

Effect of Short-Term Antimicrobial Therapy on the Tolerance and Antibiotic Resistance of Multidrug-Resistant *Staphylococcus capitis*

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Background: Bacteria undergo adaptive mutation in the host. However, the specific effect of antimicrobial use on bacterial evolution and genome mutations related to bacterial survival within a patient is unclear.

Materials and Methods: Three *S. capitis* strains were sequentially isolated from cerebrospinal fluid of a clinical inpatient. Antimicrobial susceptibility, growth rate, biofilm formation and whole blood survival of these strains were measured. Relative fitness was calculated. The virulence was examined in the *Galleria mellonella* model. Whole-genome sequencing and in silico analysis were performed to explore the genetic mechanisms of the changes in antimicrobial resistance phenotype. Hypothetical proteins are cloned, expressed and characterized by detection the susceptibility to gentamycin.

Results: The first isolate was susceptible to rifampin (MIC=0.25 µg/mL), resistant to gentamicin (MIC=16 µg/mL), while the later two isolates were resistant to rifampin (MIC >64 µg/mL), susceptible to gentamicin (MIC=4 µg/mL). For the latter two strains, compared to the first, frameshift mutation in a hypothetical protein encoding gene and base substitutions (in genes *saeR*, *moaA* and *rpoB*) were discovered. The mutation of *rpoB* gene caused rifampicin resistance. Mutations in *saeR*, *moaA* and *hypothetical* gene are associated with changes in other biological traits. Amino acid sequence-based structure and function identification of the hypothetical protein indicated that a mutation in the encoding gene might be associated with altered aminoglycoside susceptibility. Growth curve showed that the later two isolates grew faster than the first isolate with a positive fitness advantage of 13.5%, and 14.8%, accordingly. Biofilm form ability and whole blood survival of the derivative mutants were also enhanced. No significant differences of virulence in the *G. mellonella* model were observed.

Conclusion: We report here for the first time that short-term clinical antibiotic use was associated with resistance mutations, collateral sensitivity, and positive in vivo fitness advantages to *S. capitis* during infection.

Keywords: resistance, mutations, collateral sensitivity, adaptive enhancement, selective pressure

Introduction

Ever since the first use of antibiotics in the clinic, clinicians have been faced with the emergence of mutations in bacteria that cause antibiotic resistance. Resistance mechanisms allow bacteria to survive in the presence of antimicrobials and are the main reasons for antibiotic treatment failure.¹ However, how bacterial genes mutate under the selective pressure of clinical antimicrobial agents, and the effect of these mutations on bacterial resistance and virulence, remain unclear.

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The rapid development of high-throughput sequencing technology is providing insight for genomics research, enabling accurate identification of single-nucleotide variations between strains. This approach could reveal genetic evolution of a pathogen within a host, offering new opportunities for exploring the role of genetics and within-host evolution in the outcome of antimicrobial agent-bacterial pathogen interactions. Understanding how a bacterial pathogen evolves during infection of the human host is important for fighting the infection. WGS helps to reveal some important drug-responses of bacteria, such as tolerance, toxicity, and resistance patterns.² Epidemiological studies of *Streptococcus pneumoniae* from the last 25 years provide insight into how genomic plasticity within lineages of recombinogenic bacteria permits adaptation to clinical intervention.³ WGS is also used to explore molecular changes in bacterial populations evolving in vivo in relation to long-term chronic infections.⁴ Such studies revealed that *Yersinia pestis* harbors different adaptive mutations in different hosts, which increase its ability to infect the host.⁵ Further, monitoring of resistant bacteria in long-term inpatients led to the identification of recombinant on plasmids.⁶ Changes in the genome under the selective pressure of antibiotics in vitro are also investigated.^{3,7} However, few studies have focused on the impact of clinical short-term antimicrobial treatment on the pathogen and collateral sensitivity in *S. capitis* has not been explored. Mutation analysis of clinical strains isolated at different time points of infection from a patient receiving antibacterial drugs could help explain how pathogens adapt to antibacterial drugs and evolve in the host, and help understand the associated changes in bacterial pathogenicity and other biological traits.

Staphylococcus capitis is a Gram-positive coccus belonging to coagulase-negative *Staphylococcus* spp. (CoNS), which is frequently found on the human skin and mucosa, and even in the human gut. Although infections caused by this species are rare compared with those caused by *Staphylococcus aureus*, cases of *S. capitis* infections are gradually increasing. Recent reports indicate its emergence as a major pathogen causing nosocomial and bloodstream infections.⁸ Demographic and medical developments that result in increasing numbers of elderly, multimorbid, and immunocompromised patients, and the increasing use of implanted foreign bodies have contributed to the progressively increasing importance of CoNS in healthcare. Furthermore, as for nosocomial pathogens, increasing rates of antibiotic resistance are an even greater

problem for CoNS than for *S. aureus*, from a perspective of therapy, CoNS are challenging because a large proportion of methicillin-resistant strains and increasing numbers of isolates show reduced susceptibility to glycopeptides, limiting the array of available therapeutics.⁹

In the current study, three strains of *S. capitis* were isolated from the cerebrospinal fluid of a hospitalized patient. We analyzed these strains by WGS and virulence assays to explore the effect of clinical medication on antimicrobial susceptibility phenotype, virulence and the driving mutations.

Materials and Methods

Ethical Consideration

The current study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China (No: RN.2019.132). Written informed consent was obtained from patient and healthy blood donor.

Bacterial Isolates

Three strains were isolated from the cerebrospinal fluid of a clinical patient with a carotid artery rupture admitted to the First Affiliated Hospital of Zhejiang University. The patient was treated with eight antimicrobial agents such as linezolid, tigecycline, meropenem, polymyxin et al throughout their hospital stay, six antibiotics were introduced after the isolation of the first strain. The isolates were named Sca70935, Sca71187, and Sca71207. The bacteria identification was performed by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) and WGS. Isolate Sca70935 (the first isolate) was used as a reference strain for the SNPs analysis.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of each isolate to penicillin G, oxacillin, gentamicin, clindamycin, erythromycin, quinupristin/dafostine, trimethoprim/sulfamethoxazole, rifampicin, ciprofloxacin, levofloxacin, tigecycline, linezolid, and vancomycin was determined by using the agar dilution method, and interpreted according to the Clinical and Laboratory Standard Institute (CLSI) 2018 recommendations.¹⁰

Genome Sequencing and Analysis

Genomic DNA was extracted from the three *S. capitis* isolates by using the Genra Puregene Yeast/Bact Kit (Qiagen,

Hilden, Germany), according to the manufacturer's instructions. The isolated DNA was sequenced by using the Illumina Novaseq platform. The Trimmomatic software was used to filter low-quality reads. The filtered reads were assembled using the SPAdes Software. Online tools (<http://www.genomicepidemiology.org/>) were used to identify the acquired antimicrobial resistance genes and the plasmids. For genes whose function could not be annotated by using the RAST Annotation Server, other online tools (<https://predictprotein.org/>) were used. The command-line PHIGARO software was used for prophage prediction and a web tool CRISPRfinder was used to identify clustered regularly interspaced short palindromic repeats with associated genes (CRISPR/cas). The TMHMM 2.0c program (<http://www.cbs.dtu.dk/index.php>) was used to predict the presence of transmembrane helices in protein. Structurally similar homologous proteins were identified using the SWISS-MODEL website (<https://www.swissmodel.expasy.org>) to predict protein function.¹¹ The Snippy pipeline, version 3.0 (<https://github.com/tseemann/snippy>), was used for read mapping and variant calling.

Growth Assay

The growth curves of these strains were determined by measuring the optical density at a wavelength of 600 nm using an automated growth curve detector (BioTek USA). Briefly, three isolates were cultured overnight in TSB broth and diluted to an optical density at 600 nm (OD₆₀₀) of 0.01. After dilution 100 times, the bacteria were suspended in MHB medium and grown at 37 °C, with agitation at 200 rpm. The cell density was determined every 0.5 h. The growth curve was plotted using the OD₆₀₀ value.

Biofilm Assay

The biofilm assay was performed as previously described. Briefly, 1×10⁶ colony-forming units (CFU)/mL were inoculated into TSB broth in polystyrene microtiter 96-well plates and incubated at 37 °C for 24 h. The formed biofilm was stained using crystal violet, the bound dye was eluted with 95% ethanol, and the dye intensity was quantified at OD₆₀₀. The assay was performed in triplicate; in each replicate assay, the quantification was based on the analysis of eight wells per sample.

Fitness Measurements

The Fitness measurements assay was performed as previously described.^{12,13} The rifampin-sensitive (RIF-S) isolate Sca70935, the initial isolate, and the two rifampin-

resistant (RIF-R) isolates, Sca71187 and Sca71297, isolated subsequently, were diluted to 0.5×10⁶ CFU/mL. Then, equal (10 µL) volumes of Sca70935 and Sca71187 (or Sca71297) cultures were combined, and 20 µL of the mixture was added to 20 mL TSB broth and cultured at 37 °C, with agitation at 200 rpm. After 24 h, 50 µL of the subcultures was inoculated on drug-free MH agar, and 50 µL of the subcultures was inoculated on MH agar containing 8 µg/mL rifampin. The RIF-S and RIF-R colonies were counted the next day, and the adaptive difference was calculated, as follows:

$$\text{relative adaptive fitness } F = 1 + \ln \left[\left(\frac{rt/St}{(rt-1)/(St-1)} \right) \frac{1}{17} \right]$$

$$\text{fitness cost } C = (1-F) \times 100\%$$

where *rt* is the number of resistant colonies and *St* is the number of sensitive colonies.

Survival of Bacteria in Whole Blood

The survival of bacteria assay was performed as previously described.¹⁴ Cultures of the RIF-S and RIF-R *S. capitis* strains in the logarithmic growth phase (OD₆₀₀=0.5) were diluted to 2×10⁶ CFU/mL with 0.9% saline; 50 µL of each culture was mixed with 150 µL of the heparinized whole blood from a healthy volunteer in a 1.5 mL Eppendorf tube, and incubated at 37 °C with agitation at 200 rpm. After 0, 60, 120, and 180 min, 10 µL of the mixture was withdrawn, combined with 90 µL of MH broth, and plated on MH agar. Bacterial colonies were counted after overnight incubation, each strain was assayed three times.

Infection of *Galleria mellonella* Larvae

The virulence of *S. capitis* isolates was determined by infecting *G. mellonella* larvae, as described previously.¹⁵ Briefly, overnight cultures of *S. capitis* were washed with phosphate-buffered saline (PBS) and the cell concentration was adjusted with PBS to 1×10⁶ CFU/mL. Each experimental group contained randomly picked 20 *G. mellonella* larvae weighing 250 mg. Each *G. mellonella* larva was injected with 20 µL of bacterial suspension; the negative control group larvae were injected with 20 µL of sterile PBS solution. The injected *G. mellonella* larvae were placed in an incubator at 37 °C and observed every 12 h. The larval survival rate was recorded over 72 h. All experiments were performed in triplicate.

Cloning, Expression and Function

Identification of the Hypothetical Protein

Genomic DNA of Sca70935 was extracted by using the Genra Puregene Yeast/Bact Kit (Qiagen, Hilden,

Germany), according to the manufacturer's instructions. The hypothetical protein encoding gene *Anthp1* was amplified using manually designed sequence specific primers (F-GAAGGAGATATACATATGAAACTTGAAGCACAA-AAACCA

R-GTGCGGCCGCAAGCTTTTATGATATTTTCTAT-TATTATTACCGCTTC). pET32a vector were digested with Xho I and Nde I restriction enzymes. The PCR amplification was In-Fusion cloned into the digested pET 32a expression vector containing C terminal poly-histidine tag. The clone was further transformed into chemically competent *E. coli* BL21 (DE3) cells compatible to the expression vector and the integrity of the clone was verified by sequencing. Single colony were grown at 37°C in 5 mL of LB media containing 100 µg/mL ampicillin for 12h. After incubation, cultures was diluted by 1:100 to fresh 50mL LB media containing 1mM isopropyl-1-thio-β-Dgalactopyranoside (IPTG) for expression 3 hours at 37°C at 200 rpm. The fusion protein was observed using SDS-PAGE and characterized by detecting the susceptibility of *E. coli* BL21 (DE3) to gentamicin in disk diffusion assay.

Accession Numbers

The sequences of Sca70935, Sca71187, and Sca71207 have been deposited in GenBank under the accession numbers RYDS00000000, RYDR00000000 and RYDQ00000000.

Results

Basic Clinical Status of the Patient

On January 23, 2019, the patient was transferred to the First Affiliated Hospital of Zhejiang University after an emergency surgery in another hospital because of a rupture of the internal carotid aneurysm. In February 26, Sca70935 was isolated from the patient's cerebrospinal fluid. The Sca71187 and

Sca71207 were obtained on March 5 and March 10, respectively. The patient was treated with eight antimicrobial agents throughout their hospital stay, six antibiotics were introduced after the isolation of the first strain. The medication regime is shown in Figure 1. The patient was discharged on April 3.

Antimicrobial Susceptibility Profiling

The three isolates were resistant to oxacillin, penicillin G, erythromycin, clindamycin, levofloxacin, moxifloxacin, ciprofloxacin, and linezolid, but were sensitive to sulfamethoxazole, tigecycline, tetracycline, nitrofurantoin, vancomycin, and teicoplanin. Strain Sca70935 was resistant to gentamicin and quinupristin/dalfopristin but sensitive to rifampicin, while strains Sca71187 and Sca71207 were sensitive to gentamicin, intermediate to quinupristin/dalofopine, and resistant to rifampicin. The drug susceptibility data for the three isolates are shown in Table 1.

Genomic Characteristics of Three Multidrug Resistant Strains

The sizes of the assembled draft genomes of the three isolates were similar, 2.52 Mbp on average. The mean number of contigs in each draft genome was 79. The average N50 value was 22,502 bp and the mean %GC content was 32.82%. The three isolates harbored an identical resistome composed of ten genes, namely, *bla_Z* and *mecA* for β-lactam resistance; *aac(6)-Ie* and *ant(4)-Ib* for aminoglycoside resistance; *qacA*, *norA*, and *mgrA* for quinolone resistance; *ermA* for erythromycin resistance; *dfcC* for trimethoprim/sulfamethoxazole resistance; and *cfpA* for linezolid resistance. The genotypes partly explain the resistance profiles (Table 1). The *S. capitis* strains carry 3 plasmids, a Siphoviridea prophage PHAGE_Staphy_StB20_NC_019915 and no CRISPR/Cas

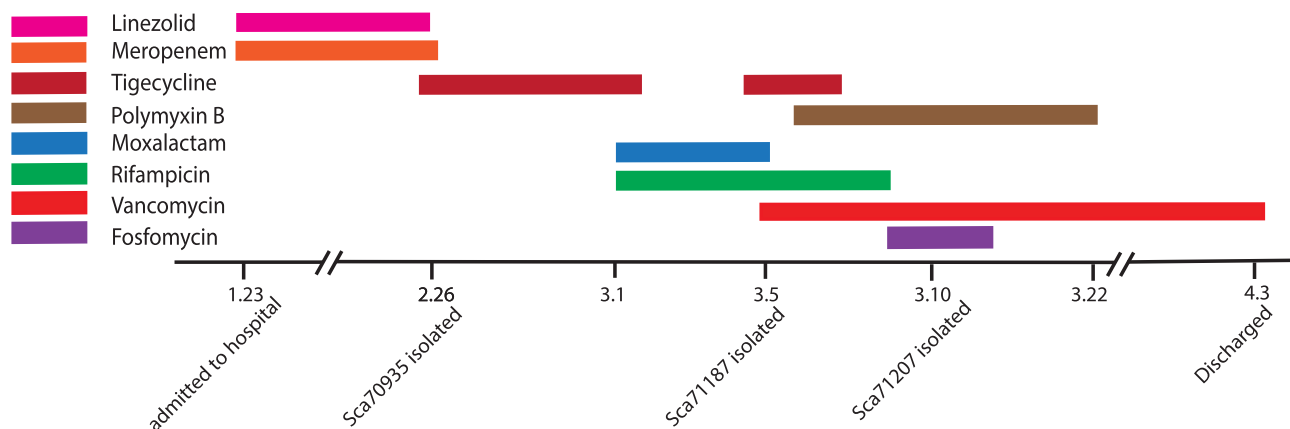


Figure 1 Overview of the antibacterial regimen and strain isolation.

Table 1 Antimicrobial Susceptibility of the *S. capitis* Isolates

Strain ID	Sca70935	Sca71187	Sca71207	Resistance Gene*
PEN	≥0.5(R)	≥0.5(R)	≥0.5(R)	blaZ
OXA	≥4(R)	≥4(R)	≥4(R)	mecA
ERY	≥8(R)	≥8(R)	≥8(R)	ErmA
LIN	≥8(R)	≥8(R)	≥8(R)	
SXT	≤10(S)	≤10(S)	≤10(S)	dfrC
QDA	4(R)	2(I)	2(I)	
LNZ	≥8	≥8	≥8	cfrA
GEN	≥16(R)	4(S)	4(S)	aac(6')-Ie,ant(4')-Ib ^Δ
TGC	0.5(S)	0.25(S)	0.25(S)	
TET	4(S)	2(S)	2(S)	
NIT	≤16(S)	≤16(S)	≤16(S)	
RIF	≤0.5(S)	≥64(R)	≥64(R)	rpoB [#]
VAN	≤0.5(S)	≤0.5(S)	≤0.5(S)	
TEC	1(s)	1(s)	1(s)	
LEV	8(R)	8(R)	8(R)	qacA
MFX	4(R)	4(R)	4(R)	norA, mgrA
CIP	≥8(R)	≥8(R)	≥8(R)	
CIR	Neg	Neg	Neg	

Notes: *The three strains carry the same resistance gene. [#]*rpoB* mutation was identified in strains Sca71187 and Sca71207. ^Δ*aac(6')-Ie* and *ant(4')-Ib* are aminoglycoside-resistance genes, but mainly result in amikacin resistance.

Abbreviations: PEN, penicillin G; OXA, oxacillin; ERY, erythromycin; LIN, lindamycin; SXT, trimethoprim/sulfamethoxazole; QDA, quinupristin/dalofopine; LNZ, linezolid; GEN, gentamicin; TGC, tigecycline; TET, tetracycline; NIT, nitrofurantoin; RIF, rifampin; VAN, vancomycin; TEC, teicoplanin; LEV, levofloxacin; MFX, moxifloxacin; CIP, ciprofloxacin; CIR, clindamycin-induced resistance.

elements in the genome, there is no loss of a plasmid during the treatment.

Mutation Analysis and Function of the Mutated Genes

Compared with the Sca70935 strain, four mutations were identified in Sca71187 and Sca71207 (the two strains harbored the same mutations). Three of these mutations were base substitutions and one was a base deletion, resulting in three missense mutations and one frameshift mutation, respectively (details are shown in Table 2). The base substitutions in *rpoB* gene and *saeR* gene were conserved substitutions, while in *moaA* gene, they were not.

The *saeR* gene encodes a 228 amino acid polypeptide with an N-terminal regulatory domain and a potential C-terminal DNA-binding domain. The SaeR protein is a member of the two-component regulatory system SaeR/SaeS and is involved in the regulation of expression of virulence genes. It regulates transcription by recognizing a specific DNA sequence near the promoter sequence of the target gene. The SaeR/SaeS system activates the expression of foreign genes and cell wall-associated genes involved in host cell adhesion and

invasion, inhibiting the production of type 5 capsular peptidoglycan.¹⁶

The second mutated gene, *moaA*, encodes a protein that catalyzes the cyclization of GTP to (8S)-3',8-cyclo-7,8-dihydroguanosine 5'-triphosphate, which plays a role in the biosynthesis of molybdenum protein. The molybdenum protein is involved in the biosynthesis of cofactors, which activate and accelerate enzymatic reactions.¹⁷ The specific involvement of MoaA in the function of specific *S. capitis* enzymes is not clear.¹⁸

The third mutated gene, *rpoB*, encodes the β-subunit of the bacterial RNA polymerase, whose main function is to transcribe DNA into RNA. In staphylococci, the *rpoB* gene contains a rifampicin resistance-determining region; mutations in that region result in resistance to rifampicin.^{19,20} The mutation in strains Sca71187 and Sca71207 occurs in a cluster I region of the drug resistance-determining region.

One identified gene encodes a hypothetical protein named Anthp1. We predicted the 3D-structure of the protein based on its amino acid sequence (Figure 2A) and identified protein with 75% sequence coverage in the SWISS-MODEL database (Figure 2B and C). The reference protein (Figure 2B) is an aminoglycosyltransferase

Table 2 Summary of the Protein Variants in the Sca71187 and Sca71207 Strains

ID	SaeR	MoaA	rpoB	hp
Sca71187	c.43A>G p.Asn15Asp	c.389T>C p.Val130Ala	c.1430C>A p.Ala477Asp	frameshift_variant c.552delA p.Lys184fs
Sca71207	c.43A>G p.Asn15Asp	c.389T>C p.Val130Ala	c.1430C>A p.Ala477Asp	frameshift_variant c.552delA p.Lys184fs

Abbreviations: hp, hypothetical protein; c., mutation of nucleic acid; p., change of coding protein.

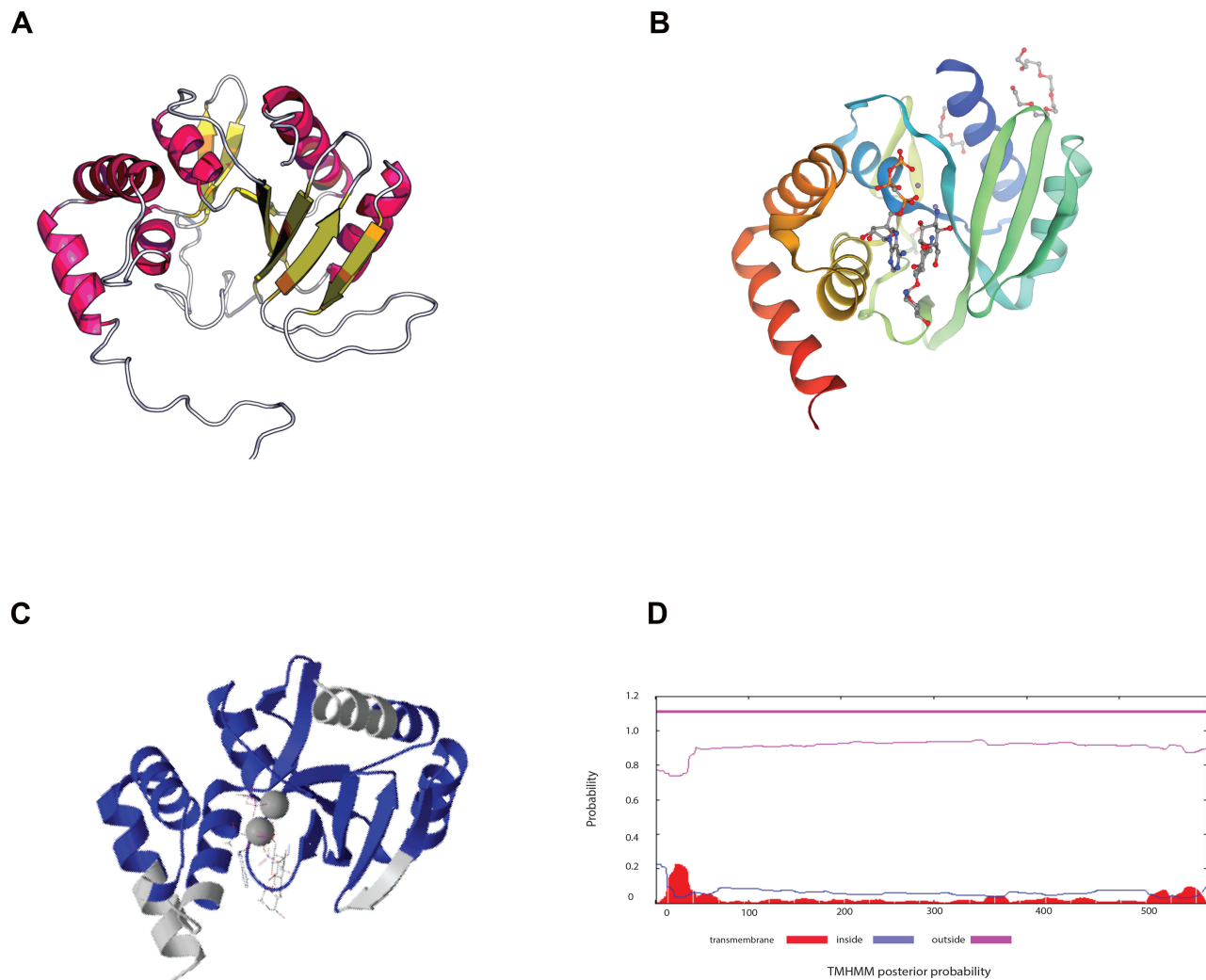


Figure 2 Predictive analysis of genes with unknown function. **(A)** Structure prediction of a hypothetical protein based on the amino acid sequence. **(B)** Structure of a homologous protein from the SWISS-MODEL database (SEQ ID NO: Suvd.1.A). **(C)** Comparison of the proteins shown in panels **(A and B)** proteins. Blue, identical amino acids in both proteins; gray, different amino acids. **(D)** Possible cellular distribution of the hypothetical protein.

involved in the resistance to gentamicin. According to the domain analysis, the encoded hypothetical protein is mainly extracellular (**Figure 2D**).²¹ Therefore, we propose that the hypothetical protein may underpin gentamicin resistance in the Sca70935 isolate because, in strains Sca71187 and Sca71207, a 13 C-terminal amino acid stretch, RIEEAVINRKIS, is replaced by NVIKKR (early termination of translation) as a result of a mutation.

Strain Growth and Biofilm Formation

Compared with the initial isolate Sca70935, the growth rate of the other two isolates was increased ($p < 0.05$, independent *t*-test). There were no significant differences between Sca71187 and Sca71207. After 6 h of growth, the growth rate of the mutant strain significantly increased and continued until plateauing, and the number of the mutant strains in plateau phase was greater than the number of Sca70935

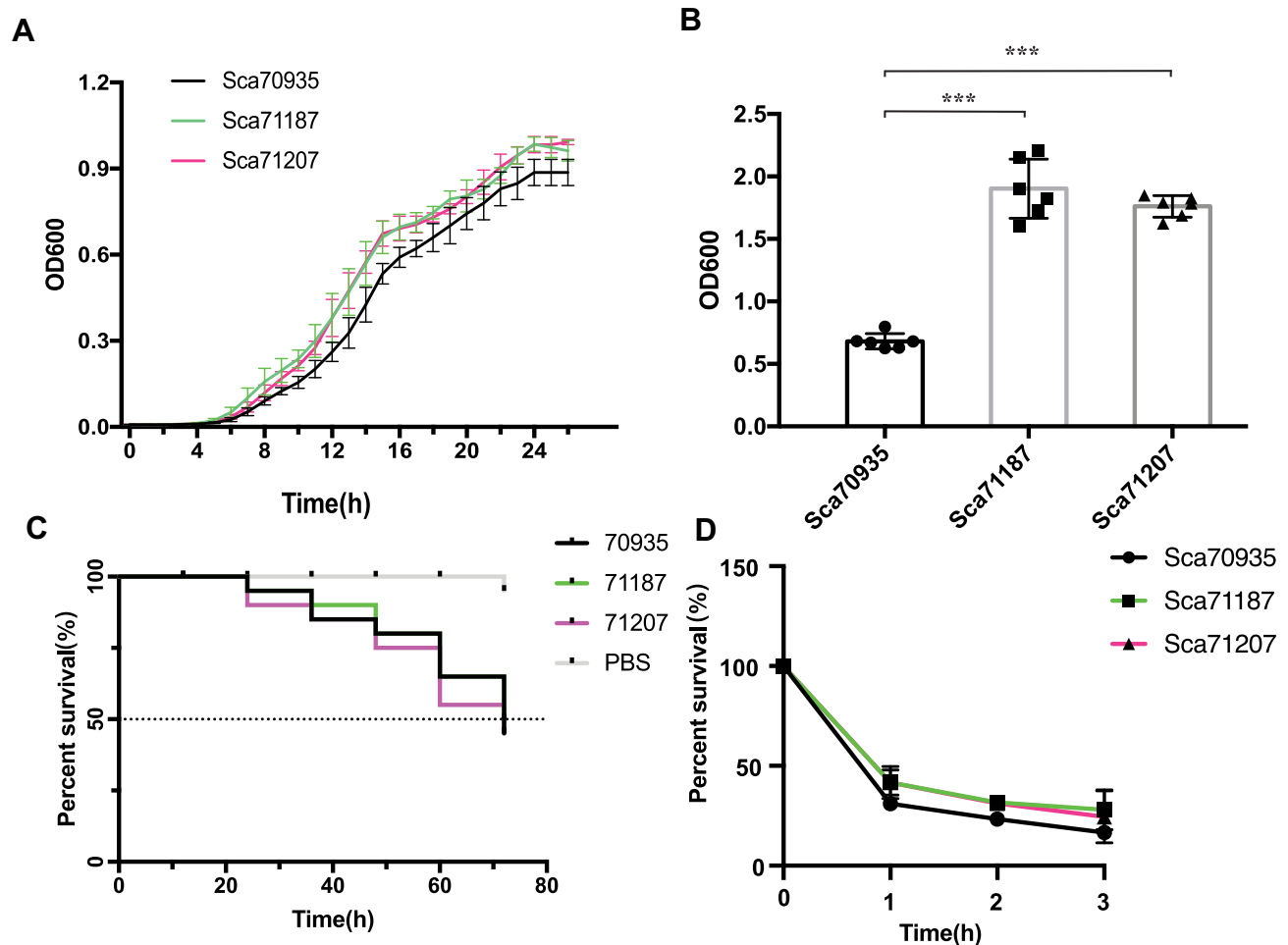


Figure 3 (A) Growth curve. (B) Biofilm formation. (C) In vivo infection of *G. mellonella* larvae. (D) survival of bacteria in whole blood *** indicated ($p < 0.001$).

strains (Figure 3A). As shown in Figure 3B, the biofilm-forming ability of the two strains was significantly higher than that of the reference isolate ($p < 0.001$, independent *t*-test).

The Fitness Cost of Mutations, Bacterial Infection of *G. mellonella* and Survival of Bacteria in Whole Blood

The relative positive fitness advantage was 13.5% for Sca71187 and 14.8% for Sca71207. Thus, the later derivative mutants all bore an adaptive advantage compared with the parental strain Sca70935. As shown in Figure 3C, *G. mellonella* died about 50% within 72 h following infection. Kaplan–Meier survival curve analysis showed that the mortality of *G. mellonella* was similar when infected with the three strains. No significant differences in virulence in the *G. mellonella* model were observed. The results of the whole blood survival assay are shown in Figure 3D. The whole blood survival of strains Sca71187

and Sca71207 was significantly higher than that of the Sca70935 isolate ($p < 0.05$).

Effect of Hypothetical Protein in BL21 Strains on Their Susceptibility to Gentamicin

An *E. coli* strain expressed the previously described hypothetical protein anthp1 (BL21-pet32a-anthp1) were successful constructed to investigate the effect of hypothetical protein on gentamicin resistance. As shown in the result of SDS-PAGE (Figure S1), the protein is clearly expressed. The disk diffusion method showed that the minimum inhibitory zone diameter of gentamicin for strains which express and not express the protein were same 18mm.

Discussion

A wide range of broad-spectrum antibacterial drugs, immunosuppressive agents, and chemotherapeutic drugs

is typically used in clinical practice, and various invasive surgeries are widely practiced. This results in an increasing isolation rate of CoNS and the bacterial antibiotic resistance.²² CoNS was once not considered to be pathogenic. However, recent clinical and laboratory tests have confirmed that the majority of normal microbiota are conditional pathogens. Consequently, CoNS has become one of the important pathogens causing hospital bloodstream infections that cannot be easily explained as contamination by opportunistic pathogens.²³ According to recent reports, *S. capitis* is now a major pathogen causing nosocomial and bloodstream infections, meningitis, prosthetic valve endocarditis, and late-onset sepsis. That is mainly because of its ability to produce slimy biofilms, enabling it to adhere to medical devices, such as prosthetic valves and catheters. Hence, the bacterium is difficult to control or clear by the immune response.²⁴ During a clinical treatment, infections caused by *S. capitis* should be monitored. In particular, the incidence of oxacillin-resistant CoNS is high, in some cases exceeding 60%.^{25–27}

These *S. capitis* strains isolated in the current study were resistant to methicillin. Of note, these strains were also resistant to linezolid. Linezolid is the first oxazolidinone antibiotic to be approved for clinical use. It is currently used to treat methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VE), and other important multidrug resistant pathogens. The acquisition of *cfi* resistance genes and mutations in the *23S rRNA* gene constitute the main mechanisms of resistance to these antimicrobials.²⁸

Antimicrobial susceptibility profiling revealed that strains Sca71187 and Sca71207 have become resistant to rifampicin during antimicrobial treatment. According to the clinical data, rifampicin was introduced only after the Sca70935 strain was isolated and confirmed sensitive to rifampicin. The mutation of the *rpoB* gene was first identified in strain Sca71187 after 5 days of treatment. The point substitution is located in the rifampicin resistance-determining region of the gene. This indicated that the use of rifampicin was associated with rifampicin resistance in *S. capitis* in a short day, suggesting that the ability of this drug to induce resistance should be monitored in the clinical setting. That is similar to the findings of O'Neill, who reported a high mutation frequency of rifampicin-resistance genes in *S. aureus*, approximately 10^{-6} – 10^{-8} , which is easily drug-inducible.²⁹

At the same time, susceptibility profiling revealed that during antimicrobial treatment, the gentamicin phenotype of

Sca71187 and Sca71207 changed from resistance to susceptibility, and quinupristin/dalofopine resistance changed to intermediate. Collateral sensitivity was first proposed by Szybalski and Bryson in the 1950s, but research on the inducing conditions and molecular mechanisms of this phenomenon has been stagnant in the last few decades.⁷ Recently, the development of drug resistance and collateral susceptibility have been studied in bacteria exposed to antibiotics via adaptive laboratory evolution, and the molecular mechanisms of collateral synergistic susceptibility were preliminarily explored by WGS. Nevertheless, the mechanism of collateral susceptibility remains unclear.³⁰ Although the three strains harbor genes for two aminoglycoside modifying enzymes, *aac* (6')-Ie and *ant* (4')-Ib, it has been shown that these two enzymes cause resistance to tobramycin, not gentamicin.^{31,32} Genome analysis performed in the current study identified another mutated gene in strains Sca71187 and Sca71207. Based on the predicted structure and function of the encoded protein, we speculated that the gene might be involved in gentamicin resistance in strain Sca70935. Although the expression of this protein in *E. coli* do not decreased the susceptibility to gentamicin, it may play a more important role in *S. capitis*. Furthermore, structure prediction shows mutation of the encoding gene altered the structure of the C-terminal binding domain of the protein, rendering it unable to bind gentamicin for further modification, and re-sensitizing the bacterium to gentamicin. Alteration of a strain's sensitivity to rifampicin that alters the sensitivity to other antimicrobial agents might be caused by collateral sensitivity. This effect is widespread during bacterial resistance evolution.³³ The mechanism of altered sensitivity to quinupristin/dalofopine observed in the current study is unclear and further research is needed. The re-sensitization of bacteria to gentamicin suggests that clinicians may develop an alternative strategy based on collateral sensitivity to treat bacterial infections, which may inhibit the rise of drug-resistant mutants and delay the development of drug resistance.^{34–36}

SNP analysis of the Sca71187 and Sca71207 genomes revealed mutation in the *saeR* gene. The *saeR* gene encodes a response regulator protein, an important element of the SaeRS two-component system. The SaeS sensor detects an environmental signal, following which SaeR binds the target molecule through the C-terminal effector domain and regulates gene transcription. The two-component system is involved in host cell adhesion and invasion, and the expression of genes encoding extracellular proteins and cell wall-associated proteins.³⁷ The

experiments performed in the current study demonstrated the increased growth rate and biofilm formation of the mutated isolates, indicating enhanced ability to survive within the host. Therefore, we speculate that an N-terminal mutation lead to more (or less) interaction of *SaeR* to *SaeS* and more (or less) expression of genes that are part of the *SaeRS* regulon. Moreover, these effects could be direct or indirect.

Rifampicin resistance and other changes in the genomes was not associated with an adaptive fitness cost but, instead, promoted bacterial growth rate, biofilm formation, and an ability to survive in the whole blood. This is consistent with other reports that rifampicin-induced resistance is not associated with an adaptive fitness cost.¹⁹ This suggests that the effect of a drug on bacterial fitness cost should be considered when using the drug. Further research is needed to investigate the effect of the mutation of the *moaA* gene on the physiology of bacteria. Mutations of bacteria within the host are key factors that determine the pathogen's adaptability to the host's immune system and drug treatment. Understanding the effect of in vivo mutations of bacterial pathogens will facilitate subsequent treatment and prognosis.

Although in the current study we only investigated the mutation of a single strain after antimicrobial treatment, the occurrence of the same mutations in two subsequent isolates indicates the consistency of these mutations. However, more strains should be analyzed in the future.

Conclusion

We reported here for the first time that the adaptive mutations of *S. capitis* in the cerebrospinal fluid of a clinical patient elicited by antibiotics result in rifampicin resistance, enhanced biofilm formation, and collateral susceptibility with fitness advantage. We preliminarily explored the correlation between antimicrobial resistance and changes in protein structure caused by genetic mutations. The results of the current study will help to elucidate the mechanism of the adaptive evolution of *S. capitis* in response to the selective pressure of antibiotics in the host, to improve the understanding of infection caused by such strains and to devise measures to prevent the emergence of drug resistance based on collateral sensitivity.

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Disclosure

The authors report no conflicts of interest in this work.

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