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Clonal Spread of *mef*-Positive Macrolide-Resistant *Streptococcus pneumoniae* Isolates Causing Invasive Disease in Adults in Germany▽

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Isolates (3,845) obtained from German adults with invasive pneumococcal disease between 1992 and 2004 were investigated. Of these, 430 isolates (11.2%) were erythromycin A nonsusceptible. Macrolide resistance genotypes and multilocus sequence types were determined. Among the isolates, 35.6% were *erm*(B) positive and 63.5% were *mef* positive. Over the study period, the frequency of resistance rose significantly from 2.2 to 17.0% ($P < 0.001$). A serotype 14, sequence type 9 clone was the most widespread.

Streptococcus pneumoniae continues to be a significant cause of morbidity and mortality in humans. The worldwide increase in antibiotic resistance in pneumococci has become a serious infectious-disease problem within the last 20 years. Macrolide resistance in *S. pneumoniae* is usually caused by the presence of the *erm*(B) or the *mef*(E) or *mef*(A) resistance determinant. The *erm*(B) gene encodes a 23S rRNA methylase that confers resistance to 14-, 15-, and 16-member-ring macrolides, lincosamides, and streptogramin B. The *mef*(E) and *mef*(A) genes, which are carried by different genetic elements, encode an efflux pump that leads to resistance to 14- and 15-member-ring macrolides (the M phenotype) (14, 20). Other rare mechanisms of macrolide resistance are changes in a highly conserved region of domain V of 23S rRNA, which plays a key role in macrolide binding, and in ribosomal proteins L4 and L22 (3, 5, 18, 21).

Multilocus sequence typing (MLST) is a recently developed technique that produces unambiguous molecular typing data by using the sequences of seven loci to obtain an allelic profile of each strain (6, 11; http://www.mlst.net). The present study used this technique to analyze the genetic relatedness of clinical erythromycin A-resistant strains of *S. pneumoniae* isolated from adults with invasive pneumococcal disease in Germany.

The German National Reference Center for Streptococci received consecutive isolates from 126 clinical microbiological laboratories throughout Germany. Inclusion criteria were isolation from an individual of >16 years and isolation from a normally sterile body site.

MIC testing, serotyping, the determination of resistance genotypes and phenotypes, and MLST of 62 randomly selected macrolide-resistant strains were performed as described previously (11, 13).

Multilocus sequence types were analyzed using the program eBURST, which displays relationships between closely related isolates of a bacterial species or population. eBURST, unlike cluster diagrams, trees, or dendrograms, uses a simple but appropriate model of bacterial evolution in which an ancestral (or founding) genotype increases in frequency in the population and, while doing so, begins to diversify to produce a cluster of closely related genotypes that are all descended from the founding genotype. This cluster of related genotypes is referred to as a clonal complex (8; http://eburst.mlst.net). A phylogenetic tree using maximum likelihood was created with the program Puzzle (19; http://genius.dkfz-heidelberg.de).

A total of 3,845 isolates were consecutively collected at 126 centers from 1992 to 2004. Of these, 3,134 strains (81.5%) were isolated from blood, 426 (11.1%) were from cerebrospinal fluid, 119 (3.1%) were from pleural fluid, and 166 (4.3%) were from other normally sterile body sites.

Macrolide-resistant *S. pneumoniae* strains showed cross-resistance to other 14- and 15-member-ring macrolides. All strains were telithromycin susceptible and were inhibited by 1 μg of telithromycin/ml (MIC at which 90% of the tested isolates were inhibited, 0.12 μg/ml); 4% of the isolates were clindamycin resistant. Over the study period (1992 to 2004), a

TABLE 1. Development of resistance among *S. pneumoniae* isolates from adults with invasive pneumococcal disease in Germany from 1992 to 2004

Yr of isolation	% of erythromycin A-resistant isolates	% of *mef*-positive isolates (% of macrolide-resistant isolates)[a]	% of *erm*(B)-positive isolates (% of macrolide-resistant isolates)[a]
1992	2.2	0.7 (31)	1.5 (69)
1993	3.9	2.2 (58)	1.7 (42)
1994	3.8	1.1 (29)	2.7 (71)
1995	6.9	3.1 (45)	3.9 (55)
1996	7.5	4.5 (60)	3.0 (40)
1997	12.5	7.7 (62)	4.8 (38)
1998	12.1	6.7 (55)	5.5 (45)
1999	17.3	10.1 (58)	6.1 (42)
2000	15.3	8.5 (56)	6.8 (44)
2001	15.2	8.2 (54)	6.7 (46)
2002	13.3	8.9 (67)	4.2 (32)
2003	15.9	11.7 (74)	4.2 (26)
2004	17.0	13.3 (78)	3.9 (22)
Total	11.0	6.7 (61)	4.2 (39)

[a] Percentage of macrolide-resistant isolates positive for the indicated gene.

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TABLE 2. Distribution of 62 STs of macrolide-resistant pneumococcal isolates from adults with invasive pneumococcal disease in Germany

ST[a]	Macrolide resistance phenotype,[b] genotype	Clone designation or description[c]	No. of isolates	% of total isolates	Predominant country(ies) of origin
9	M, *mef*(A)	England^{14}-9	15	24.2	United Kingdom, Germany
124	cMLS$_B$, *erm*(B)	PMEN global clone "clone 35"	6	9.7	Germany, The Netherlands
273	cMLS$_B$, *erm*(B)	PMEN global clone Greece^{6B}-22	5	8.1	Portugal
15	cMLS$_B$, *erm*(B)	Single-locus variant of England^{14}-9	3	4.8	Brazil
81	cMLS$_B$, *erm*(B)	Spain^{23F}-1	2	3.2	Spain
242	M, *mef*(E)	Taiwan^{23F} clone	2	3.2	Taiwan
90	cMLS$_B$, *erm*(B)	Spain^{6B}-2	1	1.6	Spain, Australia
127	cMLS$_B$, *erm*(B)		1	1.6	United Kingdom, Germany
132	cMLS$_B$, *erm*(B)		1	1.6	The Netherlands
162	M, *mef*		1	1.6	United Kingdom
176	M, *mef*		1	1.6	Poland
315	cMLS$_B$, *erm*(B)	Poland^{6B}-20	1	1.6	Poland
385	iMLS$_B$, *mef*		1	1.6	United States
392	cMLS$_B$, *erm*(B)		1	1.6	United Kingdom
440	cMLS$_B$, *erm*(B)		1	1.6	United Kingdom
507	M, *mef*		1	1.6	Finland
564	cMLS$_B$, *erm*(B)		1	1.6	Germany
663	M, no *erm*(B), no *mef*		1	1.6	United States
1435	M, *mef*	Single-locus variant of Taiwan^{23F}-15	1	1.6	Japan, Germany
1552	M, *mef*		1	1.6	Germany
1554	M, *mef*		1	1.6	Germany
1561	cMLS$_B$, *erm*(B)		1	1.6	Germany
1562	cMLS$_B$, *erm*(B)		1	1.6	Germany
1563	M, *mef*	Single-locus variant of England^{14}-9	1	1.6	Germany
1564	cMLS$_B$, *erm*(B)		1	1.6	Germany
1566	cMLS$_B$, *erm*(B)		1	1.6	Germany
1569	cMLS$_B$, *erm*(B)	Single-locus variant of Spain^{9V}-3	1	1.6	Germany
1571	M, *mef*		1	1.6	Germany
1575	cMLS$_B$, *erm*(B)		1	1.6	Germany
1577	cMLS$_B$, *erm*(B), *mef*		1	1.6	Germany
1582	cMLS$_B$, *erm*(B)		1	1.6	Germany
1584	cMLS$_B$, *erm*(B)		1	1.6	Germany
1592	M, *mef*		1	1.6	Germany
1594	cMLS$_B$, *erm*(B)		1	1.6	Germany
1595	cMLS$_B$, *erm*(B)		1	1.6	Germany
Total			62	100.0	

[a] New STs first described in the present study are highlighted in bold.
[b] cMLS$_B$, constitutive resistance to 14-, 15-, and 16-member-ring macrolides, lincosamides, and streptogramin B; iMLS$_B$, inducible resistance to14-, 15-, and 16-member-ring macrolides, lincosamides, and streptogramin B.
[c] PMEN, Pneumococcal Molecular Epidemiology Network.

statistically significant increase in the frequency of erythromycin A resistance (2.2% to 17%) was observed ($P < 0.001$) (Table 1). In total, 153 of the 430 nonsusceptible isolates (35.6%) were *erm*(B) positive, and 273 (63.5%) were *mef* positive. Two isolates were both *erm*(B) and *mef* positive, and two isolates contained neither *erm*(B) nor *mef*.

The serotyping of resistant isolates resulted in the following distribution: serotype 14, 48.8%; 6B, 9.5%; 23F, 7.7%; 9V, 4.4%; 19A, 4.0%; and 19F, 3.5%. Fewer than 10 isolates of other serotypes were encountered (data not shown).

Macrolide resistance was caused by the oligoclonal spread of some multilocus sequence types. Among those, sequence type 9 (ST 9; serotype 14; United Kingdom and Germany) was by far the most important, followed by another serotype 14 clone (ST 124) and a serotype 6B clone (ST 273). In addition, 16 macrolide-resistant clones were described for the first time in this investigation (Table 2). eBURST analysis showed ST 9 to be the cofounder of a large clonal complex also containing ST 15 (Fig. 1A). ST 124 is the founder of a very large clonal complex (Fig. 1B). ST 273 forms a complex with ST 146 and ST 385 (Fig. 1C). All isolates of ST 9 carried the *mef*(A) gene, and both isolates of ST 242 carried the *mef*(E) gene. A maximum-likelihood tree of the concatenated alleles of the 35 multilocus sequence types found in this study, developed using the program Puzzle, showed ST 9 and ST 124 to be relatively separated from all other multilocus sequence types (Fig. 2).

Within the last 10 years, macrolide resistance in *S. pneumoniae* has emerged on a dramatic scale throughout Europe (4, 16). This study shows that resistance to macrolides in Germany has drastically increased over the last 12 years. In a previous study, our group showed a clear correlation between macrolide consumption and erythromycin resistance in Germany (15). Both the *mef* and *erm* resistance determinants could be identified. In 1992, 1994, and 1995, the *erm*(B) gene was the most predominant. From 1996 until 2004, the *mef* gene was the main resistance determinant. Of note, only two of the strains were found to be *mef*(A) and *erm*(B) negative, suggesting the presence of one of the recently described novel macrolide resistance mechanisms (23S rRNA mutations and alterations in L4 and L22 ribosomal proteins).

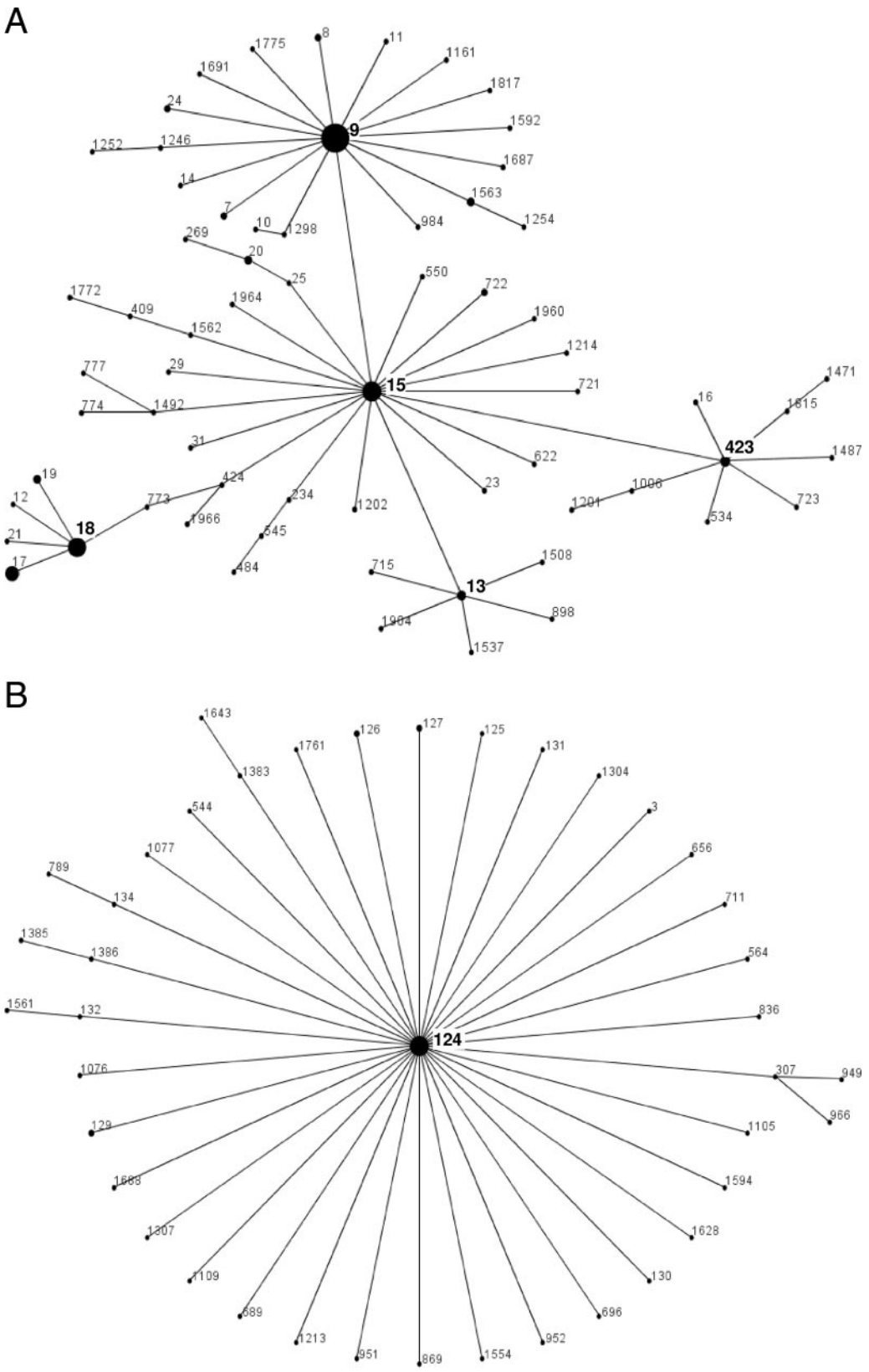

FIG. 1. (A) ST 9 is part of a clonal complex of 66 STs. The predicted founder is ST 15, but ST 9 is the founder of a large subgroup of 20 different STs. (B) ST 124 is the predicted founder of a group of 41 STs. Isolates of ST 124 from Germany, The Netherlands, Scandinavia, the United Kingdom, Australia, and Canada have been reported. All isolates in the database are reported to be penicillin G and macrolide sensitive, except for the German isolates, which are macrolide resistant. Other members of this group are mostly penicillin G and macrolide sensitive. (C) ST 273 is part of a clonal complex of 32 STs with the predicted founder ST 146. ST 273 is the cofounder of a subgroup of 11 STs.

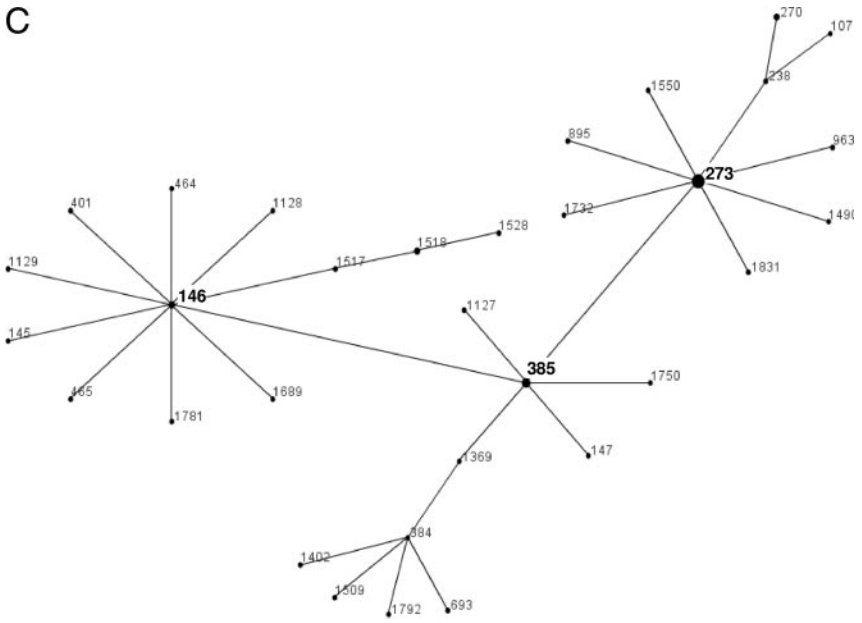

FIG. 1—*Continued.*

eBURST analysis showed that only three clones represented 58% of all macrolide-resistant isolates (ST 9 complex, ST 124 complex, and ST 273 complex). Phylogenetic analysis showed these three complexes as relatively separated subgroups in a maximum-likelihood tree. The prevalence of macrolide resistance genotypes varies substantially among countries. In a recent study, most isolates from France, Spain, Switzerland, and Poland were found to be *erm*(B) positive, whereas high levels of *mef*-positive strains from Greece and Germany were reported (17). Clones England^{14}-9 and Taiwan^{19F}-14 are the major contributors to the worldwide dissemination of M phenotype strains (7, 12; http://www.mlst.net). The England^{14}-9 clone harboring *mef*(A) has been described as being predominant among M phenotype pneumococci isolated in England, Italy, and Greece (1, 9). In contrast, the strains of the England^{14}-9 clone described in the United States carried the *mef*(E) gene (12). The England^{14}-9 clone described in this paper carried *mef*(A) and was of ST 9 exclusively, suggesting the clonal spread of this strain in Germany. In a recent report from Spain on isolates obtained from 1998 to 2003, the rate of erythromycin resistance among pneumococci was 34.4%. Interestingly, although the macrolide-lincosamide-streptogramin B resistance phenotype was the most prevalent (94.7%), the frequency of the M phenotype increased from 3.3% to 8.9%. The clonal dissemination of *mef*(E)-carrying strains of the serotype 14 variant of the Spain^{9V}-3 clone was the major contributor to this increase (2). In August 2006, a general recommendation for the 7-valent pneumococcal conjugate vaccine was issued in Germany; however, it is too early to see any effects.

In summary, the present investigation demonstrates the clonal spread of macrolide-resistant strains in Germany and underscores the high value of MLST in analyzing the genetic relatedness of antibiotic-resistant pneumococcal strains. The results are in accordance with findings of investigators from

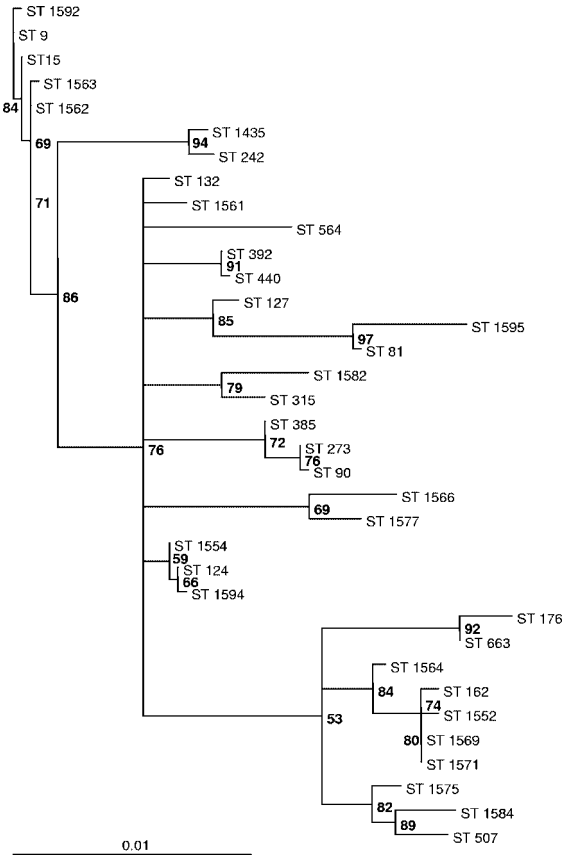

FIG. 2. Maximum-likelihood tree of 35 STs found in this study constructed by using the program Puzzle. Numbers at branching points represent the percentages of agreement in 1,000 puzzle steps. Model of substitution, HKY (10). Transition/transversion parameter (estimated from the data set), 7.17 (standard error, 1.34). Nucleotide frequencies (estimated from the data set): A, 27.8%; C, 21.5%; G, 22.6%; T, 28.1%. Expected transition/transversion ratio, 6.10. Expected pyrimidine transition/purine transition ratio, 0.96.

other countries and indicate that the horizontal spread of the *mef* gene among clinical pneumococcal isolates may be a major contributor to the emergence of macrolide-resistant pneumococci and is, therefore, a worrying infectious-disease problem with international significance.

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