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Genome Evolution to Penicillin Resistance in Serotype 3 *Streptococcus pneumoniae* by Capsular Switching

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ABSTRACT *Streptococcus pneumoniae* isolates of serotype 3 were collected from cases of invasive pneumococcal disease ($n = 124$) throughout Japan between April 2010 and March 2013. A penicillin-resistant *S. pneumoniae* (PRSP) isolate from an adult patient, strain KK0981 of serotype 3, was identified among these strains. Whole-genome analysis characterized this PRSP as a recombinant strain derived from PRSP of serotype 23F with the *cps* locus (20.3 kb) replaced by that of a penicillin-susceptible strain of serotype 3.

KEYWORDS *Streptococcus pneumoniae*, capsular switching, gPRSP, serotype 3

Streptococcus pneumoniae is the most important pathogen in community-acquired respiratory and invasive infections in all age groups.

In Japan, 7-valent pneumococcal conjugate vaccine (PCV7) was given to children under 5 years old since November 2010 until its replacement by PCV13 in November 2013. A direct vaccine effect on the incidence of invasive pneumococcal disease (IPD) in young children was obtained immediately (1, 2), followed shortly by an indirect effect on the incidence of IPD in adults (3). Similar effects were reported in the United States (4) and the European Union (5). However, serotype 3, included in PCV13, remains the leading cause of IPD in adults (5–7).

Penicillin-resistant *S. pneumoniae* (PRSP), which mostly represents serotypes 6B, 14, 19F, 23F, and 6A of the vaccine type (VT), has amino acid substitutions near or in conserved amino acid motifs in 3 penicillin-binding proteins (PBPs), PBP1A, PBP2X, and PBP2B, encoded by *pbp1a*, *pbp2x*, and *pbp2b*, respectively (2, 3). These resistances, identified by *pbp* gene analysis, are expressed as the genotype (g) (8). Clinical isolates of serotype 3 usually are penicillin-susceptible or penicillin-intermediate *S. pneumoniae* having amino acid substitutions in PBP2X (gPISP [*pbp2x*]), with susceptibility to penicillin G (PEN) ranging from 0.031 to 0.125 μg/ml (3, 8).

Of pneumococcal strains ($n = 1,317$) collected throughout Japan from April 2010 to March 2013, 124 strains were identified as serotype 3 of the mucoid type in children ($n = 10$) and adults ($n = 114$) (2, 3). All but one of the strains were PEN susceptible, having an MIC of $\leq$0.125 μg/ml, and belonged to the sequence type 180 (ST180) group. The remaining strain, non-ST180 strain KK0981, was identified as a new gPRSP belonging to ST242, which has not been reported in any other country (3).

In the present study, we used a comparative genomic approach to determine how evolution to gPRSP in that serotype 3 isolate occurred through recombination of the capsular (*cps*) locus region flanked by the *pbp2x* and *pbp1a* genes.

Table 1 shows characteristics, sequence types (STs), genotypic resistance, and β-lactam susceptibilities determined by bacteriologic methods in the 3 strains analyzed in this study; KK0981 was a new gPRSP (serotype 3, ST242), KK0381 a gPISP (serotype

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TABLE 1 Characteristics, sequence type, genotypic penicillin and macrolide resistances, and MICs of pneumococcal strains analyzed

Strain	Serotype	ST	Penicillin resistance genotype[a]	Macrolide resistance gene(s)	MICs (μg/ml) for[b]:				Disease (source)	Year of isolation	City isolated
					PEN	AMP	CTX	MEM			
KK0381 (candidate donor)	3	180	gPISP (*pbp2x*)	*erm*(B)	0.063	0.125	0.25	0.063	Pneumonia (blood)	2010	Niigata
KK0981 (putative recombinant)	3	242	gPRSP (*pbp1a* + *pbp2x* + *pbp2b*)	*mef*(A) + *erm*(B)	1	2	1	1	Bacteremia (blood)	2011	Amagasaki
KK1157 (candidate recipient)	23F	242	gPRSP (*pbp1a* + *pbp2x* + *pbp2b*)	*mef*(A) + *erm*(B)	2	2	1	1	Septic arthritis (blood)	2011	Sapporo

[a]*pbp* gene alterations identified by real-time PCR are given in parentheses.
[b]PEN, penicillin G; AMP, ampicillin; CTX, cefotaxime; MEM, meropenem.

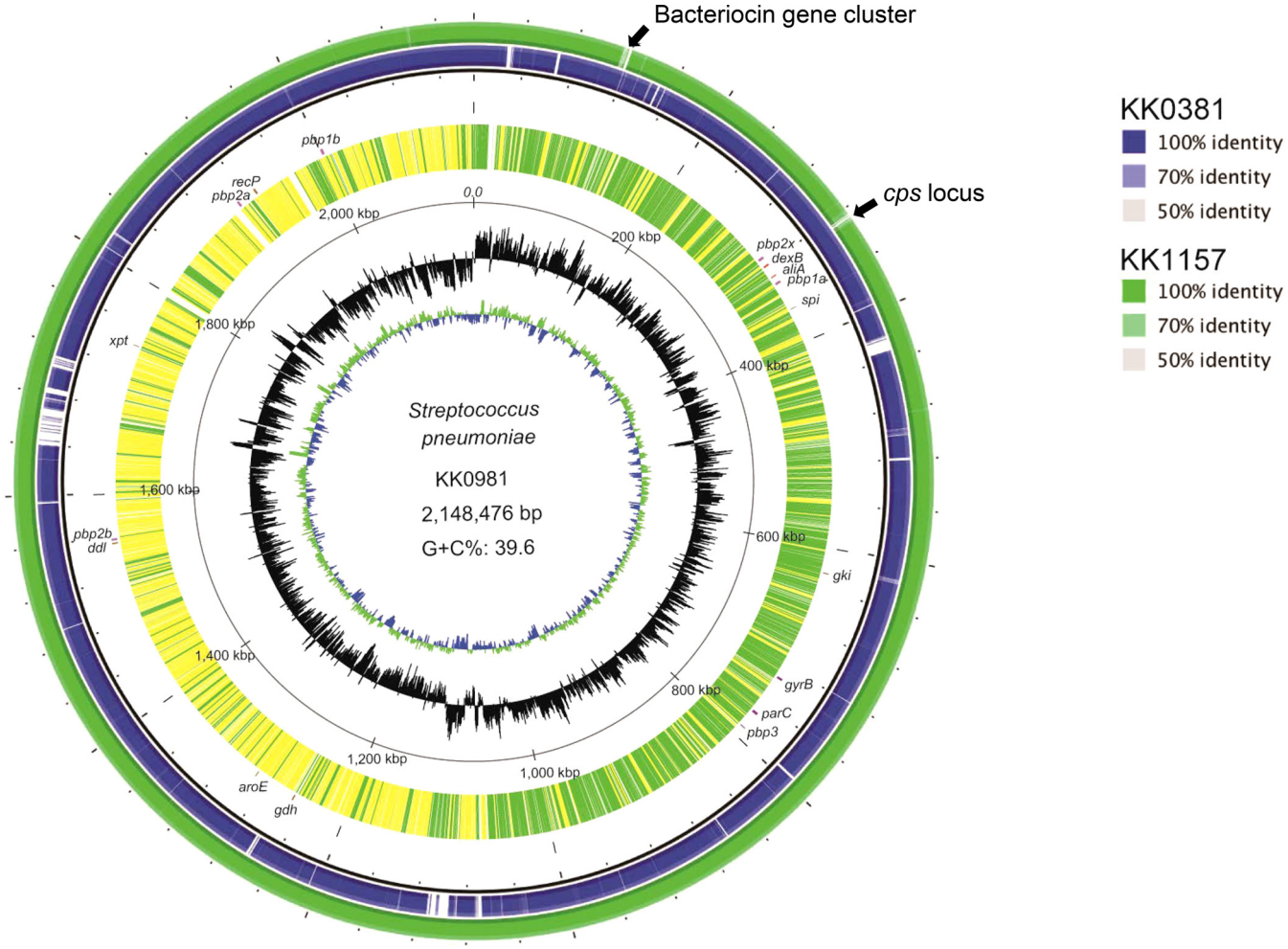

FIG 1 Circular representation of the genome of *S. pneumoniae* strain KK0981 (gPRSP/serotype 3, ST242) compared with the genomes of the KK0381 (gPISP/serotype 3, ST180) and KK1157 (gPRSP/serotype 23F, ST242) strains. KK0981 presented a chromosome 2,148,476 bp long, which contained 2164 coding sequences (CDSs), 12 rRNAs, and 58 tRNAs, showing an overall 39.6% GC content (accession number AP017971). Three contiguous sequences were obtained for KK0381, with a total length of 2,158,744 bp (i, 2,074,518 bp; ii, 43,298 bp; iii; 40,928 bp), and for KK1157, with a total length of 2,267,745 bp (i; 2,200,203 bp, ii, 36,583 bp, iii, 30,959 bp). The concentric circles represent the following information (from the outside in): circle 1, BLAST comparisons of the KK1157 draft genome against KK0981 (green); circle 2, BLAST comparisons of the KK0381 against KK0981 (blue); circle 3, annotated CDSs encoded on forward (green) and reverse (yellow) chromosomal strands, respectively; circle 4, distance from the putative origin of replication; circle 5, GC skew (black); circle 6, GC content (green/blue). Two arrows indicate the region with no homology between KK0981 and KK1157. The circular map was constructed using BRIG software and ArcWithColor software (12) with modification.

3, ST180), and KK1157 a gPRSP (serotype 23F, ST242) derived from the Taiwan^{23F}-15 clone. KK0381 was selected as a candidate donor of the *cps* locus among serotype 3 strains mostly identified as ST180 (see Fig. S1 in the supplemental material). KK1157 was selected as a candidate recipient from among serotype 23F ST242 strains identified as gPRSP ($n = 30$).

Whole-genome sequencing for these strains was performed by PacBio single-molecule real-time (SMRT) technology. The resulting sequence data were analyzed using the MiGAP pipeline (see http://www.migap.org/index.php/en) (9). The *cps* locus and its neighbors were annotated manually with the OXC141 and ATCC 700669 reference strains with GenomeMatcher (10). Gene names for KK0981 were correlated with those for OXC141 (11). Among the genomes of these strains, homology and single-nucleotide variant (SNV) comparisons were performed using BLAST Ring Image Generator (BRIG) software (12) and the MUMmer program, respectively.

The KK0981 (gPRSP/serotype 3) genome was found to have uniformly greater similarity and closer identity of SNVs with the KK1157 (gPRSP/serotype 23F) genome than with that the KK0381 (gPISP/serotype 3) genome (Fig. 1). Amino acid sequences

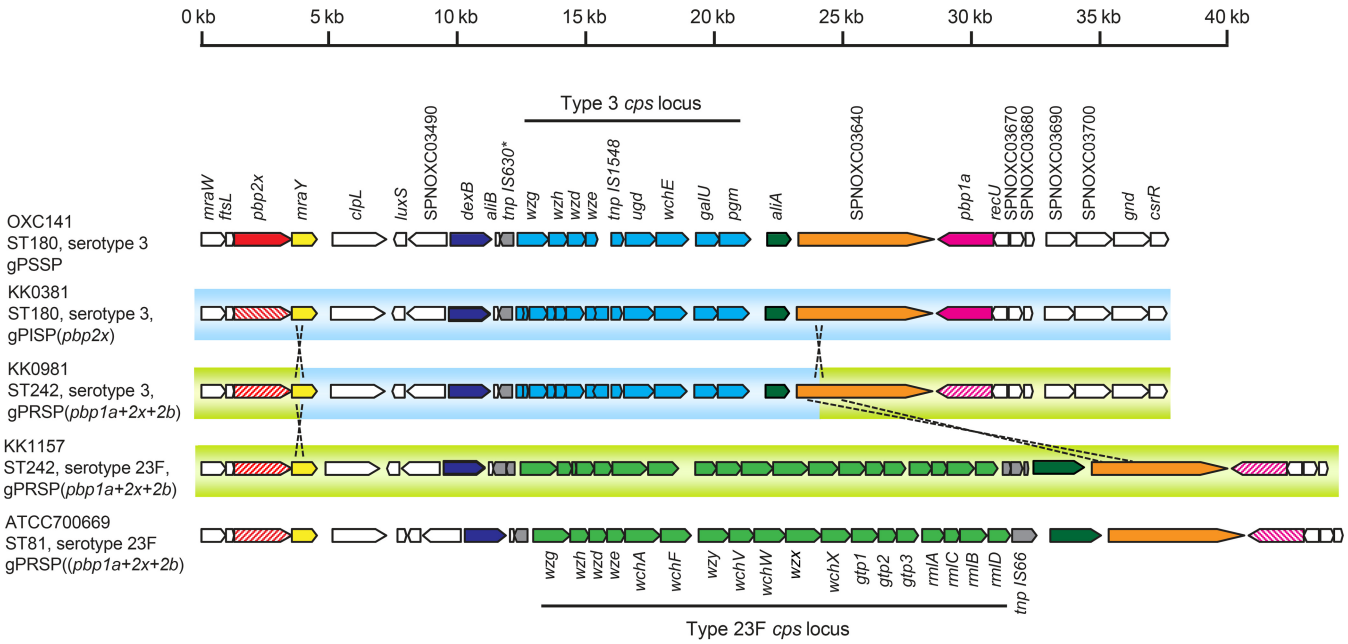

FIG 2 Comparison of the *cps* locus and its flanking region. Genomic organization of *cps* loci and their flanking regions in KK0381, KK0981, and KK1157 were aligned. OXC141 (NC_017592.1) and ATCC 700669 (NC_011900.1) were reference strains for the locus tag or gene names of type 3 and type 23F *cps* loci, respectively. Arrows represent CDSs and their functions in color: *pbp2x* (red), *mraY* (yellow), *dexB* (blue), transposase (gray), *aliA* (green), SPNOXC03640 (orange), *pbp1a* (pink), serotype 3 *cps* genes (cyan), serotype 23F *cps* genes (light green). Hatched arrows represent genotypic (g) resistant *pbp* genes. KK0981 of gPRSP of serotype 3 and ST242 was identified definitely as a transformant caused by recombination in regions from *mraY* to the SPOXC03640 gene of the serotype 3 pneumococcal strain of ST180 as a donor type and KK1157 of gPRSP of serotype 23F and ST242 derived from the Taiwan^{23F}-15 clone as a recipient type. The cyan or light green bar behind the CDSs represents the genetic background of KK0381 or KK1157, respectively, based upon SNV analysis. The alignment was generated with GenomeMatcher software (10); *tnpIS630* is a pseudogene.

of *pbp1a*, *pbp2x*, and *pbp2b* genes of KK0981 were identical with those of KK1157, which showed the same ST242 but a different serotype (see Fig. S2 in the supplemental material). These data indicated that the KK0981 and KK1157 strains share the same genetic background. Specifically, these original data suggest that recombination of the *cps* locus region flanked by *pbp2x* and *pbp1a* genes occurred between the genomes of KK0381 and KK1157.

To identify the recombinant position in the KK0981 (gPRSP/serotype 3) genome, nucleotide sequence alignments from *pbp2x*, the *cps* locus, and *pbp1a* (total length, 29,629 bp) were compared with those of KK1157 (gPRSP/serotype 23F) and KK0381 (gPISP/serotype 3) (Fig. 2). The 20,291-bp length from bp 312701 located in the *mraY* gene to bp 332991 located in the SPNOXC03640 gene (encoding endo-alpha-*N*-acetylgalactosaminidase) of KK0981 was identical to that of KK0381 (see Fig. S3 and S4 in the supplemental material). Sequences of the *cps* loci and their flanking regions in the 3 strains were aligned with those of the reference strains OXC141 (NC_017592.1; PEN susceptible, serotype 3, ST180) and ATCC 700669 (NC_011900.1; PEN resistant, serotype 23F, ST81). No traces of phage or insertion sequences (IS) were clearly identified in the near vicinity of the recombination position.

From the results described above, KK0981 (gPRSP/serotype 3 and ST242) was concluded to occur by homologous recombination of the 20.3-kb region from the *mraY* gene to the SPOXC03640 gene, with a pneumococcal strain of serotype 3 and ST180 as a donor type and a PRSP strain of serotype 23F and ST242 derived from a Taiwan^{23F}-15 clone as a recipient type.

Details of polysaccharide structures and capsule biosynthetic genes have been elucidated (13). Homologous recombination is known to occur in the *cps* locus and its flanking regions, changing serotypes from 4 to 19A (14), from 6A to 6C, or from 7B to 9N (15). Characteristically, the *pbp2x* and *pbp1a* genes involved in β-lactam resistance adjoin the 2 ends of the *cps* locus. Recombination of a large DNA fragment including the *cps* locus and *pbp* genes with lengths from 19.0 kb to over 58.2 kb have been noted

in 11 events occurring in 36 independent cases of capsular switching (15). Previously, a pneumococcal strain of serotype 3 found to be PEN nonsusceptible (MIC $\geq$ 1 μg/ml), which belonged to the PMEN clone Spain^{23F}-1 (CC81 and ST81), was isolated from a patient with IPD in the United Kingdom (16). While a recombination event was reported to occur at the *cps* locus region involving *pbpX* (*pbp2x*), variations in *pbp1a* and *pbp2b* genes affecting β-lactam resistance are unexplained.

In our study, KK1157 (gPRSP/serotype 23F, ST242), the recipient type, belonged to ST242 of the Taiwan^{23F}-15 clone registered in the Pneumococcal Molecular Epidemiology Network (PMEN). Most ST242 pneumococcal strains registered in multilocus sequence type (MLST) databases (see http://pubmlst.org/spneumoniae/) were from Asia; approximately half of them were not susceptible to PEN (MIC $\geq$ 1 μg/ml). The present study is the first report of a PRSP derived from a Taiwan^{23F}-15 clone prevalent in Asia that acquired a *cps* locus region encoding a type 3 capsule by homologous recombination, resulting in occurrence of PRSP of serotype 3. Additionally, this recombinant event most likely occurred in recent years because the SNVs in the recombination region of KK0981 were completely consistent with those of KK0381 of serotype 3.

Evolution to penicillin resistance by capsular switching will continue to occur in *S. pneumoniae* strains not included in the present vaccine type because of selection pressures involving both the spread of pneumococcal vaccination and excessive use of chemotherapeutic agents. Ongoing high-quality molecular epidemiologic surveillance is essential.

Accession number(s). The whole-genome sequences of the 3 strains have been registered in the DNA Data Bank of Japan (DDBJ) under the accession numbers AP017971 (KK0981), AP018043 (KK0381), and AP018044 (KK1157).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00478-17.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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