



# Whole-Genome Sequencing Analysis of Multidrug-Resistant Serotype 15A *Streptococcus pneumoniae* in Japan and the Emergence of a Highly Resistant Serotype 15A-ST9084 Clone

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**ABSTRACT** Since the introduction of pneumococcal conjugate vaccines (PCVs), an increase in the incidence of disease attributable to serotype 15A-ST63 (sequence type 63) pneumococci has been observed in many regions worldwide. We conducted a nationwide pediatric pneumococcal infection surveillance study between 2012 and 2014 in Japan. In the surveillance study, we detected multidrug-resistant serotype 15A-CC63 (clonal complex 63) strains (resistant to macrolides, penicillin, cefotaxime, and meropenem); in this study, we analyzed these resistant isolates to determine the dynamics and mechanism of resistance using whole-genome sequencing. In most of the penicillin-, cefotaxime-, and meropenem-resistant strains, recombination occurred in the *pbp2x* region, resulting in the acquisition of cefotaxime resistance in addition to penicillin and meropenem resistance. In the multidrug-resistant serotype 15A-CC63 strains, we identified a specific clone with ST9084, and all of the isolates were recovered from the Yamaguchi prefecture in Japan. All of the serotype 15A-ST9084 isolates had a novel *pbp2x* type (*pbp2x*-JP3) that was inserted by recombination events. The conserved amino acid motif profiles of *pbp1a*, *pbp2b*, and *pbp2x* of the strains were identical to those of serotype 19A-ST320. A Bayesian analysis-based date estimation suggested that this clone emerged in approximately 2002 before the introduction of the PCV in Japan. This clone should be monitored because serotype 15A is not contained in the currently used 13-valent PCV (PCV13), and it was resistant to beta-lactams, which are often used in a clinical setting.

**KEYWORDS** 15A, Japan, PCV13, PCV7, ST63, ST9084, *Streptococcus pneumoniae*, cefotaxime, multidrug resistance, serotype shift

*Streptococcus pneumoniae* is a common bacterial pathogen that causes infections such as pneumonia, otitis media, occult bacteremia, and meningitis. Global estimates suggest that pneumococcus was responsible for 826,000 deaths among children under 5 years of age in 2000 (1). To prevent infectious pneumococcal disease, 7-, 10-, and 13-valent pneumococcal conjugate vaccines (PCVs) that target a subset of pneumococcal serotypes have been introduced in many countries, which has resulted in a dramatic decrease in the number of invasive pneumococcal disease (IPD) cases. However, the identification of cases of non-PCV serotype pneumococcus has increased (2–8). One of the increasing non-PCV serotypes is serotype 15A, and most of these isolates belonged to sequence type 63 (ST63) (9–17). The serotype 15A-ST63 strain was submitted as Sweden<sup>15A-25</sup> in the Pneumococcal Molecular Epidemiology Network (PMEN) and is highly resistant to macrolides (<http://web1.sph.emory.edu/PMEN/>) (18).

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**TABLE 1** Serotype 15A isolates from Japan in 2012 to 2014 and MICs for meropenem and cefotaxime<sup>a</sup>

Sequence type	No. of MEM-R isolates (n = 10) with MIC for CTX ( $\mu\text{g/ml}$ ) of:			No. of MEM-IR isolates (n = 54) with MIC for CTX ( $\mu\text{g/ml}$ ) of:			No. of MEM-S isolates (n = 22) with MIC for CTX ( $\mu\text{g/ml}$ ) of:		
	$\leq 0.5$	1.0	$\geq 2.0$	$\leq 0.5$	1.0	$\geq 2.0$	$\leq 0.5$	1.0	$\geq 2.0$
63	3	3	1	32	4	1	16	1	1
9084	0	0	2	0	0	8	0	0	0
Other	0	1	0	5	2	2	4	0	0

<sup>a</sup>MEM-R, meropenem resistant (MIC of  $\geq 1.0$   $\mu\text{g/ml}$ ); MEM-IR, meropenem intermediate resistant (MIC of 0.5  $\mu\text{g/ml}$ ); MEM-S, meropenem susceptible (MIC of  $\leq 0.25$   $\mu\text{g/ml}$ ); CTX, cefotaxime.

In Japan, the 7-valent PCV (PCV7) was licensed in February 2010 and used on a voluntary basis until April 2013. During this period, the estimated rates of PCV7 vaccination in children under 5 years of age increased from  $<10\%$  in 2010 to 80% to 90% in 2012 (2). In April 2013, PCV7 was approved as a routine vaccine in Japan, and the vaccine was switched to PCV13 in October 2013. We conducted a nationwide pediatric pneumococcal infection surveillance study in Japan during the PCV13 era between 2012 and 2014 (19). According to the results of the surveillance study, the incidence of meropenem-resistant (MEM-R) pneumococci increased during the surveillance period, which was primarily caused by an increased incidence of isolates of the meropenem-resistant serotypes 15A and 35B. Previous studies demonstrated that penicillin-binding protein (PBP) typing using the *pbp1a*, *pbp2b*, and *pbp2x* transpeptidase domain sequences is useful for predicting beta-lactam resistance (3, 20, 21). The relationship between the three PBP sequences and beta-lactam susceptibility has been reported by the CDC (22). Using this typing methodology, we demonstrated that the meropenem resistance in serotype 15A-ST63 strains emerged due to recombination events overlapping the *pbp1a* region, which caused an insertion of the meropenem resistance-related *pbp1a* type 13 (*pbp1a*-13) sequence in Japan (23). This *pbp1a*-13 sequence was primarily identified in serotype 19A-ST320 isolates in the United States, which are well-known highly multidrug-resistant clones (3, 24). Thus, a detailed understanding of the dynamics of pneumococcal strains, especially antimicrobial-resistant clones, can be gained using the PBP typing method in combination with multilocus sequence typing (MLST).

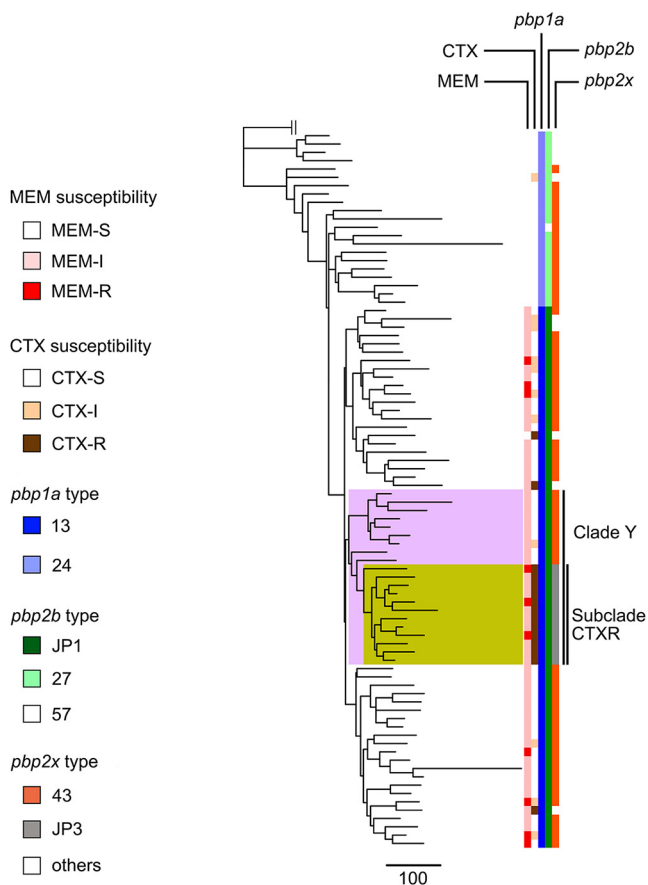
In the treatment of such macrolide-, penicillin-, and meropenem-resistant serotype 15A-ST63 pneumococcal infections, cefotaxime (CTX) is effective in most cases and represents a key drug. Most of the Japanese serotype 15A-ST63 isolates with penicillin and meropenem resistance analyzed in a previous study were susceptible to cefotaxime (MIC,  $\leq 0.5$   $\mu\text{g/ml}$ ). In this study, we demonstrated the emergence of cefotaxime resistance, in addition to macrolide, penicillin, and meropenem resistance, in serotype 15A isolates in Japan. In addition, we demonstrated the prevalence of the multi-beta-lactam-resistant (MBLR) serotype 15A-ST9084 (single-locus variant [SLV] of ST63) clone in one prefecture in Japan. We analyzed the isolates using whole-genome sequencing and present here their genetic characteristics.

## RESULTS

All 86 serotype 15A isolates analyzed in this study were penicillin G resistant (PG-R).

Among the 86 serotype 15A isolates, 62 were of ST63, 10 were of ST9084, and 14 were of other STs (Table 1). Of the 10 ST9084 isolates, 2 were assigned to ST10244, and 2 others were assigned to a new sequence type in a previous study (19). Among the 24 MBLR isolates, 9 were of ST63, 10 were of ST9084, and the other 4 isolates were of other STs.

The 10 serotype 15A-ST9084 isolates were collected from different cases in the Yamaguchi prefecture between May 2012 and November 2014 (see Fig. S1 in the supplemental material). The 10 cases were diagnosed in three different cities. All of

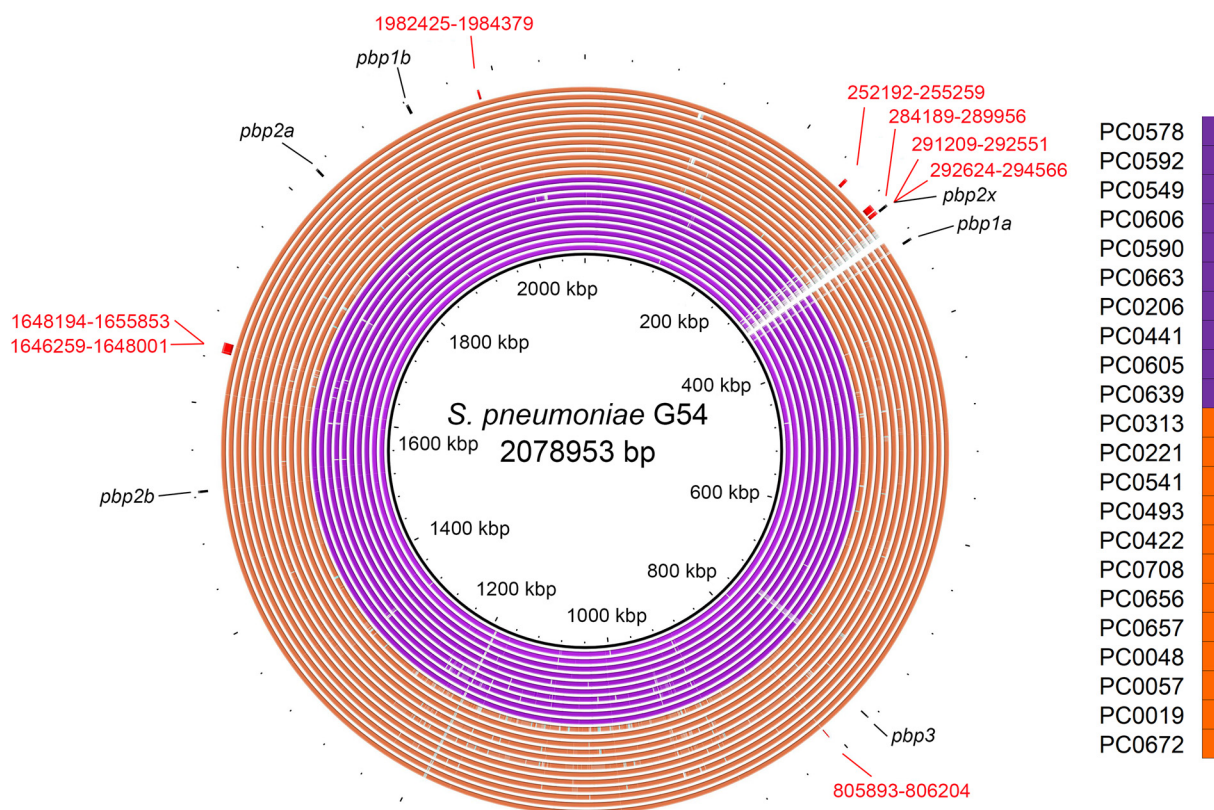


**FIG 1** Phylogenetic tree, susceptibilities to cefotaxime and meropenem, and PBP profiles for all serotype 15A-CC63 isolates derived from Japan between 2012 and 2014. Clade Y is highlighted in pink, and subclade CTXR is highlighted in yellow. Subclade CTXR consists of all serotype 15A-ST9084 isolates and one isolate each of serotype 15A-ST63 and serotype 15A-ST9644. The phylogenetic tree was created by Gubbins using *Streptococcus pneumoniae* G54 as an outgroup isolate. MEM, meropenem; CTX, cefotaxime.

the cases except for one were diagnosed as having otitis media, and the pathogens were recovered from the middle-ear fluid. The remaining case was diagnosed as having pneumococcal meningitis, and the isolate was recovered from cerebrospinal fluid.

**Whole-genome sequencing statistics.** Sequencing statistics are shown in Data Sets S1 and S2 in the supplemental material. The 52 isolate genomes that were newly tested in this study were sequenced at an average depth of 60.37 (standard deviation [SD], ±19.06) and an average coverage of 98.72% (SD, ±0.38%) using the 2,078,953-bp *S. pneumoniae* G54 chromosome as a reference sequence (GenBank accession no. [NC\\_011072.1](https://www.ncbi.nlm.nih.gov/nuccore/NC_011072.1)). The average number of contigs and the average  $N_{50}$  (base pairs) value for these isolates were 70.3 (SD, ±5.9) and 53,180 (SD, ±5,298), respectively.

**Phylogenomics.** To determine the relationship between Japanese serotype 15A-CC63 isolates and foreign isolates, we created a mapping-based phylogenomic tree using serotype 15A-CC63 isolates from Japan, the United Kingdom, and the United States. In the tree, 82 of 86 Japanese serotype 15A-CC63 isolates were included in the same Japanese isolate-specific clade (Fig. S2). In addition, an MBLR serotype 15A-ST9084 isolate-specific clade was generated within the Japanese serotype 15A-CC63 isolate-specific clade; this result implied that the ST9084 lineage originated from the Japanese ST63 lineage. The tree created using only Japanese serotype 15A-CC63 isolates also revealed a serotype 15A-ST9084-specific subclade (subclade CTXR) that was included in a clade that specifically contained serotype 15A-CC63 isolates from the Yamaguchi prefecture (clade Y) (Fig. 1).



**FIG 2** Genomic similarities between *Streptococcus pneumoniae* G54 (GenBank accession no. [NC\\_011072.1](https://www.ncbi.nlm.nih.gov/nuccore/NC_011072.1)) and multi-beta-lactam-resistant serotype 15A-ST9084-specific recombination sites predicted by Gubbins. The specific recombination sites show the predicted recombination sites identified within all subclade CTXR isolates (orange rings) that were not identified within any remaining clade Y isolates (purple rings). Colored segments indicate >95% similarity, and gray segments indicate >90% similarity, as indicated by a BLAST comparison between each isolate genome and *Streptococcus pneumoniae* G54. Red numbers indicate the sequence coordinates of the recombination sites using *Streptococcus pneumoniae* G54. Black arrows indicate each penicillin-binding protein region.

**Prediction of recombination sites potentially responsible for additional cefotaxime resistance.** Using all the clade Y isolates, we predicted the recombination sites that could cause the additional cefotaxime resistance observed in the serotype 15A-ST9084 lineage.

The predicted recombination sites present in all subclade CTXR serotype 15A-ST9084 isolates included eight sites, with one presenting a 1,943-nucleotide overlap of the *pbp2x* region of *S. pneumoniae* G54 (from positions 292624 to 294566 of the reference sequence) (Fig. 2 and Fig. S3 and S4) that seemed to be responsible for the additional cefotaxime resistance. The genes that included all of the recombination sites are listed in Table S1 in the supplemental material.

**PBP profile and three conserved amino acid motifs. (i) *pbp1a*.** Sixty-five isolates, including all 24 MBLR isolates, presented *pbp1a*-13, which was identified as the cause of meropenem resistance in serotype 15A-ST63 strains in Japan in a previous study (23) (Table 2 and Fig. 1). The other 21 isolates contained *pbp1a*-24, which was identified in Sweden<sup>15A-25</sup> (PMEN15A-25). Most of the isolates with *pbp1a*-24 were PCG-R (21/21 isolates), cefotaxime susceptible (CTX-S) (19/21 isolates), and meropenem susceptible (MEM-S) (21/21 isolates). The three conserved amino acid motifs of *pbp1a*-13 were 370SSMK, 466SSN, and 557KTG (Table S2). The sequences of the PBP transpeptidase domains that were not reported in the CDC database but were identified among the Japanese isolates are listed in Data Set S3 in the supplemental material.

**(ii) *pbp2b*.** Sixty-five isolates presented *pbp2b*-JP1 with *pbp1a*-13, and they included all 24 MBLR serotype 15A isolates. The other 21 isolates, except for one, contained

**TABLE 2** PBP profile of each antimicrobial susceptibility pattern<sup>a</sup>

MEM susceptibility	CTX susceptibility	<i>pbp1a</i> type: <i>pbp2b</i> type: <i>pbp2x</i> type (no. of strains)
MEM-S	CTX-S	24:27:43 (15), 24:27:28 (3), 24:27:JP1 (1), 24:57:43 (1)
	CTX-IR	24:27:112 (1)
	CTX-R	13:JP1:JP19 (1)
MEM-IR or -R	CTX-S	13:JP1:43 (40)
	CTX-IR	13:JP1:43 (8), 13:JP1:JP2 (2)
	CTX-R	13:JP1:JP3 (12), 13:JP1:147 (1), 13:JP1:JP6 (1)

<sup>a</sup>MEM-R, meropenem resistant; MEM-IR, meropenem intermediate resistant; MEM-S, meropenem susceptible; CTX-R, cefotaxime resistant; CTX-IR, cefotaxime intermediate resistant; CTX-S, cefotaxime susceptible.

*pbp2b*-27 (Table 2 and Fig. 1). This *pbp2b*-JP1 isolate was also identified in the meropenem-intermediate-resistant (MEM-IR) or MEM-R serotype 15A-ST63 strains in Japan in a previous study (23). Of the 65 isolates with *pbp2b*-JP1, 40 were CTX-S. The three conserved amino acid motifs of *pbp2b*-JP1 were 391SVVK, 448SSNA, and 620KTG (Table S3).

(iii) ***pbp2x***. The *pbp2x* types of the 24 MBLR serotype 15A isolates were more diverse than those of the other two PBPs and consisted of five different types. The most common *pbp2x* type was *pbp2x*-JP3 ( $n = 12$ ), followed by *pbp2x*-43 ( $n = 8$ ) and *pbp2x*-JP2 ( $n = 2$ ) (Table 2 and Fig. 1). Ten of the 12 isolates with *pbp2x*-JP3 showed an MIC of 4  $\mu\text{g/ml}$  for CTX; in contrast, all 8 isolates with *pbp2x*-43 showed an MIC of 1  $\mu\text{g/ml}$ . All of the serotype 15A-ST9084 isolates contained *pbp2x*-JP3. In the reference *pbp2x* type database, *pbp2x*-33 was closest to *pbp2x*-JP3, which possessed 2 amino acid (aa) sequence mutations. Although all MBLR serotype 15A isolates contained the 337SAFK or SAMK motif, all 13 isolates with the SAFK motif were CTX-R; in contrast, 10 of 11 isolates with SAMK were CTX-IR (Table S4). In addition, all MBLR serotype 15A isolates had the 395SSN and 547KSG motifs.

**Other resistance genes and pilus prevalence.** All of the Japanese serotype 15A-CC63 isolates contained the *tetM* and *ermB* genes and were negative for the *ermTR*, *tetO*, *mefA*, and *mefE* genes; the *folA* mutation; and the *folP* insertion. In addition, all Japanese serotype 15A-CC63 isolates lacked the pilus determinants PI-1 and PI-2 (Data Set S2).

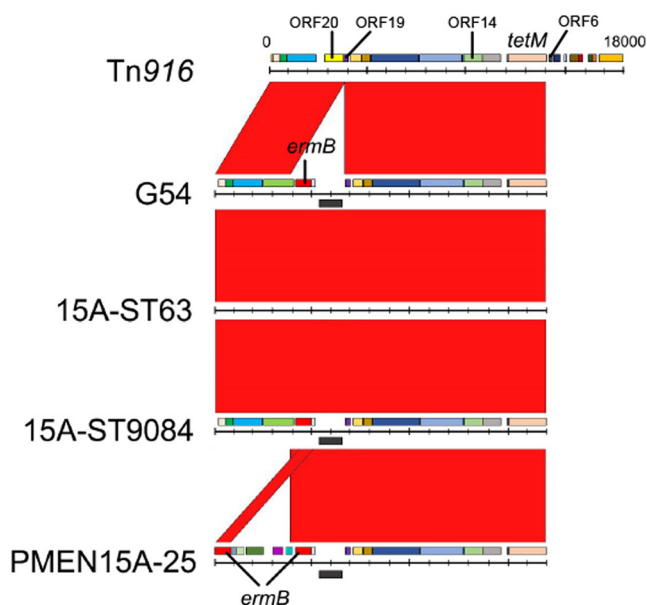
**Genome comparison via core-genome analysis.** A core-genome analysis was performed, and we identified one MBLR serotype 15A-ST9084 isolate-specific gene that was submitted as a pneumococcal hypothetical protein under accession no. [WP\\_087671931.1](https://www.ncbi.nlm.nih.gov/nuccore/WP_087671931.1) in the NCBI database (see the supplemental material).

**Dating the origin of the multi-beta-lactam-resistant serotype 15A-ST9084 clone.** The date of origin for the subclade CTXR lineage was estimated using BEAST, which predicted a most recent lineage origination date (year) of 2001.8 and a 95% highest posterior density (HPD) interval between 1964.8 and 2010.8 (Fig. S5).

**Variations in the serotype 15A-CC63 *cps* loci.** We obtained 15,432 bp of aligned *cps* locus sequences and created a maximum likelihood tree. There were variations in this region even among serotype 15A-CC63 isolates (Fig. S6); only 4 of 86 isolates from Japan and one PMEN15A-25 isolate contained the same sequence. Six of 10 serotype 15A-ST9084 isolates were clustered; all isolates in the cluster were from the Yamaguchi prefecture.

**Structure of the Tn916-like integrative conjugative element (ICE).** Among the 86 serotype 15A-CC63 isolates from Japan, the *ermB* gene was inserted between open reading frame 19 (ORF19) and ORF20 of Tn916, as in Tn6002 (25); 6 of the 86 isolates had a 40-amino-acid deletion in ORF14 that was annotated as *iap* (Fig. 3). PMEN15A-25 had the same *ermB* insertion as the serotype 15A-CC63 isolates in Japan; however, the components upstream of the *ermB* 3' end were different from those of serotype 15A-CC63 isolates from Japan, and PMEN15A-25 contained another *ermB* gene, such as in Tn6003. All analyzed isolates, including PMEN15A-25, lacked the sequence from ORF6 to *int* of Tn916.





**FIG 3** Comparison of the Tn916-like integrative conjugative elements (ICE) of serotype 15A isolates and the *S. pneumoniae* G54 strain. Red bands between the sequences indicate BLASTN matches. The *ermB* element was inserted between ORF19 and ORF20 of Tn916. The ICE structures were homologous among the G54, serotype 15A-ST63, and serotype 15A-ST9084 strains; however, the PMEN15A-25 strain had different insertions at the same positions as the other strains.

## DISCUSSION

Although the incidence of invasive pneumococcal infections dramatically decreased after the introduction of PCVs (2, 7, 8, 26, 27), the emergence and spread of resistant clones have been problematic (3, 23, 28–31). In particular, bacterial resistance to penicillin, cephalosporins, and carbapenems may affect the outcomes of pneumococcal infections because these antimicrobials are commonly used in many clinical settings. In the PCV13 era, increases in serotype 15A-ST63 pneumococcal infections and colonization have been observed in many countries (9–17, 19), and only isolates derived from Japan were previously shown to be resistant to meropenem (23). In addition, although most of these serotype 15A-ST63 isolates were susceptible to cefotaxime, we realized the emergence of additional cefotaxime-resistant isolates in this lineage; hence, we aimed to elucidate the mechanism of this resistance in this study.

We analyzed 24 MBLR (resistant to penicillin, cefotaxime, and meropenem) serotype 15A-ST63 isolates and another 62 serotype 15A-ST63 isolates using whole-genome sequencing. The results demonstrated that the additional cefotaxime resistance correlated with recombination events in the *pbp2x* region, and most of the MBLR isolates had a *pbp2x* type other than *pbp2x*-43, which was present mainly in CTX-S isolates. Of note, the isolates with *pbp2x*-JP3 showed higher MIC values for cefotaxime than those with the other *pbp2x* type, and most of these isolates belonged to ST9084.

All of the serotype 15A-ST9084 (SLV of ST63) isolates were MBLR, and this clone appears to have originated from the prevalent serotype 15A-ST63 clone according to results of the phylogenomic analysis. In the two phylogenetic trees, one was created using global serotype 15A-ST63 isolates, and the other was created using only Japanese isolates. The serotype 15A-ST9084 isolates clustered exclusively and were included with the Japanese clusters. The identification of Japan-specific *pbp2b*-JP1 and *pbp2x*-JP3 in the serotype 15A-ST9084 strains may support this finding.

These serotype 15A-ST9084 isolates were derived from different cases between 2012 and 2014 in three cities in the Yamaguchi prefecture in Japan. The ages of the patients and dates of the cases indicated that these cases were not caused by an outbreak event but rather were caused by small outbreaks and sporadic incidents; therefore, this MBLR lineage may have already spread within the Yamaguchi prefecture. In addition, we are

concerned about the future spread of this clone throughout Japan because the currently used PCV13 does not contain serotype 15A. A previous study in Japan demonstrated that the number of pediatric infection cases attributable to serotype 15A increased during the period between 2014 and 2016 (32). Moreover, resistance to multiple drugs (penicillin, cefotaxime, meropenem, and macrolides) may allow the clone to spread rapidly, as previously observed in the United States for a multidrug-resistant serotype 19A-CC320/271 clone (24).

The recombination site predictions in this study demonstrated that the additional cefotaxime resistance in serotype 15A-CC63, especially in the serotype 15A-ST9084 lineage, was caused by recombination events in the *pbp2x* region. We believe that developing a greater understanding of the mechanism of recombination events that cause beta-lactam resistance, serotype switching, and increases/decreases in fitness cost, etc., is very important for the control of pneumococcal infections. With regard to antibiotic resistance, recombination events that occur in the PBP regions (especially in the *pbp1a*, *pbp2b*, and *pbp2x* regions) are involved in beta-lactam resistance (33). Although the direct influence of the pneumococcal environment, such as antibiotic pressure and vaccine pressure, etc., on the recombination events is not well known, recent antibiotic use and pediatric serotypes (i.e., serotypes commonly found in children) were suggested as risk factors for the development of resistance among IPD cases (34–36). *S. pneumoniae* is naturally transformable by the uptake and chromosomal integration of DNA from other pneumococcal strains and other related nonpathogenic mitis group species. Therefore, we believe that future studies on pneumococcal genetic ecology, including the mechanisms underlying genetic evolution, should focus on both species.

With respect to conserved amino acid motifs in the PBP transpeptidase domains, all MBLR serotype 15A-ST9084 isolates had the 337SAFK motif in *pbp2x*-43. Although this 337SAFK motif was identified in only the MBLR serotype 15A-ST9084 isolates in this study, we observed the same sequence in the multidrug-resistant serotype 19A-ST320 isolates from a previous U.S. data set (3). Comparing the other *pbp1a*, *pbp2b*, and *pbp2x* motifs between serotype 15A-ST9084 isolates in Japan and serotype 19A-ST320 isolates in the United States, all eight motifs also had the same sequences, suggesting that the basic mechanism of cephalosporin resistance in these two clones is identical. However, this match must be coincidental because these strains are not closely related based on the phylogenetic tree reported in a previous study (23), and the PBPs are associated with certain distances in the whole-genome sequence.

Estimation of the date of origin for the most recent common ancestor (MRCA) of the MBLR serotype 15A-ST9084 clone showed that the clone emerged in approximately 2002, prior to the introduction of PCVs. This finding may explain the dynamics of the resistant clone, as the clone developed naturally by chance in the pre-PCV era and subsequently spread in response to PCV pressure and/or other factors, such as antimicrobial use and changes in lifestyle (including child care systems). Interestingly, Obolski et al. and Lehtinen et al. demonstrated that vaccination impacts antibiotic resistance frequencies in pneumococci by removing competition from susceptible strains and prolonging the duration of carriage of resistant isolates (37, 38). The results of these studies clearly support the results of this study.

Tn916-like ICE analysis demonstrated that all serotype 15A-CC63 isolates in Japan had Tn6002-like ICEs; this result was consistent with data from a previous publication by Santoro et al. that demonstrated that *S. pneumoniae* G54, the predicted ancestor of the serotype 15A-ST63 strain, had a defective Tn6002-like ICE (39). Although our analysis was based on assembled contigs, which resulted in a lack of data concerning the downstream sequences of *tetM*, all Tn6002-like ICEs identified in serotype 15A-CC63 isolates in Japan seemed to lack genes from ORF6 to *int* of Tn6002. Interestingly, the PMEN15A-25 isolate that was recovered in 1998 in Portugal had a Tn916-like ICE different from that of the Japanese isolates (i.e., a defective Tn6003-like ICE); however, PMEN15A-25 also lacked the sequences downstream of *tetM*. Considering that *S. pneumoniae* G54 was isolated in 1991 before the PMEN15A-25 strain was recovered, the

*ermB* insertion region of *S. pneumoniae* G54 may be replaced by that of PMEN15A-25; thus, the defective Tn6003-like ICE was inserted in the PMEN15A-25 isolate.

In this study, we identified a PBP motif associated with cefotaxime resistance in an emerging lineage of MBLR serotype 15A-ST9084 strains in Japan. Although the incidence of this strain was identified in only one prefecture in Japan, additional and careful monitoring is warranted due to its high level of resistance. To perform multifaceted surveillance, the global PBP profile database should be enhanced.

## MATERIALS AND METHODS

**Patient data and bacterial isolates.** In a previous nationwide prospective surveillance study of pediatric invasive pneumococcal disease (IPD) ( $n = 343$ ) and non-IPD ( $n = 286$ ) isolates collected from January 2012 to December 2014 from 154 Japanese medical institutions, 87 of 92 serotype 15A isolates belonged to MLST clonal complex 63 (CC63) (19). We analyzed all 87 isolates except one, which did not grow from the stock. Patient data were collected during the surveillance period using a standardized questionnaire, as described previously.

**Antimicrobial susceptibility definition.** Based on 2008 Clinical and Laboratory Standards Institute (CLSI) guidelines (52), we performed susceptibility testing for penicillin, cefotaxime, meropenem, and erythromycin using the broth microdilution method, as described previously (19). Penicillin G-susceptible (PG-S) and penicillin G-resistant (PG-R) isolates were defined as having MICs of  $\leq 0.06$  and  $\geq 0.12$  mg/liter, respectively. Cefotaxime-susceptible (CTX-S), cefotaxime-intermediate-resistant (CTX-IR), and cefotaxime-resistant (CTX-R) isolates were defined as having MICs of  $\leq 0.5$ , 1.0, and  $\geq 2.0$  mg/liter, respectively. Meropenem-susceptible (MEM-S), meropenem-intermediate-resistant (MEM-IR), and meropenem-resistant (MEM-R) isolates were defined as having MICs of  $\leq 0.25$ , 0.5, and  $\geq 1.0$  mg/liter, respectively. Multi-beta-lactam resistance (MBLR) was defined as PG-R, CTX-R or -IR, and MEM-R or -IR.

**Whole-genome sequencing.** Of the 86 serotype 15A-CC63 isolates described above, 34 were analyzed in our previous study using whole-genome sequencing (23). Therefore, we obtained additional whole-genome sequencing data for the 52 remaining isolates in this study. Total genomic DNA was extracted, and sequence libraries were prepared using a QIAamp DNA minikit (Qiagen, Hilden, Germany) and a Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA). We multiplexed and sequenced the samples on an Illumina NextSeq system for 300 cycles (2- by 150-bp paired-end reads). Isolates with  $N_{50}$  values of  $< 20,000$  were excluded from subsequent analysis. The resulting sequences were assembled using SPAdes v3.12.0 (40), followed by MLST (<http://pubmlst.org/spneumoniae/>) using BLAST+ v2.6.0 (41).

**PBP typing, antimicrobial resistance genes, and pilus detection.** We allocated PBP transpeptidase domain type numbers to the extracted *pbp1a*, *pbp2b*, and *pbp2x* transpeptidase domain sequences of the examined isolates. The type numbers originated from the CDC's PBP types, which were reported in previous studies (3, 20–22). PBP types that were not previously reported in the CDC database (22) were numbered with the prefix "JP," such as in *pbp1a*-JP1. Three conserved amino acid motifs (SXXK, SXN, and KT/SG) of the three PBPs were identified according to the R6 amino acid sequence (GenBank accession no. [NC\\_003098.1](https://www.ncbi.nlm.nih.gov/nuclot/NC_003098.1)). In addition, we identified the presence of the *ermB*, *ermTR*, *mefA*, *mefE*, *tetM*, *tetO*, *rrgA1* (pilin 1), and *pitB1* (pilin 2) genes and searched for mutations within the *folA* and *folP* genes in the assembled contigs (3).

**Phylogenetic analysis.** To predict recombination sites and create a phylogenetic tree, we used Genealogies Unbiased by Recombinations in Nucleotide Sequences (Gubbins) v1.4.6 (42), which identifies recombination sites and constructs a phylogenetic tree using an algorithm that iteratively identifies loci containing increased densities of base substitutions while constructing a phylogenetic tree based on putative point mutations outside the recombination regions. *S. pneumoniae* G54 (GenBank accession no. [NC\\_011072.1](https://www.ncbi.nlm.nih.gov/nuclot/NC_011072.1)) was used as a reference sequence because a previous study suggested that this strain is closely related to serotype 15A-ST63 isolates (43). To elucidate the relationships among the serotype 15A-CC63 lineages in Japan, the United Kingdom, and the United States, we downloaded the foreign isolates' whole-genome read data from the Sequence Read Archive (SRA) database (<https://www.ncbi.nlm.nih.gov/sra/>) (see Data Set S2 in the supplemental material) (44, 45) and created a phylogenetic tree using all of the isolates. With regard to the foreign isolates, serotypes and STs were examined by extracting the corresponding genetic regions with BLAST+ v2.6.0, as previously described (23). Following this process, we created additional trees, one tree using all the data for the Japanese isolates and another tree using isolates included in the serotype 15A-ST9084-specific clade, to predict recombination sites that could cause cefotaxime resistance. The genes identified at the recombination sites were annotated using Prokka v1.12 (46), and the protein sequences were searched in the NCBI database using ACT v18.0.0 (47).

**Dating the origin of the multi-beta-lactam-resistant serotype 15A-ST9084 clone.** The date of the most recent common ancestor (MRCA) of the MBLR serotype 15A-ST9084 clone was estimated using BEAST v1.10.4 with isolate alignment data obtained from the read data (48). In this analysis, we used serotype 15A-ST9084-specific clade isolates. The tree was used to analyze the final maximum likelihood tree and the alignment of base substitutions occurring outside putative recombination regions using an uncorrelated relaxed-clock model. The tree was calibrated using the isolation dates (month and year) of the strains. The coalescent exponential population was used as the tree prior, and the length of the chain value was set such that all output values had an effective sample size of  $> 200$ .

**Genome comparison by core genome analysis.** To identify the genes that were specific to the MBLR serotype 15A-ST9084 isolates, we performed pangenome and core genome analyses using



get\_homologues (49). To prepare the input data, the assembled contigs of each isolate were annotated using Prokka v1.12 (46). The base sequences of the identified genes that were specific to the MBLR serotype 15A-ST9084 isolates were searched in the NCBI database using BLAST<sup>+</sup> v2.6.0.

***cps* locus and Tn916-like integrative conjugative element comparison.** A phylogenetic tree was constructed for the serotype 15A *cps* locus using RAxML v8.2.10 (50) by mapping short reads of each isolate to previously reported reference sequences (51). To clarify the structure of the Tn916-like integrative conjugative element (ICE), the corresponding regions were extracted from the *de novo* strain assemblies. The sequences were compared using ACT v18.0.0 (47) with standard parameters in BLAST<sup>+</sup> v2.6.0.

Details of the genomic analysis process are described in the supplemental material.

**Data availability.** The accession numbers of the sequence data are available in Data Set S1 in the supplemental material.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.02579-18>.

**SUPPLEMENTAL FILE 1**, PDF file, 1.3 MB.

**SUPPLEMENTAL FILE 2**, XLSX file, 0.02 MB.

**SUPPLEMENTAL FILE 3**, XLSX file, 0.02 MB.

**SUPPLEMENTAL FILE 4**, XLSX file, 0.01 MB.

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