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Isolation and first draft genome sequence of a linezolid-dependent *Staphylococcus aureus* clinical strain

Víctor A. García-Angulo[1], Beatrice Herve[2], Joel Melo[3], Camila Sanhueza[1], Sebastián De la Fuente[2], Luis Lozano Aguirre[4], Chris Baysdorfer[5] & María Teresa Ulloa*[,1]

[1]Microbiology & Mycology Program, Biomedical Sciences Institute, School of Medicine, University of Chile, Santiago 8380453, Chile

[2]Laboratorio Clínico, Clínica Las Condes, RM, Santiago 8340518, Chile

[3]Departamento de Medicina Interna, Clínica Las Condes, RM, Santiago 8340518, Chile

[4]Unidad de Análisis Bioinformáticos, Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca 62209, México

[5]Department of Biological Sciences, California State University, East Bay, Hayward, CA 94542, USA

*Author for correspondence: Tel.: +56 2978 6145; mtulloa@uchile.cl

Background: Antibiotic-dependent pathogenic bacteria are sporadically isolated from patients that received prolonged antibiotic treatments. Evolution of antibiotics dependence and its clinical implications are scarcely studied. **Materials & methods:** A linezolid-dependent *Staphylococcus aureus* strain was isolated from a cystic fibrosis patient. A draft genome sequence was obtained and searched for known antibiotics resistance determinants and virulence factors. **Results:** The genome was assembled into 79 contigs for a total of 2.83 Mbp. This strain is a sequence type 5 methicillin-resistant *Staphylococcus aureus* with a type I SCC*mec* cassette also conserving the Panton–Valentine leukocidin. The G2576T substitution, conferring linezolid resistance, was harbored by all five copies of the 23S rRNA. **Conclusion:** The linezolid-dependent strain is related to a strain circulating in Latin America that acquired a mutation conferring linezolid resistance.

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Keywords: antibiotic dependence • cystic fibrosis • genome sequencing • linezolid resistance • *Staphylococcus aureus*

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most prevalent pathogens in people with cystic fibrosis, with an infection rate of up to 26% in the USA [1]. In these strains, resistance to β-lactam antibiotics is provided by the staphylococcal chromosomal cassette *mec* (SCC*mec*), for which several types have been described [2]. The evolution of resistance in MRSA has made necessary novel antibiotics to treat these infections. Linezolid, a member of the oxazolidinone family of antibiotics is used to treat hospital-acquired infections by MRSA and methicillin-sensitive *S. aureus* as well as infections caused by other multidrug-resistant bacteria in critical care [1,3,4]. This antibiotic inhibits bacterial protein synthesis by preventing the formation of the translation initiation complex after direct binding to the 50S ribosomal subunit [3]. Given that this is one of the most recently developed antibiotics for human use, a low incidence of resistance (0.05 %) is found in clinical *S. aureus* isolates, although this incidence is much higher in patients who received linezolid for extended times [5]. The mechanisms of linezolid resistance in staphylococci known to date encompass chromosomal mutations in the 23S rRNA gene, including the G2447T, T2500A and G2576T substitutions. Also, deletions or substitutions in the ribosomal L3, L4 and L22 proteins are associated with resistance, in spite that these proteins assemble in a ribosomal site away from the linezolid-binding site [4,6–9]. Methylation of A2503 in the 23S rRNA by the plasmid-encoded Cfr enzyme also confers resistance [4,6].

Beyond antibiotic resistance, a striking development in pathogenic bacteria is antibiotic dependence. This refers to the requirement of an antibiotic to grow or to a significant improvement in bacterial growth in the presence of an antibiotic [10–13]. Antibiotic-dependence phenotypes appeared in strains submitted to *in vitro* antibiotic-resistance

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Table 1. *S. aureus* Bcl050618 antimicrobial susceptibility testing.

Antimicrobial agent	MIC (μg/ml)	Interpretative categories
Cefoxitin	+[†]	R
Oxacillin	≥4	R
Gentamicin	≥16	R
Ciprofloxacin	≥8	R
Moxifloxacin	2	R
Erythromycin	≥8	R
Clindamycin	≥8	R
Linezolid	≥8	R
Daptomycin	0.25	S
Teicoplanin	≤0.5	S
Vancomycin	≤0.5	S
Tetracycline	≤1	S
Tigecycline	≤0.12	S
Nitrofurantoin	32	S
Chloramphenicol	≥64	R
Rifampin	≤0.5	S
Trimethoprime-sulfamethoxazole	≤10	S

[†]Methicillin resistance.

development [14] and in natural clinical isolates [10,15,16]. Clinical generation of dependence has been described for a series of antibiotics such as vancomycin, streptomycin, terramycin, aureomycin and chloromycetin [10,12,15,17]. Linezolid dependence was first identified in a set of highly resistant *Staphylococcus epidermidis* strains isolated from blood [13]. Later, the only known case to date of linezolid dependence in *S. aureus* was described in an isolate from a respiratory sample in a cystic fibrosis case [18]. Here, we report the isolation of a linezolid-dependent *S. aureus* strain from a surgical site infection in a cystic fibrosis patient. In order to further characterize it, full genome sequencing was applied to this strain. Know linezolid resistance determinants were searched and other genomic characterizations performed.

Materials & methods

A bipulmonary transplant surgical wound secretion sample from a cystic fibrosis patient was inoculated on tryptic soy agar and chocolate agar PolyViteX plates and incubated at 35°C. After 24 h, no growth was observed but after 48 h of incubation, small-colony development was detected. Identification was performed by MALDI-TOF (VITEK MS, bioMérieux). Antimicrobial susceptibility testing was performed using VITEK® AST-P618 with VITEK XL bioMérieux SA for the antibiotics indicated in Table 1. In the case of linezolid, VITEK determined that the strain could grow in a concentration equal to or greater than 8 μg/ml. This result, although not providing an exact MIC, indicated that the isolate is linezolid-resistant. The linezolid resistance observed by VITEK was corroborated by E-test® (bioMérieux) and Kirby–Bauer disk diffusion test [19] as per clinical laboratory protocol for unusual resistances. Maintenance of the linezolid dependence was monitored by passages in Mueller–Hinton (MH). In the Kirby–Bauer test, sporadic growth of two types of colonies, white and yellow, was observed. These colonies were further characterized by MALDI-TOF and antibiotic susceptibility tests. During the passages in MH agar, the same pattern of colonies derived from an isolated linezolid-dependent colony was consistently detected.

For DNA sequencing, bacterial DNA was sonicated with a Bioruptor UCD 200 (Diagenode) and a sequencing library was constructed using the IonXpress Plus gDNA Fragment Library kit (Thermo Fisher Scientific, MA, USA). Size selection in the range of 250–350 bp was performed with a Blue Pippen (Sage Science) and DNA quantity and quality was assessed with a Bioanalyzer 2100 (Agilent). Sequencing was conducted on an Ion Torrent personal genome machine (PGM) (Thermo Fisher Scientific) using a single 318 chip. Reads were trimmed using the Trim sequences tool from Galaxy [20], assembled using SPAdes and annotated using the rapid annotation using subsystem technology (RAST) tool kit from the PATRIC bacterial bioinformatics resource center [21]. After assembly, indels were corrected by hand where possible. Sequence alignments were performed using the Clustal

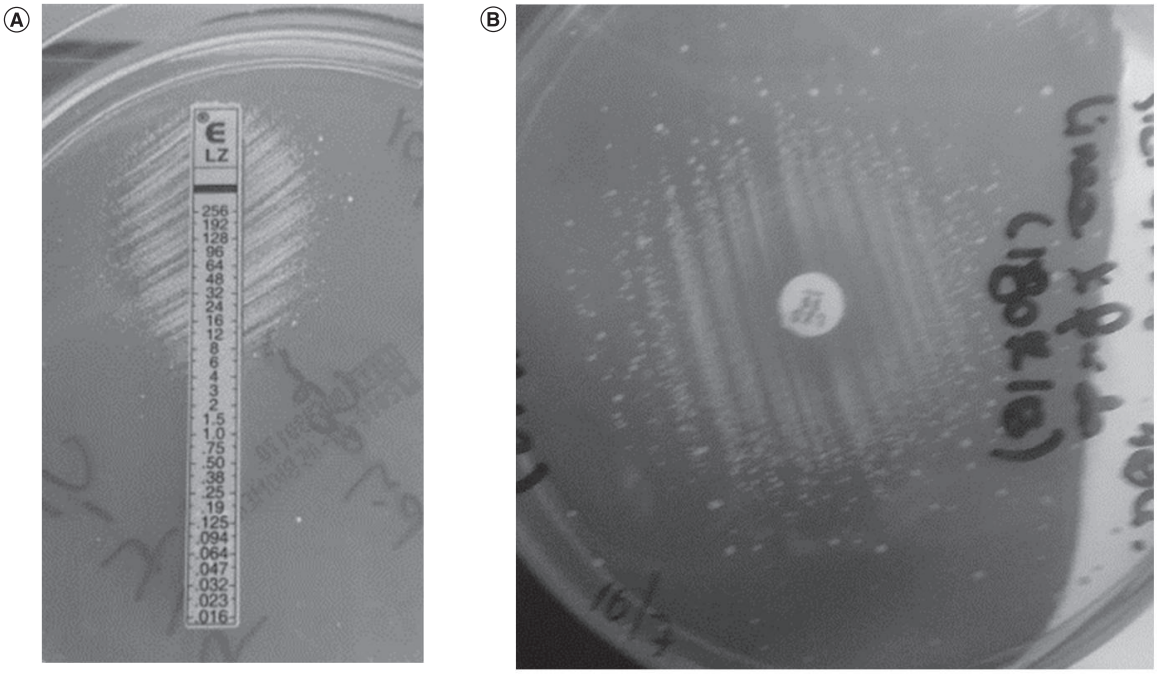

Figure 1. Susceptibility tests for the *S. aureus* Scl050618 isolate. (A) E-test with a linezolid strip incubated for 24 h. **(B)** Kirby–Bauer-susceptibility test with a linezolid disk, incubated for 24 h.

Omega accessory application of BioEdit. The search and retrieval of specific gene sequences were performed by local BLAST using BioEdit.

Results

On 5 May 2018, a successful bipulmonary transplant was performed on a 7-year old female patient with a diagnosis of cystic fibrosis at 4 months of age with pulmonary, sinus and pancreatic involvement. The patient has a history of chronic infection with multidrug resistant *Pseudomonas aeruginosa*, MRSA and extended-spectrum beta-lactamase-producing *Escherichia coli* that evolved with recurrent exacerbations since 2017, respiratory failure and need for noninvasive motion ventilation. Multiple antibiotic schemes had been used to treat these chronic infections, including triassociated (meropenem, colistin + vancomycin or meropenem, colistin + linezolid), plus aztreonam or inhaled colistin. In postoperative ambulatory controls, a sample of the surgical wound in the anterior thoracic zone was taken. Sample was platted on tryptic soy agar and PolyViteX, and slow-growing colonies were obtained, identified as *S. aureus* by MALDI-TOF and denominated Scl050618. An automated susceptibility test was performed revealing that this strain was resistant to methicillin and linezolid (Table 1). As resistance to linezolid is infrequent among clinical *S. aureus* isolates, this result prompted a confirmation using E-test. Strikingly, bacterial growth was exclusively observed around the strip above 6 μg/ml (Figure 1A). This growth pattern is similar to that reported for other antibiotic-dependent bacteria, including linezolid-dependent *S. aureus* [18]. Twenty passages in MH agar were performed and maintenance of the linezolid-dependence by E-test was corroborated each five passages. In Kirby–Bauer susceptibility tests with 30 μg linezolid disk, growth around the disk was observed (Figure 1B), with a small inhibition zone. Notably, sporadic growth of colonies far from the disc was observed. In these few colonies, two different morphologies, namely white and yellow colonies, were detected. One colony of each morphology from this area was characterized by the Kirby–Bauer diffusion method. The white colony was found to be nondependent linezolid-resistant, while the yellow colony was linezolid-susceptible. Both strains were confirmed as *S. aureus* by MALDI-TOF.

In order to characterize this linezolid-dependent isolate, its genome was sequenced and assembled into 79 contigs for a total of 2.82 Mbp (deposited in the PATRIC database, genome ID 1280.22564). The sequence contained 2953 predicted genes with a GC content of 32%. In accordance with the antibiotic susceptibility studies performed with VITEK (Table 1), the AMR phenotype function of PATRIC predicted Scl050618 to be resistant

to methicillin, erythromycin, ciprofloxacin, clindamycin, gentamicin and penicillin, but susceptible to tetracycline and trimethoprim/sulfamethoxazole, thus confirming that Scl050618 is a MRSA strain. A search for the SCC*mec* cassette using the SCC*mec*Finder-1.2 server [22] in this genome identified the presence of a type I SCC*mec*. Multilocus sequence typing identified this strain as belonging to the sequence type 5. In total, this strain encoded 78 virulence factor genes from the Virulence Factors Database, including genes for capsular type 8 and the genes for the Panton–Valentine leukocidin.

A screening for known mutations conferring linezolid resistance in *Staphylococcus* identified the G2576T substitution harbored by all 5 copies of the 23 S rRNA. No additional mutations were identified in any copy of the 23 S rRNA. The ribosomal proteins L3, L4 and L22 were identical to those of *S. aureus* NCTC 8325 linezolid-susceptible reference strain. A search for related genomes using the Similar Genome Finder service of PATRIC identified the *S. aureus* strain DAR5897 (genome ID 1409753.3) as the closest genome (distance 0.000385) followed by DAR3153 (distance 0.0004834, genome ID 1422283.3), MRSA blood isolates from Venezuela and Argentina, respectively [23]. Importantly, neither of the copies of the 23 S rRNA in each related strain has the G2576T substitution, suggesting that the linezolid resistance-associated mutation rose specifically in Scl050618.

Discussion

The linezolid-dependent MRSA characterized here belongs to the sequence type 5 and has a type I SCC*mec*, both of which are hallmarks of the Chilean/Cordobes MRSA clone that is reportedly more prevalent in Chile [23,24]. Nonetheless, this strain harbors the Panton–Valentine leukocidin genes, which are absent in the typical Chilean/Cordobes clone strains. Moreover, at the genome level this strain is closely related to strains from the USA 100 clone, the second most prevalent clone in Chile [23].

The most notable characteristic of Scl050618 is its dependence to linezolid. This was observed in E-test and Kirby–Bauer tests. E-test showed that this strain required up to 6 μg/ml for fast growth. Nonetheless, the formation of a small inhibition zone around the antibiotic disk in the Kirby–Bauer diffusion test suggests that high linezolid concentrations can hamper the growth of this strain. Similar inhibition at high concentrations of antibiotic has been reported for strains dependent on linezolid, vancomycin or teicoplanin among other [11,18,25,26]. Clinical antibiotic dependence has been proposed to represent the ultimate step in the evolution of antibiotic resistance [11]. Bacteria with this trait usually develop in patients with long antibiotic treatments. Previously, a linezolid-dependent MRSA was isolated from a respiratory sample of a cystic fibrosis patient who received multiple, long lasting cycles of linezolid and trimethoprim/sulfamethoxazole [18]. Our patient also received several linezolid treatments, the last one lasting 53 days. The relationship between resistance and dependence is not clear. It has been hypothesized that dependence provides a selective advantage upon long time exposure to antibiotic treatments [12,13]. Alternatively, it may be possible that dependence is a fitness cost related to the acquirement of resistance mutations. However, neither has been fully proven. *S. epidermidis* linezolid-dependent strains harbor specific mutations when compared with linezolid-resistant-only isolates from the same hospital, namely the T2504A/C2534T substitutions in the 23S and mutations in the L3 protein [13]. Notably, the ribosomes of one of these strains require linezolid for proper assembly and function *in vitro* [27]. In our strain, a different mutation was found in the 23S with no changes in ribosomal proteins. Moreover, in the previously reported *S. aureus* linezolid-dependent strain, no linezolid resistance-associated mutations at all were found [18]. Thus, no specific mutations are consistently associated with the dependence phenotype. This suggests that at least for *S. aureus*, linezolid resistance mutations that lead to linezolid dependence are different to those known to date. Alternatively, linezolid resistance and linezolid dependence are two independent traits.

The clinical relevance of antibiotic dependence has not been assessed. In *S. epidermidis* it was shown that dependence to linezolid was present in an overwhelming majority of the linezolid-resistant clinical strains [28,29]. Thus, linezolid-dependent strains could be quite common in patients but may be overlooked by current protocols. Our strain developed both linezolid plain resistant and susceptible colonies *in situ* during Kirby–Bauer tests. In several passages of an isolated linezolid-dependent colony, the same pattern of growth and resistance of the derived colonies was detected, ruling out the possibility of contamination. Rather, the production of these colonies seems to be related to a capacity to produce revertant derivatives. Relatively fast reversion from linezolid dependence to just resistance and *vice versa* in *S. epidermidis* exists [29]. Moreover, the ability of other antibiotic-dependent pathogens to produce revertants both *in vivo* and *in vitro* has been reported [30–32]. Thus, to assess the prevalence of antibiotic-dependent strains among patients and to determine the effect of antibiotic in the infection outcome in this kind of *S. aureus* strains given its ability to revert, is of outmost importance. Currently, our group projects

to fully sequence the genome of the revertant derivatives to perform genomic comparisons in order to identify mutations associated with linezolid-dependence in MRSA.

The molecular basis for bacterial antibiotic dependence and its clinical implications are poorly understood. This first genomic sequence of a linezolid-dependent *S. aureus* strain may serve as the basis for comparative genomic studies between sensitive, resistant and dependent strains aimed to identify traits related to this remarkable and understudied phenotype.

Conclusion

In this work, the Scl050618 linezolid-dependent MRSA was isolated from a surgical wound of a fibrosis cystic patient. This MRSA is genetically related to strains currently prevalent in Chile and conserved the G2576T substitution in the 23S rRNA gene copies, which is a mutation known to confer linezolid resistance in staphylococci. The two strains phylogenetically closest to Scl050618 lack this linezolid resistance determinant. This mutation was also absent from a linezolid-dependent MRSA described before. The Scl050618 strain generated revertant derivatives *in vitro*. The genetic basis for linezolid dependence in MRSA as well as the effect of linezolid in the outcome of infection with this kind of strains are not straightforward to predict and require further extensive research.

Future perspective

Antibiotic dependence is a fascinating but rather understudied trait. Of equally particular interest are human pathogen isolates dependent to the antibiotics of last resort vancomycin and linezolid. Although the molecular basis for vancomycin dependence has been unveiled to a certain degree, much is ignored regarding linezolid resistance. Thus, further research will be aimed at studying the evolution of linezolid dependence. Much of the knowledge obtained in this area will be critical for the further understanding of the mechanisms of antibiotic resistance, one of the main challenges humankind will face during the upcoming decade. Previous reports demonstrated that linezolid-dependence is a common but underrated trait in clinical *S. epidermidis* strains. Thus, research is urgently needed to understand the contribution to infections of these strains and their apparent feasibility to develop revertant isogenic strains. Such knowledge becomes paramount to correctly integrate antibiotics use to therapies when this kind of strains is involved.

Summary points

- Linezolid dependence was detected in antibiotic susceptibility tests for a *Staphylococcus aureus* strain isolated from a surgical site infection of a Chilean cystic fibrosis patient.
- Genome sequencing identified this strain as a sequence type 5 methicillin-resistant *S. aureus* with a type I SCC*mec* cassette related to strains circulating in Latin America.
- This strain has the G2576T substitution, known to confer linezolid resistance, in the 5 copies of the 23S rRNA, but lacks additional mutations that could be associated to linezolid dependence.

Author contributions

VA Garcia-Angulo: investigation, formal analysis, writing-original draft; B Herve: resources, conceptualization, writing-review and editing; J Melo: resources, writing-review and editing; C Sanhueza: investigation, formal analysis; SDL Fuente: investigation, formal analysis; LL Aguirre: data curation, methodology; C Baysdorfer: investigation, data curation, writing-review and editing; MT Ulloa: conceptualization, funding acquisition, supervision, formal analysis, writing-review and editing.

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The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional approval from the Ethical Committee of Las Condes Clinic.

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