochin susceptibility and bile solubility tests were performed as described elsewhere (1). Quellung reaction tests with pneumococcal antisera (Statens Serum Institut, Copenhagen, Denmark) were performed in accordance with the manufacturer's protocol. MLST was performed according to the method for *S. pneumoniae* described on the MLST website (<https://pubmlst.org/spneumoniae>). Sequences of the *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl* alleles were assembled and compared with the registered sequences listed on the website.

Whole-Genome Sequencing and Phylogenetic Analysis

After single-colony isolation, genomic DNA from ASP0312-Sp and SP2752 was purified by using the DNeasy Blood & Tissue Kit (QIAGEN, <https://www.qiagen.com>). Genomic DNA

libraries were constructed by using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and then sequenced by using a MiSeq system (Illumina). Genome assembly was performed by using SPAdes version 3.13.1 with the --careful option and a read coverage cutoff value of 10 (2). For species identification, a phylogenetic analysis of the whole-genome sequence was performed. The sequences of 86 streptococcal strains (*Streptococcus oralis* subsp. *oralis*, n = 44; *S. oralis* subsp. *dentisani*, n = 9; *S. oralis* subsp. *tigurensis*, n = 18; *S. pneumoniae*, n = 2; *S. mitis*, n = 5; *S. infantis*, n = 5; and *S. pseudopneumoniae*, n = 3) for which draft genome sequences have been published previously were obtained from the public database (3,4) and used as references. The species and subspecies of the reference strains were based on the results of the phylogenetic tree analysis of Velsko et al. (4). Annotation of the draft genomes was performed by using the DDBJ Fast Annotation and Submission Tool (5). A core gene alignment was generated by using Roary with the -e-mafft option and used for the determination of phylogenetic relationships. Phylogenetic trees were reconstructed with the maximum likelihood method by using IQ-TREE with 1,000 ultrafast bootstrap replicates (6,7). The trees were visualized by using iTOL version 3 (8). Sequences surrounding the capsule cluster were extracted from contigs of the draft genomes of ASP0312-Sp and SP2752. If necessary, PCR primer sets designed to cover gaps between the contigs and amplicon sequences were confirmed by using Sanger sequencing.

Homology Search of Pneumococcal Genes

Homology searches of 36 known pneumococcal virulence genes within the genomes of ASP0312-Sp and SP2752 were performed by using GENETYX (GENETYX, Tokyo, Japan). The

36 genes (encoded factors) were: *bgaA* (β -galactosidase), *cbp* (choline binding protein) *A*, *cbpC*, *cbpD*, *cbpE*, *cbpF*, *cbpG*, *cbpI*, *cbpJ*, *cbpL*, *cbpM*, *endoD* (endo- β -N-acetylglucosaminidase), *eno* (enolase), *fic* (Fic domain protein), *htrA* (high-temperature requirement protein A), *hyl* (hyaluronate lyase), *iga* (immunoglobulin A1 protease), *lyt* (autolysin) *A*, *lytB*, *nan* (neuraminidase) *A*, *nanB*, *pavA* (pneumococcal adhesion and virulence A), *pcpA* (pneumococcal choline binding protein A), *pfbA* (plasmin- and fibronectin-binding protein A), *pht* (polyhistidine triad protein) *A*, *phtB*, *phtD*, *phtE*, *ply* (pneumolysin), *psaA* (pneumococcal surface antigen A), *pspA* (pneumococcal surface protein A), *spxB* (pyruvate oxidase), *strH* (β -N-acetylglucosaminidase), *sub* (subtilase family protein), *zmpB* (zinc metalloproteinase B), and *zmpC* (zinc metalloproteinase C).

Nucleotide Sequence Accession Numbers

For ASP0312-Sp and SP2752, we deposited the whole-genome sequences (GenBank accession nos. DRX277946 and DRX232035, respectively), sequences around the capsule (GenBank accession nos. LC601604 and LC601606, respectively), and *hyl* sequences (GenBank accession nos. LC602142 and LC602144, respectively) in the DNA Data Bank of Japan (<https://www.ddbj.nig.ac.jp>).

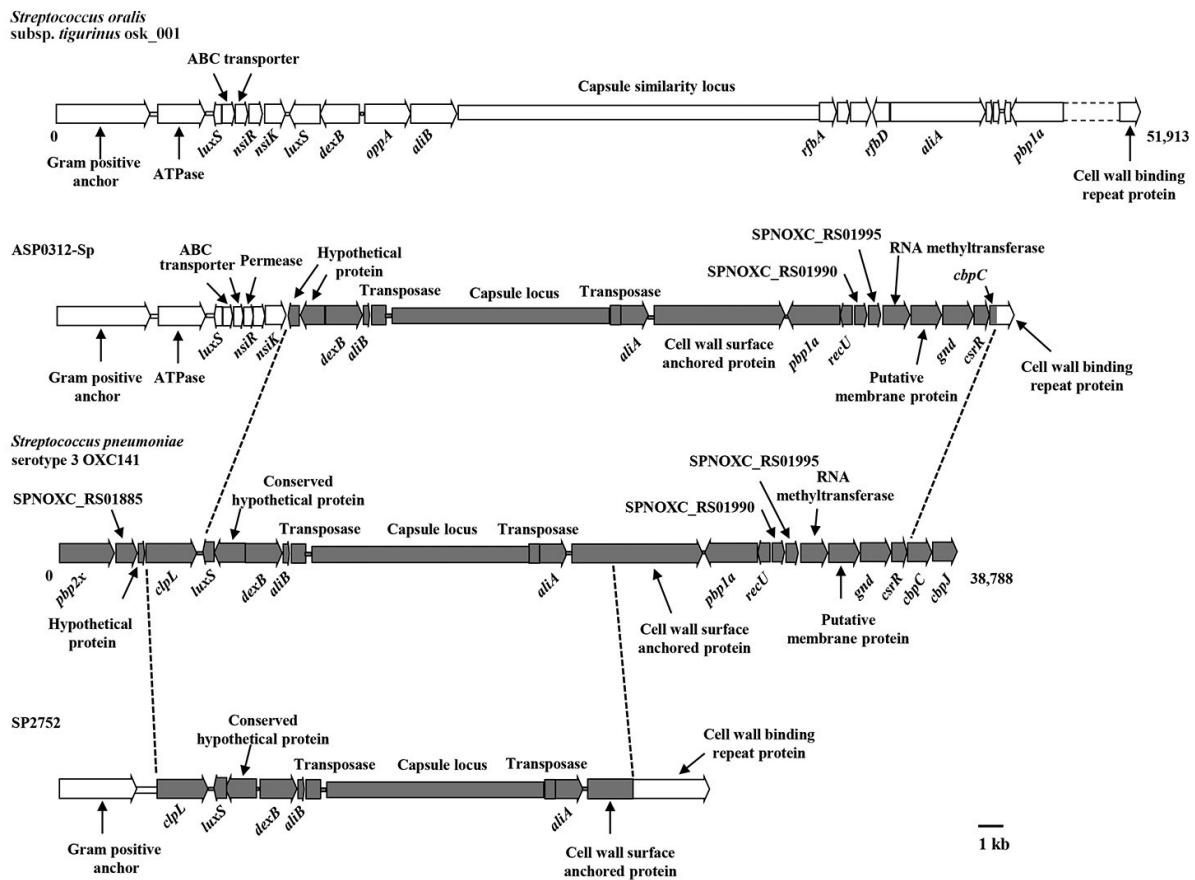
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Appendix Figure. Linear genetic rearrangements of invasive *Streptococcus oralis* expressing serotype 3 pneumococcal capsule from 2 adult patients, Japan. Genetic rearrangements are shown between genes encoding the gram-positive anchor and cell wall binding repeat protein in strains ASP0312-Sp and SP2752. Gene clusters encoding the *Streptococcus pneumoniae* serotype 3 capsule are included in the region. Sequences at the corresponding locations in *S. oralis* subsp. *tigurinus* osk_001 (GenBank accession no.

AP018338.1) and *S. pneumoniae* serotype 3 OXC141 (GenBank accession no. FQ312027) were obtained from the National Center for Biotechnology Information database (<https://www.ncbi.nlm.nih.gov>) and used to determine the sites of recombination. All gene names used here are identical to those from the osk_001 and OXC141 strains. The white rectangles indicate the sequences of *S. oralis* subsp. *tigurinus* osk_001, and the gray rectangles indicate the sequences of *S. pneumoniae* serotype 3 OXC141. The recombination points are indicated by dashed lines. SPNOXC represents *Streptococcus pneumoniae* OXC41; the RS number after SPNOXC is the GenBank locus tag.