Transferable Multidrug Resistance Plasmid Carrying cfr Associated with \texttt{tet(L)}, \texttt{ant(4')}-\texttt{Ia}, and \texttt{dfrK} Genes from a Clinical Methicillin-Resistant \textit{Staphylococcus aureus} ST125 Strain

Enrique Ruiz de Gopegui, Carlos Juan, Laura Zamorano, José L. Pérez, and Antonio Oliver
Servicio de Microbiología, Hospital Universitari Son Espases, Palma de Mallorca, Spain

A multidrug resistance (MDR) conjugative plasmid of ca. 50 kb (designated pERGB) was detected in a linezolid and methicillin-resistant \textit{Staphylococcus aureus} strain with sequence type 125 (ST125-MRSA-IVc). This strain was detected in two patients with chronic obstructive pulmonary disease, previously treated with multiple antimicrobials, including linezolid. \text{pERGB} was transferable by conjugation and carried the resistance genes \texttt{cfr} (oxazolidinones, phenicols, lincosamides, pleuromutilins, and streptogramin A), \texttt{ant(4')}-\texttt{Ia} (tobramycin), \texttt{tet(L)} (tetracycline), and \texttt{dfrK} (trimethoprim). A novel genetic structure, linking all of these resistance genes for the first time, was elucidated through sequencing of a 15,259-bp fragment from pERGB. Active surveillance to prevent the dissemination of such highly concerning MDR transferable elements is needed.

\text{Linezolid was the first oxazolidinone approved for the treatment of Gram-positive bacteria and is still the only antimicrobial in this class released for clinical use. It has been commercialized in the United States since 2000 and in Spain since 2002. Linezolid resistance in staphylococci is very uncommon, although it has increased in the recent years.}

The most frequently reported mechanism of linezolid resistance in staphylococci is a point mutation within the central loop of domain V of the 23S rRNA gene (9), followed by mutation in the ribosomal protein L3 or L4 (17). Both forms of resistance are mainly caused by prolonged exposure to linezolid and are not transferable between strains. A third mechanism of resistance is determined by the \texttt{cfr} gene, encoding an rRNA methyltransferase that methylates the adenosine at position A2503 (\textit{Escherichia coli} numbering) in the 23S rRNA (13). This enzyme confers cross-resistance to linezolid and four other classes of antimicrobial agents: phenicols, lincosamides, pleuromutilins, and streptogramin A. The \texttt{cfr} gene is often located in a plasmid which may be horizontally transferred (18). It was originally identified on the pSCSF1 plasmid from a \textit{Staphylococcus sciuri} isolate obtained from the nasal swab of a florfenicol-treated calf with a respiratory infection in 1997 (26). In humans, the first clinical isolate documented to carry the \texttt{cfr} gene was a methicillin-resistant \textit{Staphylococcus aureus} (MRSA) strain isolated from a patient with respiratory infection in Colombia in 2005 (29). The first outbreak by MRSA carrying the \texttt{cfr} gene was reported in 2008 and involved 15 patients admitted in the intensive care unit of a hospital from Madrid, Spain (20).

Here we describe two cases of respiratory infection by \texttt{cfr}-positive MRSA isolates detected in Majorca, Spain, occurring in patients with chronic obstructive pulmonary disease (COPD) admitted in August 2010 in the same unit of the main tertiary hospital of the island. Moreover, we show that \texttt{cfr} was located in a novel transferable plasmid together with multiple other antibiotic resistance determinants.

The linezolid-resistant MRSA isolates from the two patients had the same disk diffusion susceptibility profile. According to Clinical and Laboratory Standards Institute (CLSI) (4) breakpoints, both isolates were resistant to penicillin, oxacillin, cefoxi-
rifampin-resistant mutant of ATCC 29213 resistant MRSA isolate from patient 1 was used as a donor, and aing the protocol described by Shore et al. (27). The linezolid-

TABLE 1 Resistance profiles and genes detected in linezolid-susceptible and -resistant MRSA clinical isolates from patients 1 and 2 and in the transconjugant derivative

<table>
<thead>
<tr>
<th>Patient or description</th>
<th>Date</th>
<th>Isolate no.</th>
<th>Sequence type</th>
<th>Resistance phenotype*</th>
<th>Presence of gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>June 2009</td>
<td>50115</td>
<td>ST125-IVc</td>
<td>OXA, CIP, TOB</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>February 2009</td>
<td>44207</td>
<td>ST22-IVh</td>
<td>OXA, CIP, TOB, CLI, LIN, CLO, TET, TS</td>
<td>+</td>
</tr>
<tr>
<td>Transconjugant</td>
<td>August 2010</td>
<td>69333</td>
<td>ST125-IVc</td>
<td>OXA, CIP, TOB, CLI, LIN, CLO, TET, TS</td>
<td>+</td>
</tr>
</tbody>
</table>

* OXA, oxacillin; CIP, ciprofloxacin; TOB, tobramycin; ERY, erythromycin; CLI, clindamycin; iCLI, inducible clindamycin resistance (erythromycin-clindamycin D-zone test positive); LIN, linezolid; CLO, chloramphenicol; TET, tetracycline; TS, trimethoprim-sulfamethoxazole (cotrimoxazole); RIF, rifampin.

(HC08 strain) from the outbreak reported in Madrid (20) was included for comparative purposes. The linezolid-resistant isolates from both patients and interestingly also the first linezolid-susceptible isolate from patient 1 had indistinguishable electrophoretic band patterns, which corresponded to the ST125-MRSA-IVc clone, one of two MRSA clones predominant in Majorca (1, 24) and also the most prevalent MRSA clone in Spain since 1998 (30). On the other hand, the linezolid-susceptible isolate from patient 2 belonged to ST22-MRSA-IVh (EMRSA-15 clone), also prevalent in Majorca, whereas the HC08 strain corresponded to ST228-MRSA-I.

To determine the mechanism of resistance to linezolid, a PCR assay to detect the presence of the cfr gene (14) was performed with genomic DNA extracted using the QIAamp DNA minikit (Qiagen, Germany). The HC08 strain (20) was used as a positive control. The PCR assay for the cfr gene was negative for the linezolid-susceptible MRSA isolates and positive for those showing resistance to the drug (Table 1).

Conjugation experiments were then performed to assess whether the cfr gene was located on a transferable plasmid, adapting the protocol described by Shore et al. (27). The linezolid-resistant MRSA isolate from patient 1 was used as a donor, and a rifampin-resistant mutant of ATCC 29213 S. aureus strain was used as a recipient. The transconjugants were selected in brain heart infusion (BHI) agar plates with rifampin (20 μg/ml) and chloramphenicol (15 μg/ml) and checked through the analysis of plasmid and susceptibility profiles, cfr PCR assay (14), and PFGE.

The antimicrobial susceptibility profiles of linezolid-resistant MRSA from patient 1 (donor), rifampin-resistant ATCC 29213 S. aureus (recipient), and a selected transconjugant for several antibiotics were determined by Etest and/or broth microdilution, and results are presented in Table 2. Remarkably, the transconjugant had acquired resistance or reduced susceptibility to clindamycin, chloramphenicol, and linezolid (as expected for cfr transfer) but also to tetracycline, tobramycin, and cotrimoxazole. The tigecycline MIC was also slightly enhanced.

The PCR assay for the cfr gene in the plasmid DNA (14) extracted from the transconjugant using the Qiagen Plasmid Midi kit was also positive. The size of the plasmid was determined through analysis of the EcoRI restriction fragments, with a result of ca. 50 kb. The plasmid was larger than the two first cfr plasmids characterized, both detected in animals, plasmids pSCFS1 (17.1 kb) (12) and pSCFS3 (35.7 kb) (14), but the size was similar to that of other cfr plasmids: pSCFS6 (43 kb, also detected in animals) (15) and pSCFS7 (45 kb, detected in humans) (27).

For the detection of the additional resistance genes transferred,

TABLE 2 Antimicrobial resistance profiles of isolate 69371 (cfr positive) from patient 1 (donor), a rifampin-resistant (Rifr) ATCC 29213 S. aureus strain (recipient), and its transconjugant derivative

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Susceptibility testing method</th>
<th>MIC (μg/ml) for strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>Etest</td>
<td>69371 (donor)</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>Etest</td>
<td>ATCC 29213 Rifr (recipient)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>Etest</td>
<td>Transconjugant</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>Etest</td>
<td>0.5</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Etest</td>
<td>0.5</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole (1/19)a</td>
<td>Etest</td>
<td>0.064</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>Etest</td>
<td>0.75</td>
</tr>
<tr>
<td>Teicoplanin</td>
<td>Etest</td>
<td>2</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>Etest</td>
<td>0.75</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>Etest</td>
<td>0.25</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Etest</td>
<td>0.25</td>
</tr>
<tr>
<td>Linezolid</td>
<td>Etest</td>
<td>0.38</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>Etest</td>
<td>3</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Broth microdilution</td>
<td>0.25</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Broth microdilution</td>
<td>0.25</td>
</tr>
<tr>
<td>Rifampin</td>
<td>Broth microdilution</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≤0.06</td>
</tr>
</tbody>
</table>

*a* The value on the MIC scale refers to the first component of the combination.
PCR analysis was carried out with the plasmid DNA extract of the transconjugant and clinical isolates, and results are shown in Table 1. The PCR assays for the ant(4’)-Ia gene (also known as aadD) which confers resistance to tobramycin, amikacin, kanamycin, and neomycin (25), for the tet(L) gene (tetracycline resistance) (22), and for the dfrK gene (6) (trimethoprim resistance) were positive for the transconjugant derivative and for the two linezolid-resistant MRSA isolates (Table 1). On the other hand, the PCR assays were negative in all cases for the tet(K) and tet(M) genes (tetracycline resistance) (8), the vga(C) gene (pleuromutilin/lincomamide/streptogramin A resistance) (6), and the fexA gene (which codifies an efflux protein of phenicol) (14).

To determine the proximity of the cfr determinant to the other resistance genes, DNA fragments resulting from EcoRI digestion from the transconjugant derivative were cloned into pUCP24 (21). The resulting plasmids were then transformed into the CaCl₂-competent (21). The resulting plasmids were then transformed into the Escherichia coli XL1 Blue strain. Transformants were selected on Luria-Bertani (LB) agar plates supplemented with 100 μg/ml gentamicin. Plasmid DNA, purified from several transformants with the Qiagen plasmid minikit, was screened for cfr presence through PCR assays (14), yielding a positive result in one of them. The cloned 8,675-bp EcoRI fragment was fully sequenced through primer walking and contained the resistance genes cfr, ant(4’)-Ia, and tet(L) (Fig. 1). Left and right ends were extended to obtain a 15,259-bp fragment through diverse PCR assays, followed by sequencing, based on available GenBank records: plasmid pSCFS3 (accession number AM086211.1) (14) for the left end and pKKS825 (accession number FN377602.2) (11) and pKKS2187 (accession number FM207105.1) (12) for the right end.

The initial segment of the sequenced fragment corresponded to an entire copy of insertion sequence IS21-558, with a homology of 100% with pSCFS3 (14), followed by the cfr gene and a noncoding region of 688 bp, also identical to sequence of pSCFS3. Nevertheless, adjacent to this noncoding region, a novel structure was detected, including a putative resolvase (72% nucleotide sequence identity with the res gene from Bacillus megaterium, GenBank accession number CPO03018.1) and a putative transposase (amino acid identity of 63% with a transposase of Enterococcus faecium, accession number ZP_03980121.1). Next, an IS431-flanked genetic structure was found; an inverted copy of insertion sequence IS431 was followed by a truncated repU gene, with 99% identity to the nucleotide sequence of plasmid pUB110 (23) and very similar to that previously described in pKKS825/pKKS2187 plasmids (10, 11). Downstream, the ant(4’)-Ia, tet(L), and dfrK resistance genes were identified, with sequences nearly identical to those described for the pKKS825 plasmid (11). Further downstream of dfrK, two more open reading frames (ORFs) were detected: the pre/mob gene (97% identical to pKKS2187) and an entire copy of the repU gene. At the end of the sequenced fragment, a direct copy of IS431 was documented (Fig. 1).

In summary, we describe a new plasmid (designated pERGB) that combines several relevant antibiotic resistance determinants, leading to a MDR transferable element that is highly concerning, particularly when acquired by MRSA strains as described in this work. Thus, active surveillance to prevent the dissemination of such MDR elements and strains is urgently needed.

**Nucleotide sequence accession number.** The described 15,259-bp sequence of plasmid pERGB from MRSA strain 69371 has been deposited in the GenBank database under accession number JN970906.

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**REFERENCES**


