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Polyphyletic Emergence of Linezolid-Resistant Staphylococci in the United States▽

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Since the year 2000, linezolid has been used in the United States to treat infections caused by antimicrobial-resistant Gram-positive cocci. At present, linezolid-resistant (Lin^r) *Staphylococcus aureus* and *Staphylococcus epidermidis* strains are rare and the diversity of their genetic backgrounds is unknown. We performed sequence-based strain typing and resistance gene characterization of 46 Lin^r isolates that were collected from local and national sources between the years 2004 and 2007. Resistance was found to occur in at least three clonal complexes (CCs; lineages) of *S. aureus* and in at least four subclusters of a predominant, phylogenetically unstable CC of *S. epidermidis*. New candidate resistance mutations in 23S rRNA and the L4 riboprotein were identified among the *S. epidermidis* isolates. These findings suggest that linezolid resistance has emerged independently in multiple clones of *S. aureus* and with a variety of ribosomal mutations in multiple clones of *S. epidermidis*.

Linezolid is the first antimicrobial from the synthetic oxazolidinone class to be introduced clinically (14). Approval was granted in the United States in the year 2000 for linezolid treatment of skin and soft tissue infections and pneumonia caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus* spp. and for infections caused by vancomycin-resistant enterococci (VRE). Linezolid also has activity *in vitro* against *Staphylococcus epidermidis* (14), which is a leading cause of infections associated with indwelling medical devices (35). The growing number of infections caused by multidrug-resistant staphylococci in the United States (11) has necessitated the use of new antimicrobials, such as linezolid.

The unique antimicrobial mechanism of linezolid occurs through binding to the peptidyltransferase center of the 50S ribosomal subunit and preventing the initiation of bacterial protein synthesis (1). Currently, linezolid resistance occurs in <1% of *S. aureus* isolates and <2% of *S. epidermidis* isolates from the United States (9). The most frequently reported mechanism of resistance in staphylococci is a G2576T point mutation within domain V of 23S rRNA (9). Additionally, G2447T, T2500A, and C2534T resistance mutations in 23S rRNA are known from various clinical and laboratory-derived staphylococcal isolates, and still other resistance mutations in 23S rRNA are known from enterococci (16, 18, 26, 38). Furthermore, a previous study demonstrated that the deletion of 2 amino acids in a conserved region of the L4 riboprotein, which is encoded by the *rplD* gene, conferred cross-resistance to oxazolidinones, macrolides, and chloramphenicol in *Streptococcus pneumoniae* (37). No mutations in the L4 riboprotein have yet been identified among linezolid-resistant (Lin^r) staphylococci. Finally, a methylase that is encoded by the *cfr* gene, which may be horizontally transferable, targets A2503 of 23S rRNA and simultaneously confers resistance to linezolid and four other classes of antimicrobials (31). *cfr* was originally identified in *Staphylococcus sciuri* isolates from animals (28), but it has recently been identified in *S. aureus* and *S. epidermidis* clinical isolates from humans (17, 31).

An understanding of the molecular epidemiology of Lin^r staphylococcal infections requires knowledge of both the bacterial isolates' resistance mechanisms and their genetic backgrounds. The Lin^r isolates reported so far have been found to be closely related on the basis of strain typing tools, such as phage typing, pulsed-field gel electrophoresis, ribotyping, and repetitive-element PCR (8, 10, 25, 32, 36). However, the diversity of the genetic backgrounds of Lin^r staphylococci remains unknown because few comparisons have been made beyond outbreak settings and between isolates from different hospitals. In the study described here, we utilized portable, sequence-based strain typing tools to facilitate evolutionary comparisons of locally and nationally sampled Lin^r *S. aureus* and *S. epidermidis* isolates.

MATERIALS AND METHODS

Bacterial isolates. A total of 46 Lin^r staphylococcal isolates were included in this study. We obtained 17 Lin^r *S. epidermidis* isolates from the years 2006 and 2007 from Westchester Medical Center (WMC), a 620-bed acute-care hospital located in Valhalla, NY. At that institution, linezolid resistance was first detected among coagulase-negative staphylococci in 2005. Additionally, 23 Lin^r *S. epidermidis* isolates and 6 Lin^r *S. aureus* isolates were obtained from the years 2004 to 2007 from national sources, including the Linezolid Experience and Accurate Determination of Resistance (LEADER) surveillance program (9). These na-

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tional isolates were kindly provided by Pfizer Inc., Groton, CT. For resistance gene comparisons, we included 16 linezolid-susceptible (Lin^s) *S. epidermidis* isolates from WMC of the same multilocus sequence types (STs) as the Lin^r isolates. Redundant WMC isolates, which were genetically identical to each other and obtained from the same patient, were excluded from this study.

All Lin^r isolates had linezolid MICs of >4 μg/ml, on the basis of testing with a MicroScan Gram-positive MIC susceptibility panel and a MicroScan Walk-Away system (Dade Behring, Inc.) or by broth microdilution assays. Etest (AB Biodisk) was used to confirm the MICs of select isolates. These methods were done according to the manufacturers' instructions. Isolates were routinely grown on tryptic soy agar overnight at 37°C. Isolates were stored long-term at −80°C in a solution of tryptic soy broth and 15% glycerol. Bacterial genomic DNA was isolated by using a DNeasy kit, according to the manufacturer's (Qiagen) instructions. Species identification was confirmed by PCR amplification and sequencing of both strands of a portion of the *tuf* gene, as described previously (7).

MLST and analysis. Multilocus sequence typing (MLST) was used to identify the genetic backgrounds of the isolates. MLST was performed according to the methods published for *S. aureus* (3) and *S. epidermidis* (30). Briefly, internal fragments of seven standard housekeeping genes were amplified by PCR, and both strands were sequenced. Alleles and STs were determined from the *S. aureus* and *S. epidermidis* MLST databases (http://www.mlst.net/).

The eBURST program (http://eburst.mlst.net/) was used to infer the evolutionary relatedness of the STs (6). Briefly, STs were assigned to clonal complexes (CCs; lineages), which represent groups of closely related STs, using the stringent criterion of requiring identity at six of seven MLST loci to another ST within the CC. Nonparametric bootstrapping of CC and subcluster founder assignments was done by using 1,000 replicates.

***aap* typing of *S. epidermidis* isolates.** The short sequence repeat region of the *aap* (accumulation-associated protein) gene was used as an additional marker of the *S. epidermidis* genetic background. This repeat region was amplified by PCR, and both strands were sequenced by using the primer pair and conditions described previously (22) and new primers AAP-F2 (5′-CTTTTTCTGTTGATTTACCTTCGC) and AAP-R2 (5′-AGATCCGACTAAAGTTCCCTCATT). For the new primer pair, the thermal cycling conditions were 95°C for 2 min, 35 cycles of 95°C for 30 s and 55° for 30 s, and an extension at 72°C for 1 min. *aap* types were assigned as described previously (22).

Resistance gene characterization. To identify putative mechanisms of linezolid resistance, we amplified by PCR and sequenced domain V of 23S rRNA and both strands of all of *rplD*. PCR was used to screen for the presence of *cfr*. Strains 1243-07 and 1257-07 from Ohio, kindly provided by Pfizer Inc., were used as positive and negative *cfr* controls, respectively. All primers that were used for amplification and sequencing of 23S rRNA, *rplD*, and *cfr* were described previously (31).

The staphylococcal chromosomal cassette *mec* (SCC*mec*) genetic element carries the *mecA* locus, which confers methicillin resistance. The SCC*mec* type has been used as one of the markers that define both MRSA and methicillin-resistant *S. epidermidis* (MRSE) clones (4, 19). The SCC*mec* types were identified by PCR methods that score the *mec* class and *ccr* allelic group. The primers of Robinson and Enright (27) were used to identify SCC*mec* type I (SCC*mec* I) to SCC*mec* IV. Components of SCC*mec* V and VI, including the *ccrC* gene and the *ccrAB4* allele, were detected with the primers of Kondo et al. (12).

Nucleotide sequence accession numbers. Unique *aap*, 23S rRNA, and *rplD* sequences have been deposited in the GenBank database with accession numbers GQ995195 to GQ995213.

RESULTS

Genetic backgrounds of Lin^r staphylococci. All six available Lin^r *S. aureus* isolates were MRSA and were obtained from national sources. MLST of these isolates revealed five STs: ST5, ST8, ST36, ST105, and ST1189 (Table 1). ST1189 had not been previously recorded in the MLST database, as of November 2008. Some of the isolates were indistinguishable from the common hospital- and community-acquired MRSA clones circulating in the United States, including ST5-SCC*mec* II (USA100), ST8-SCC*mec* IV (USA300), and ST36-SCC*mec* II (USA200) (4, 11). These clones are classified within three lineages, CC5, CC8, and CC30, respectively, which do not share a unique common ancestor (2, 4).

Of the 40 available Lin^r *S. epidermidis* isolates, all but 1 were MRSE, 17 were collected locally, and 23 were collected from national sources. MLST of these isolates revealed eight STs: ST2, ST5, ST6, ST22, ST23, ST87, ST185, and ST186 (Table 1). ST185 and ST186 had not previously been recorded in the MLST database as of November 2008. ST2, ST5, ST22, and ST23 were present in local and national isolate collections. Forty-five percent of the *S. epidermidis* isolates were ST2, and ST2 was the most frequent ST in both isolate collections. We note that local Lin^s isolates were also ST2, ST5, ST22, and ST23 (data not shown) and that ST2, ST5, ST6, ST22, and ST87 of unknown linezolid susceptibilities have previously been identified in international isolate collections (20). Nine of the *S. epidermidis* isolates harbored nontypeable SCC*mec* elements, and six isolates had *ccrAB4* alleles, in addition to SCC*mec* type III components (Table 1). eBURST analysis of the eight STs along with all 182 *S. epidermidis* STs in the MLST database revealed that linezolid resistance occurs within one predominant lineage (Fig. 1), known as CC2 (19, 20).

Further investigations of *S. epidermidis* genetic backgrounds. A reliable clone phylogeny can provide a framework for studying the evolution of antimicrobial resistance. However, it has been suggested that *S. epidermidis* has an epidemic population structure, in which the evolutionary origins of clones are obscured over time by recombination (20). For recombinant bacterial species, it has been noted that predominant lineages, inferred by eBURST analysis of MLST data, may depict unreliable links between subclusters (34). As the MLST database for *S. epidermidis* has expanded, we observed that the predicted founders and the composition of some subclusters have also proven unreliable. We describe these observations below.

First, the predicted founder of CC2 has changed from ST2 to ST6; bootstrap support for the ST2 founder has dropped from 91% to 37%, and bootstrap support for ST6 has increased from 19% to 60% (Table 2). It has been suggested that the predicted founder should be the ST with the lowest average distance, in terms of pairwise locus differences, to all other STs in the CC (6). Interestingly, this average distance statistic has consistently supported the ST6 founder assignment (Table 2).

Second, it was proposed that *S. epidermidis* CC2 can be subdivided into clusters I and II and that cluster II can be further subdivided into subclusters II-5, II-6, II-85, and II-89 (19). Lin^r *S. epidermidis* strains occur in four of these five subclusters, but the overall numbers and the compositions of some of these subclusters have changed. For example, ST14 and its descendants, previously within subcluster II-85 (19), are now within subcluster II-89 (Fig. 1). ST14 is a single-locus variant (SLV) of both ST85 and ST89. The eBURST algorithm links ST14 to the ST that has the largest number of SLVs or double-locus variants (DLVs) in the case of a tie. Over time, more SLVs and DLVs of ST89 rather than ST85 have been deposited in the MLST database (Table 2); therefore, ST89 is now linked to ST14 and its descendants. ST23 has also moved from minor CC status (19) to the periphery of subcluster II-89 (Fig. 1).

To attempt to corroborate the relationships among Lin^r *S. epidermidis* isolates as depicted by eBURST analysis, we used the short sequence repeats of the *aap* gene as an additional marker of the genetic background. Eight *aap* types plus a null type were detected among the isolates (Table 1). Thirty-three

TABLE 1. Genetic characteristics of 46 Lin^r staphylococci[a]

Species	Source (no. of isolates)[b]	MLST			*aap* type	*aap* repeats	23S rRNA sequence	L4 riboprotein sequence	SCC*mec* type (no. of isolates)
		CC	ST	Allelic profile					
S. aureus	N (1)	5	5	1-4-1-4-12-1-10	NA	NA	G2447T	wt	II (1)
S. aureus	N (1)	5	5	1-4-1-4-12-1-10	NA	NA	wt	wt	II (1)
S. aureus	N (1)	5	105	1-4-1-4-12-1-28	NA	NA	G2576T	wt	II (1)
S. aureus	N (1)	30	36	2-2-2-2-3-3-2	NA	NA	G2576T	wt	II (1)
S. aureus	N (1)	8	8	3-3-1-1-4-4-3	NA	NA	G2576T	wt	IV (1)
S. aureus	N (1)	8	1189	3-186-1-1-4-4-3	NA	NA	G2576T	wt	II (1)
S. epidermidis	L (7)	2-I	2	7-1-2-2-4-1-1	32	1-2-3-2-4-3-1-1-2-4-3-2-3-2-3-2-3-5	G2576T	wt	III (7)
S. epidermidis	L (2), N (2)	2-I	2	7-1-2-2-4-1-1	32	1-2-3-2-4-3-1-1-2-4-3-2-3-2-3-2-3-5	G2576T	$_{71}$GGR$_{72}$	III (4)
S. epidermidis	N (1)	2-I	2	7-1-2-2-4-1-1	36	2-3-2-3-5-7-6-8-36	G2576T	wt	NT (1)
S. epidermidis	N (4)	2-I	2	7-1-2-2-4-1-1	43	1-1-2-3-2-3-2-3-1-1-1-2-3-2-3-5	G2576T	wt	III (4)[c]
S. epidermidis	N (1)	2-I	2	7-1-2-2-4-1-1	43	1-1-2-3-2-3-2-3-1-1-1-2-3-2-3-5	G2576T	K68N	III (1)[c]
S. epidermidis	L (1)	2-I	2	7-1-2-2-4-1-1	43	1-1-2-3-2-3-2-3-1-1-1-2-3-2-3-5	wt	wt	III (1)[c]
S. epidermidis	N (1)	2-I	22	7-1-2-2-4-7-1	32	1-2-3-2-4-3-1-1-2-4-3-2-3-2-3-2-3-5	C2534T, G2576T, G2631T	N158S	III (1)
S. epidermidis	L (1)	2-I	22	7-1-2-2-4-7-1	32	1-2-3-2-4-3-1-1-2-4-3-2-3-2-3-2-3-5	C2534T	L108S, N158S, $_{71}$GGR$_{72}$	III (1)
S. epidermidis	N (4)	2-I	185	27-1-2-2-4-1-1	39	1-1-2-8-36	G2447T	wt	III (2), NT (2)
S. epidermidis	N (2)	2-I	185	27-1-2-2-4-1-1	40	1-1-2-3-2-3-2-8-36	G2447T	wt	III (2)
S. epidermidis	N (1)	2-I	186	7-1-2-2-4-7-23	37	1-2-3-2-4-3-1-1-2-4-3-2-3-5	wt	N158S, $_{71}$GGR$_{72}$	III (1)
S. epidermidis	L (1)	2-II-5	5	1-1-1-2-2-1-1	41	1-1-2-3-2-3-2-3-3-1-1-2-3-2-3-5	G2576T	wt	IV (1)
S. epidermidis	N (1)	2-II-5	5	1-1-1-2-2-1-1	42	1-1-2-3-2-3-2-3-1-1-1-2-4-3-5	G2447T	wt	NT (1)
S. epidermidis	N (2)	2-II-5	5	1-1-1-2-2-1-1	43	1-1-2-3-2-3-2-3-1-1-1-2-3-2-3-5	T2504A	wt	IV (1), NT (1)
S. epidermidis	L (1)	2-II-5	87	7-1-1-2-2-1-1	41	1-1-2-3-2-3-2-3-3-1-1-2-3-2-3-5	G2576T	wt	NT (1)
S. epidermidis	L (1)	2-II-6	6	1-1-2-2-2-1-1	41	1-1-2-3-2-3-2-3-3-1-1-2-3-2-3-5	G2576T	wt	IV (1)
S. epidermidis	L (1), N (3)	2-II-89	23	7-1-2-1-3-3-1	NT	NT	G2576T	N158S	I (1), IV (2), S (1)
S. epidermidis	N (1)	2-II-89	23	7-1-2-1-3-3-1	NT	NT	C2534T, G2576T	N158S	NT (1)
S. epidermidis	L (2)	2-II-89	23	7-1-2-1-3-3-1	NT	NT	G2576T	wt	NT (2)

[a] NA, not applicable; NT, nontypeable; S, susceptible; wt, wild type and based on the characteristics of *S. aureus* COL (for ST8 and ST1189), MRSA252 (for ST36), *S. aureus* N315 (for ST5 and ST105), or *S. epidermidis* RP62a (for all *S. epidermidis* STs).
[b] Isolates from local (L) and national (N) collections.
[c] Detection of *ccrAB4* alleles, in addition to SCC*mec* III components.

percent of the isolates were *aap* type 32, and *aap* type 32 was the most frequent *aap* type overall and in the local collection. *aap* from ST23 isolates was nontypeable (i.e., not detected) by PCR methods. Two *aap* types were shared among STs from the same cluster: *aap* type 32 was present in ST2 and ST22 from cluster I; and *aap* type 41 was present in ST5, ST6, and ST87 from cluster II (Table 1). However, one *aap* type was shared among STs from different clusters: *aap* type 43 was present in ST2 from cluster I and in ST5 from cluster II. In summary, while *aap* typing did not clarify the relationships between subclusters, it did reveal additional genetic diversity among Lin^r *S. epidermidis* isolates.

Putative mechanisms of linezolid resistance. Previously identified resistance mutations in domain V of 23S rRNA, including G2447T, C2534T, and G2576T, were the most frequent resistance mutations among the Lin^r staphylococcal isolates that we studied (Table 1). One or more of these known mutations occurred in 41 of 46 isolates. In addition, two novel mutations, T2504A and G2631T, were identified in the 23S rRNA of Lin^r *S. epidermidis* isolates (Table 1). The T2504A mutation occurred in two genetically identical isolates from the national collection. T2504A represents a candidate resistance mutation because it was the only ribosomal mutation detected in isolates with that mutation (Table 1) and it was not found among 16 Lin^s *S. epidermidis* isolates of the same STs as the Lin^r isolates (data not shown). The G2631T mutation occurred in one isolate that also harbored C2543T and G2576T mutations.

We identified a total of four different mutations in the L4 riboprotein of Lin^r *S. epidermidis* isolates, including the substitutions K68N, L108S, and N158S and the insertion $_{71}$GR$_{72}$ to $_{71}$GGR$_{72}$ (Table 1). An alignment of the L4 riboprotein amino acid sequences shows that two of the newly identified mutations, K68N and $_{71}$GGR$_{72}$, occurred within a conserved amino acid region that is responsible for oxazolidinone, macrolide, and chloramphenicol cross-resistance in *S. pneumoniae* (37) (Fig. 2). The $_{71}$GGR$_{72}$ insertion represents a candidate resistance mutation because it was the only ribosomal mutation other than N158S that was detected in one Lin^r isolate, and it occurred in a total of six Lin^r isolates and none of the Lin^s isolates. The K68N and L108S mutations occurred in isolates that also harbored 23S rRNA mutations. The N158S mutation was found among Lin^s *S. epidermidis* isolates (data not shown); therefore, it is probably a clonal marker rather than a resistance mutation.

The *cfr* gene was not detected in any of the isolates that we studied. We found one isolate each of Lin^r *S. aureus* and *S.*

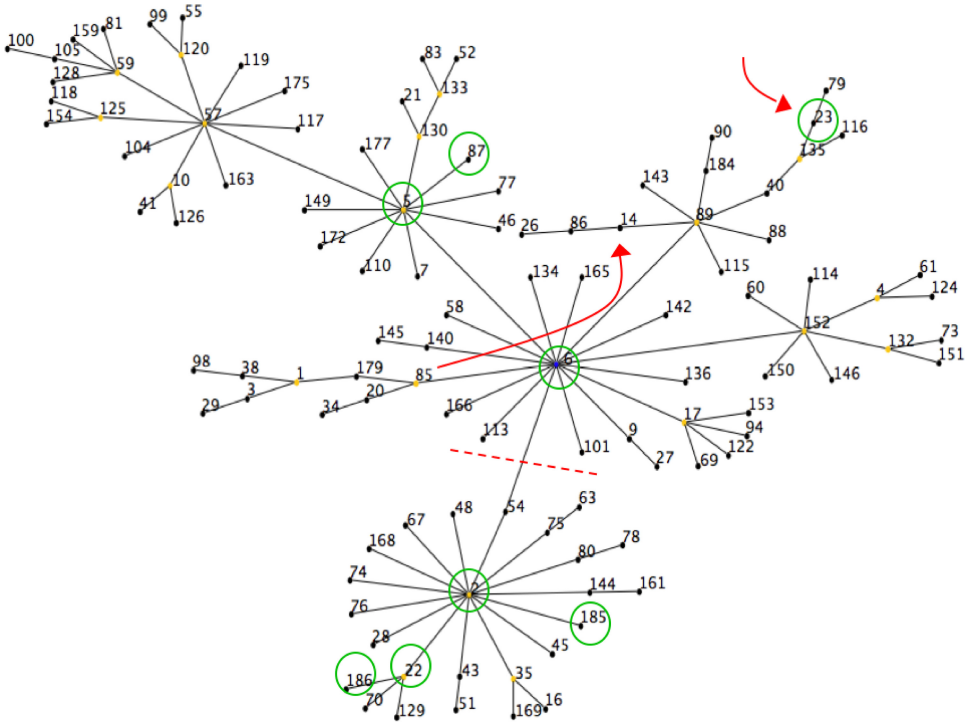

FIG. 1. eBURST diagram of *S. epidermidis* CC2 on the basis of analysis of all 182 STs in the MLST database as of November 2008. Each ST is represented by a dot; lines connect SLVs. The blue dot, ST6, represents the putative founder of CC2. Yellow dots represent putative secondary founders. Green circles indicate the eight STs with Lin^r isolates identified here. The red dotted line indicates the previously described division of CC2 into cluster I (below line) and cluster II (above line). Red arrows indicate select changes in the linkage of STs by eBURST over time, as described in the text.

epidermidis that did not present a known mechanism of resistance (Table 1). Linezolid resistance was confirmed by Etest in both of these isolates and in the isolates with the new candidate resistance mutations described above.

DISCUSSION

Linezolid is a representative of the first new antimicrobial class to be introduced clinically since the 1980s (21). Since linezolid is a purely synthetic antimicrobial, it was thought that preexisting mechanisms of resistance to linezolid would not be common in nature and, thus, that resistance would be slow to emerge (39). However, the target site of linezolid is not novel. As with other ribosome-targeting antimicrobials, resistance can occur via single nucleotide mutations and via the acquisition of genes that modify the target site of the antimicrobial. The first reported case of clinical Lin^r staphylococci appeared within 1 year after linezolid was approved for use for treatment (33). Although linezolid resistance remains rare among staphylococci (9), we have shown that resistance already occurs in multiple clones of both *S. aureus* and *S. epidermidis*.

Our results are consistent with a dynamic of sporadic linezolid resistance emergence in methicillin-resistant clones. Previously, linezolid resistance has been observed to develop not only in patients with prolonged exposure to the antimicrobial (10, 15) but also in cases without obvious exposure (24). The growth of resistant mutants in linezolid-free medium can result in a return to susceptibility, likely facilitated by the reduced fitness of resistant mutants (15). However, the persistence of resistant mutants during growth in linezolid-free medium has also been reported (24). An important epidemiolog-

TABLE 2. Changes in statistical support for *S. epidermidis* CC2 population structure on the basis of data set

ST	Analysis of 74 STs[a]					Analysis of 182 STs[a]				
	No. of SLVs	No. of DLVs	Avg distance	% CC founder bootstrap support[b]	% Subcluster founder bootstrap support[b]	No. of SLVs	No. of DLVs	Avg distance	% CC founder bootstrap support[b]	% Subcluster founder bootstrap support[b]
ST2	11	7	2.60	91.2 (1.5)	99.0 (0.0)	15	17	3.21	36.8 (1.9)	98.9 (0.3)
ST5	6	8	2.76	13.4 (1.3)	70.9 (1.9)	15	30	2.80	33.6 (1.6)	98.0 (0.5)
ST6	6	15	2.34	19.3 (1.9)	62.8 (1.6)	16	37	2.55	60.2 (1.8)	98.7 (0.5)
ST85	5	12	2.55	4.3 (0.8)	25.6 (1.7)	8	29	2.83	0.0 (0.0)	38.3 (2.2)
ST89	5	9	2.73	6.1 (1.6)	43.0 (2.5)	10	36	2.75	2.0 (0.5)	84.3 (1.2)

[a] The 74 STs used previously (20); there were 182 STs in the MLST database as of November 2008.
[b] Data represent the average (standard deviation) based on 10 runs of eBURST with 1,000 replicates each.

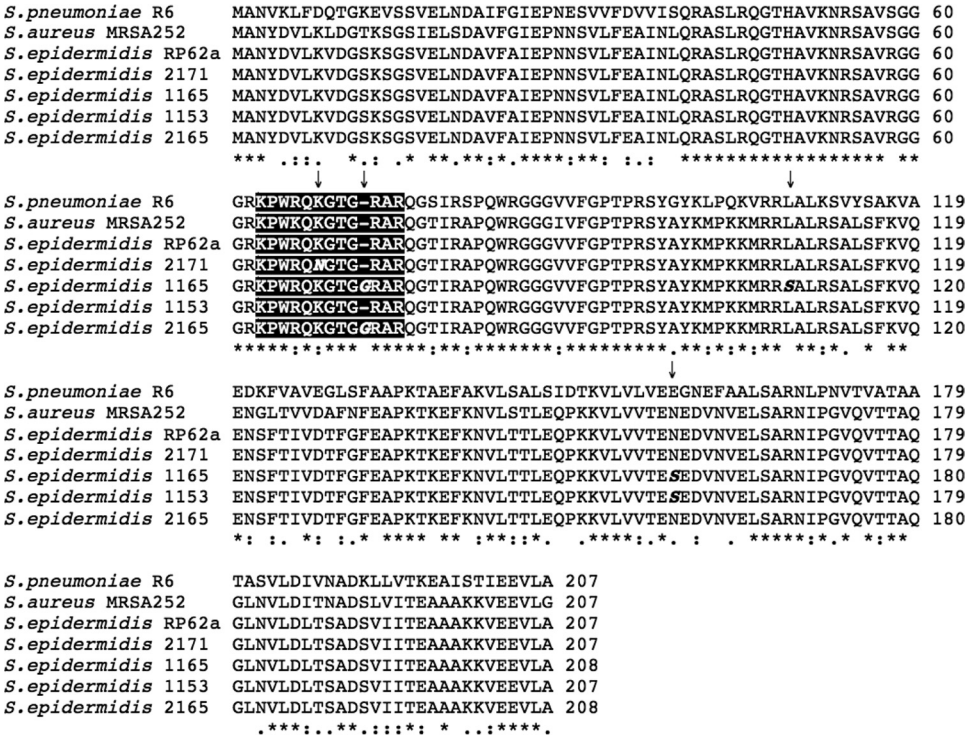

FIG. 2. Alignment of L4 riboprotein amino acid sequences. *, identical amino acids; colons, conserved substitutions; periods, semiconserved substitutions. The sequences of Lin^s strains *S. pneumoniae* R6, *S. aureus* MRSA252, and *S. epidermidis* RP62a (GenBank accession numbers NP_357783.1, YP_041689.1, and YP_189393.1, respectively) are shown for comparison. Black highlighting shows the previously identified 12-amino-acid region involved in oxazolidinone, macrolide, and chloramphenicol cross-resistance in *S. pneumoniae*. Amino acids in boldface italics and amino acids pointed to with arrows show examples of the variations observed among Lin^r *S. epidermidis* isolates, as described in the text.

ical question is whether resistant mutants that arise *de novo* in hospitals can remain resistant long enough to spread geographically. Importantly, we observed that linezolid resistance has arisen in MRSA clones with a proven ability to spread. We also observed two instances in which Lin^r *S. epidermidis* isolates of the same ST and *aap* type and with the same putative resistance mutations (ST2:*aap* type 32:G2576T and $_{71}$GGR$_{72}$ and ST23:*aap* null type:G2576T) occurred in different U.S. states.

The case for a polyphyletic origin of Lin^r *S. aureus* is strong, because the six isolates studied here belong to three different CCs that do not share a unique common ancestor (2, 4). The current understanding of the *S. epidermidis* population structure is not as detailed as that of *S. aureus* population structure, although recent advances have been made (20). As anticipated (19), some of the structure within *S. epidermidis* CC2 is not reliable. In particular, the founder assignments and the compositions of some subclusters inferred by eBURST have changed with the expansion of the MLST database. While *aap* typing was able to distinguish between some Lin^r *S. epidermidis* isolates with the same STs and resistance mutations, it did not resolve the issues regarding the population structure. Further study of the *S. epidermidis* population structure is clearly needed. The case for a polyphyletic origin of Lin^r *S. epidermidis* can be based simply on the observations that six of eight STs have the G2576T resistance mutation (Table 1) and that this mutation also occurs in different species of staphylococci and enterococci and is even reported to arise *in vitro* (23, 26); it is therefore biologically plausible that this mutation could arise on multiple occasions within *S. epidermidis*. Alternatively, recombinations involving either MLST loci or resistance loci could have occurred. For example, STs that appear to be distantly related in the eBURST diagram could have originated from a common resistant ancestor by recombination at MLST loci. Susceptible isolates are available locally for four of these eight STs (data not shown), and five of these STs are known to have international geographic distributions (20), so this alternative hypothesis for a single origin of resistance would still require multiple *ad hoc* assumptions. Finally, the resistance mutations could have originated in one ST, followed by multiple recombinations between STs.

We identified new candidate resistance mutations in *S. epidermidis*, including the T2504A mutation in 23S rRNA and the $_{71}$GGR$_{72}$ insertion in the L4 riboprotein. The T2504A mutation is adjacent to other previously described sites that have been demonstrated to confer linezolid resistance; A2503 is methylated in staphylococci by *cfr* (31), and G2505A is a known resistance mutation in *Enterococcus faecium* (26). Moreover, T2504C has been associated with *in vitro* linezolid resistance in *S. aureus* (13). In *S. pneumoniae*, oxazolidinone, macrolide, and chloramphenicol cross-resistance is caused by deletions of 2 amino acids within a conserved amino acid region of the L4 riboprotein (37). We determined that the $_{71}$GGR$_{72}$ insertion occurred within this previously described region, which is approximately 12 amino acids in length and which may interact with 23S rRNA (29). Experiments are

needed to verify whether these two new candidate resistance mutations alone are sufficient to cause linezolid resistance. Two Lin^r isolates without any detectable mechanism of linezolid resistance were also identified. Mutations in the L22 riboprotein, which have been found in macrolide-resistant *S. pneumoniae* isolates (5), should be investigated as a possible source of linezolid resistance.

In summary, although linezolid resistance among staphylococci remains rare, resistant isolates of multiple clones sampled from around the United States were identified. The case for a polyphyletic origin of linezolid resistance in *S. aureus* is stronger than that in *S. epidermidis* because of uncertainty concerning the evolutionary origins of *S. epidermidis* clones. Our results also indicate that the pool of fitness-tolerable linezolid resistance mutations is likely deeper than was previously thought. The continued judicious use of linezolid and surveillance of staphylococci are needed to preserve the therapeutic efficacy of this important antimicrobial.

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ADDENDUM IN PROOF

During the review process, a research letter was published by Liakopoulos et al. (J. Antimicrob. Chemother. **64:**206–207, 2009) showing the T2504A mutation in 23S rRNA from a linezolid-resistant *S. epidermidis* isolate. These independent findings strengthen the notion that T2504A is associated with linezolid resistance.

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