LCT-EF258 with S17I Mutation in DprA Exhibits Horizontal Gene Transfer Deficiency After Spaceflight

Yi Yu; De Chang; Qiang Guo; Junfeng Wang; Changting Liu

BACKGROUND: Space is a special environment in which microgravity and cosmic rays are the primary factors that induce gene mutations of microorganisms. In our previous studies, a single point mutation in the gene dprA was found in an *Enterococcus faecium* strain of LCT-EF258 after spaceflight. DNA processing protein A (DprA) plays a prominent role in the horizontal transfer of genes among bacteria (such as *Streptococcus pneumoniae, Helicobacter pylori, Bacillus subtilis,* and *Rhodobacter capsulatus*). However, the function of DprA in *E. faecium* remains unknown. Furthermore, *E. faecium* could acquire antibiotic resistance through the horizontal transfer of antibiotic resistance genes, but it is unclear whether dprA mutants could affect this process in *E. faecium*.

METHODS: In this study, we constructed a plasmid containing the vancomycin resistance gene vanA and then transferred the gene vanA into the dprA-mutant strain LCT-EF258 and the control strain LCT-EF90 using the electroporation technique. We then used Discovery Studio^{TM} software to construct the 3D protein structure.

RESULTS: The results showed that the horizontal transfer efficiency of the vancomycin resistance gene vanA in the dprA-mutant *E. faecium* decreased. And the hydrophobic core of the mutant DprA became stable and the binding affinity between the mutant DprA and ssDNA reduced.

DISCUSSION: This study is an exploration of bacterial gene mutation after spaceflight. The dprA mutant could affect the ability of *E. faecium* to acquire exogenous resistance gene vanA, which offered us an interesting path to block the dissemination of resistance genes between strains.

KEYWORDS: dprA, mutant, *Enterococcus faecium*, space environment.

Yu Y, Chang D, Guo Q, Wang J, Liu C. *LCT-EF258 with S171 mutation in DprA exhibits horizontal gene transfer deficiency after spaceflight*. Aerosp Med Hum Perform. 2019; 90(2):116–122.

Space is a special environment, differing from Earth in its microgravity, cosmic radiation, and elevated carbon dioxide levels. Some previous space microbiology studies showed that the space environment could affect the biological phenotype, genome, transcription, or proteome of microorganisms.[6,14,20] Other previous space microbiology studies showed that microgravity and cosmic rays were the primary factors that induce gene mutations in microorganisms in space.[7,28,31] However, the mechanisms underlying these changes are still unclear.

In 2011, we loaded an *E. faecium* strain (named LCT-EF258) in the Shenzhou-8 spacecraft. After 397 h of spaceflight, the LCT-EF258 were subjected to genomic, transcriptome, and proteome analyses [using the Illumina Hiseq2000 next-generation sequencing (NGS) platform].[5] We found the LCT-EF258 had a single point mutation in the gene dprA.[5] The dprA mutation drew our interest for the fact that DNA processing protein A (DprA) plays a key role in exogenous horizontal gene transfer in bacteria.

DprA, encoded by the gene dprA, is a widespread and conserved protein that plays a prominent role in exogenous horizontal gene transfer in bacteria.[18] DprA binds exogenous linear single-stranded DNA (ssDNA) and protects ssDNA from nucleases.[18] DprA also interacts with Recombinase A (RecA) and promotes the loading of RecA on ssDNA,[15] alleviating the ssDNA-binding protein (SSB) barrier[18] and regulating the shut-off competence.[17] Through exogenous horizontal gene transfer, bacteria generate genetic diversity to drive evolution, repair

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From the Nanlou Respiratory Diseases Department, Chinese PLA General Hospital, Beijing, China, and the Laboratory of Vaccine and Antibody Engineering, Beijing Institute of Biotechnology, Beijing, China.

This manuscript was received for review in March 2018. It was accepted for publication in September 2018.

Address correspondence to: Changting Liu, Nanlou Respiratory Diseases Department, Chinese PLA General Hospital, No. 28 Fuxing Rd., Beijing 100853, China; changtingliu2016@163.com.

DOI: https://doi.org/10.3357/AMHP.5120.2019

damaged DNA, and sometime acquire nucleic acids directly from the environment when nutrients are lacking.[25,26] Although the function of DprA has been verified by previous studies in *Streptococcus pneumoniae, Helicobacter pylori, Bacillus subtilis,* and *Rhodobacter capsulatus*,[3,10,27] no studies have investigated the function of DprA in *E. faecium*. It is still unclear whether the single point mutation of dprA in LCT-EF258 could affect the exogenous horizontal gene transfer of the *E. faecium* strain LCT-EF258.

E. faecium is Gram-positive bacteria that was once considered harmless commensals in the gastrointestinal tract of humans and animals. They are of growing concern because of their ability to cause antibiotic resistant hospital infections.[4,11,19] Antibiotic resistance has been acquired and disseminated throughout *E. faecium* through the horizontal transfer of mobile genetic elements.[21] Acquired resistance to the last-line antibiotic vancomycin is common.[13] Vancomycin resistance results from acquisition of transposon-associated complex operons.[24] There are several van operons that vary in the type of enzymes encoded.[16] The most widely distributed in clinical strains is vanA.[12] The vanA gene cluster is described as part of Tn1546-type transposons, generally carried on plasmids and thus effectively disseminated by horizontal gene transfer.[1] Although previous studies have confirmed that *E. faecium* could acquire the vancomycin resistance gene vanA through horizontal gene transfer, and DprA plays a prominent role in horizontal gene transfer in bacteria, it is still unknown whether the dprA mutant could affect the horizontal gene transfer of vanA in the *E. faecium* strain LCT-EF258.

In this study, we constructed a plasmid containing the gene vanA and then transferred the exogenous gene vanA into the dprA-mutant strain LCT-EF258 and the control strain LCT-EF90, using the electroporation technique, to compare the horizontal transfer efficiencies of the exogenous resistance gene vanA in the two strains. We then attempted to explain why the horizontal transfer efficiencies were different between the dprA-mutant strain and the wild-type strain from the prospective of their 3D protein structure using Discovery Studio™ (Biovia Co., Ltd., San Diego, CA) software.

METHODS

Materials

The *E. faecium* strain CGMCC 1.2136 was loaded in the Shenzhou-8 spacecraft as a stab culture named LCT-EF258. After spaceflight from November 1 to 17, 2011, the LCT-EF258 was stored at −80°C until further use.[5] The same strain CGMCC 1.2136 was cultured on the ground as a control named LCT-EF90.[5] With the exception of spaceflight, all other culture conditions, such as time, temperature, humidity, oxygen content, and culture medium, were identical between the two groups.[5] The whole-genome sequences of LCT-EF90 and LCT-EF258 used in this study (sequenced by the Illumina Hiseq2000 NGS platform) have been deposited in DDBJ/EMBL/GenBank under the accession numbers AJKH00000000 and ANAJ00000000, respectively.

The plasmid containing the P23 promoter was provided by Dr. Qiang Guo. The vancomycin-resistant *E. faecium* (VREF) was provided by Nanlou Clinical Laboratory of the Chinese PLA General Hospital.

Procedure

Both the mutant strain LCT-EF258 and the control strain LCT-EF90 were grown in Todd-Hewitt (TH) medium at 37°C. The DNA was prepared using conventional phenol-chloroform extraction methods. The sequences of AJKH00000000 and ANAJ00000000 were aligned using Vector NTI Advance™ software (Invitrogen Corporation, Carlsbad, CA). All the different points between AJKH00000000 and ANAJ00000000, including the dprA mutation, were amplified from the prepared genomic DNA of LCT-EF90 and LCT-EF258 using polymerase chain reaction (PCR) and sent to Taihe Biotechnology Co., Ltd., Beijing, China, for sequencing. The sequencing results were aligned using Vector NTI Advance™ software. Then, LCT-EF258 and LCT-EF90 were passaged 10 times after returning to Earth, without the stress of the space environment. The dprA fragments of the strains after 10 passages were also amplified, sequenced, and aligned. The dprA nucleotide sequences of LCT-EF258 and LCT-EF90 were translated into amino acid sequences. The amino acid sequences were aligned.

The minimum inhibitory concentrations (MICs) of vancomycin were determined using the twofold agar dilution method. The antibiotic concentrations in TH agar medium were 64, 32, 16, 8, 4, 2, 1, 0.5, and 0 $\mu g \cdot ml^{-1}$. Bacterial samples (100 μl, 0.5 CFU $\cdot$ ml^{-1}) were applied to agar media supplemented with the indicated antibiotic concentrations and then incubated at 37°C for 48 h. The minimum concentration that presented no colony growth was considered the MIC. The MIC test was performed with three replicates. The test showed the MICs of vancomycin of LCT-EF258 and LCT-EF90 were both 2 $\mu g \cdot ml^{-1}$, and that of VREF was 32 $\mu g \cdot ml^{-1}$. Therefore, 2 $\mu g \cdot ml^{-1}$ vancomycin was used to screen the resistant strains in the subsequent experiments.

A total of 1 ml of the bacterial sample was added to 100 ml of TH broth and incubated at 37°C with shaking. TH broth alone was used as the control group. Samples were collected every 2 h and the optical density (OD) was measured at a wavelength of 600 nm (OD_{600}). The initial OD value was used as the starting point and the data were recorded for up to 20 h. The test was performed with three replicates, then the average values of ODs were used to draw growth curves.

We performed PCR assay to obtain DNA fragments of the P23 promoter from the P23-containing plasmid with primers for P23-F and P23-R-StuI, the vancomycin resistance gene vanA from the VREF with primers for VanA-F-Blant and VanA-R-XbaI, and the homology sequence Homo from LCT-EF90 with primers for Homo-F-EcoRI and Homo-R. **Table I** lists the primer sequences used for PCR. The 1% agarose gel electrophoresis of P23, vanA, and Homo is shown in **Fig. A** online (https://doi.org/10.3357/amhp.5120sd.2018). The sequences of P23, vanA, and Homo are listed in **Appendix A** online (https://doi.org/10.3357/amhp.5120sd.2018). We then sequentially inserted P23, vanA, and Homo into the pMD18-T vector

Table I. Primers Used for PCR.

PRIMER	SEQUENCE (5′ TO 3′)
DprA-F	ATGTATCAAATAGAAGAAAATTTATTGAAA
DprA-R	TTATTCTTTAAATTCTGCCAAGATATC
VanA-F	ATGAATAGAATAAAAGTTGCAATACTGT
VanA-R	TCACCCCTTTAACGCTAATACG
P23-F	TCGAAAAGCCCTGACAACC
P23-R	TATATTTGGCCTCCCTTTTTAATTTA
Homo-F	TGGAGCTTGTGACCGAGGAG
Homo-R	GACCTCCACCTCCATATTCATCTG
P23-R-StuI	ACTTAGGCCTTTTTAATTTAATTCTAATACT
VanA-F-Blant	GAGGCCAAATATAATGAATAGAATAAAAGTTGCAATACTGTT
VanA-R-XbaI	AAGGTCTAGATCACCCCTTTAACGCTAATACG
Homo-F-EcoRI	GACTGAATTCTGGAGCTTGTGACCGAGGAG

Table II. Primers Used for Real-Time Quantitative PCR.

GENE & PRIMER	SEQUENCE
vanA	
vanA-F	5′- CGCGGTGCATTAGCTAGTTG -3′
vanA-R	5′- CCCTCTCAGGTGCGGCTAT -3′
16SrRNA	
16S-F	5′- CTGTGAGGTCGGTTGTGCG -3′
16S-R	5′- TTTGGTCCACCTCGCCA -3′

(TaKaRa Bio Inc., Kusatsu, Japan) through a repeated procedure that included enzyme digestion, linking, transformation into DH5α-competent cells (TaKaRa Bio Inc.), and plasmid extraction. Finally, we constructed the integrated plasmid pMD18T-P23-vanA-Homo. This plasmid was extracted, sequenced, concentrated (concentration, 2760 $\mu g \cdot ml^{-1}$), and stored at –20°C until further use. The 0.7% agarose gel electrophoresis of the plasmid pMD18T-P23-vanA-Homo is showed in **Fig. B** online (https://doi.org/10.3357/amhp.5120sd.2018). The sequence of the plasmid pMD18T-P23-vanA-Homo is listed in Appendix A online.

The strains LCT-EF258 and LCT-EF90 were subjected to PCR with primers for VanA-F and VanA-R to confirm that vanA was absent from the genome before gene transformation (**Fig. C** online; https://doi.org/10.3357/amhp.5120sd.2018). Then competent cells were produced: the strains LCT-EF258 and LCT-EF90 were grown in 250 ml of TH broth and allowed to reach logarithmic phase at OD_{600} values of approximately 0.2–0.3. The cells were then harvested by centrifugation (4°C, 5 min, 8000×g) and washed three times with 40 ml of ice-cold transfer buffer (0.5 M sucrose containing 10% glycerol). The cells were suspended in 4 ml of ice-cold transfer buffer and the OD_{600} values of the two strains were adjusted to the same final value to ensure the cell densities were the same. The numbers of 100 μl of competent cells were counted by plating dilutions on unselective media. Finally, the cells were stored in 200-μl aliquots at −80°C until further use. All steps were performed on ice.

The competent cells were thawed on ice and then 100 μl of the competent cell suspension was mixed with 2 μg of pMD18T-P23-vanA-Homo DNA and transferred to an ice-cold electroporation cuvette. One pulse was performed at a field strength of 2500 V using a 200-Ω resistor to produce a time constant of 5 ms. Directly after the pulse, 1 ml of TH broth was added to the 2-mm electroporation cuvette, and the cell/medium mixture was placed into sterile test tubes that were incubated at the respective growth temperatures for 6 h to allow for the phenotypic expression of antibiotic resistance. Subsequently, 100 μl of the mixture was plated onto TH agar plates containing 2 $\mu g \cdot ml^{-1}$ vancomycin. The transformants were detected 48 h after plating, named VREF90 and VREF258. The numbers of bacterial colonies growing on the TH agar plates containing 2 $\mu g \cdot ml^{-1}$ vancomycin were recorded. The test was repeated three times and the three replicates were named Test1, Test2, and Test3. We also electroporated both strains without pMD18T-P23-vanA-Homo DNA to determine whether there were any spontaneous

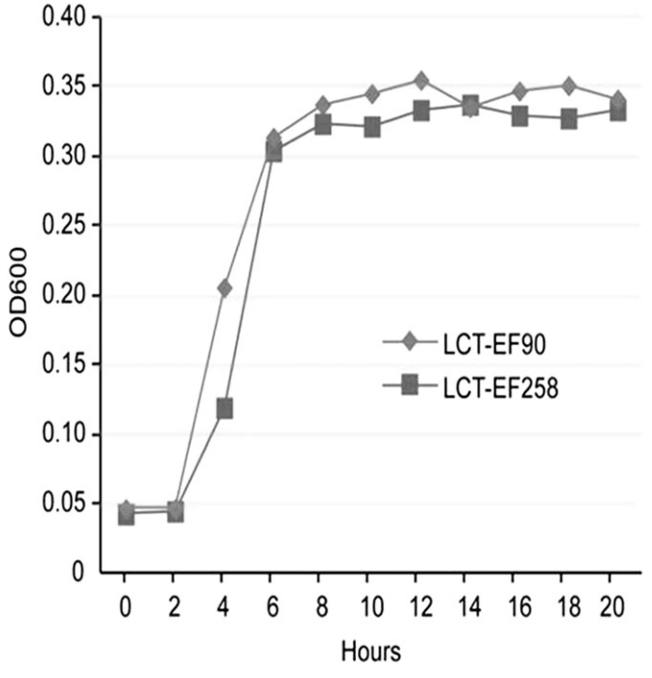

Fig. 1. The bacterial growth curve.

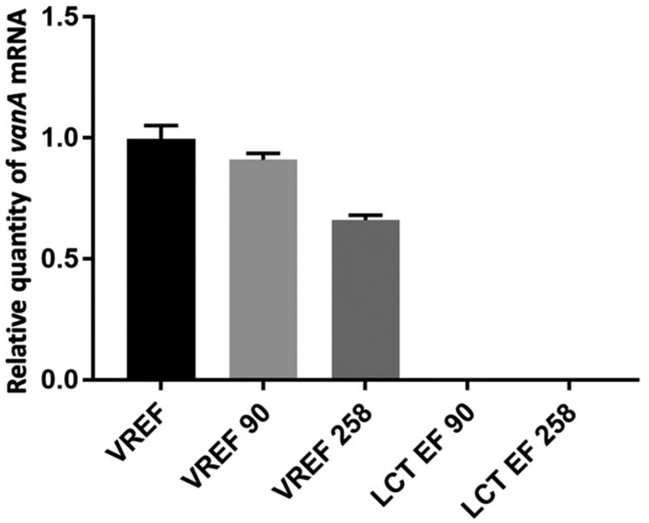

Fig. 2. Relative quantity of vanA mRNA. The relative quantity of vanA mRNA of the VREF90 group is 0.91 ± 0.022. The relative quantity of vanA mRNA of the VREF258 group is 0.66 ± 0.016. The relative quantity of vanA mRNA of VREF is 1.00 ± 0.045. The relative quantities of vanA mRNA of the LCT-EF90 group and LCT-EF258 group are 0.00. The difference between the VREF90 group and VREF258 group is considered significant at $P = 0.002$ ($P < 0.01$).

mutations conferring antibiotic resistance to vancomycin.

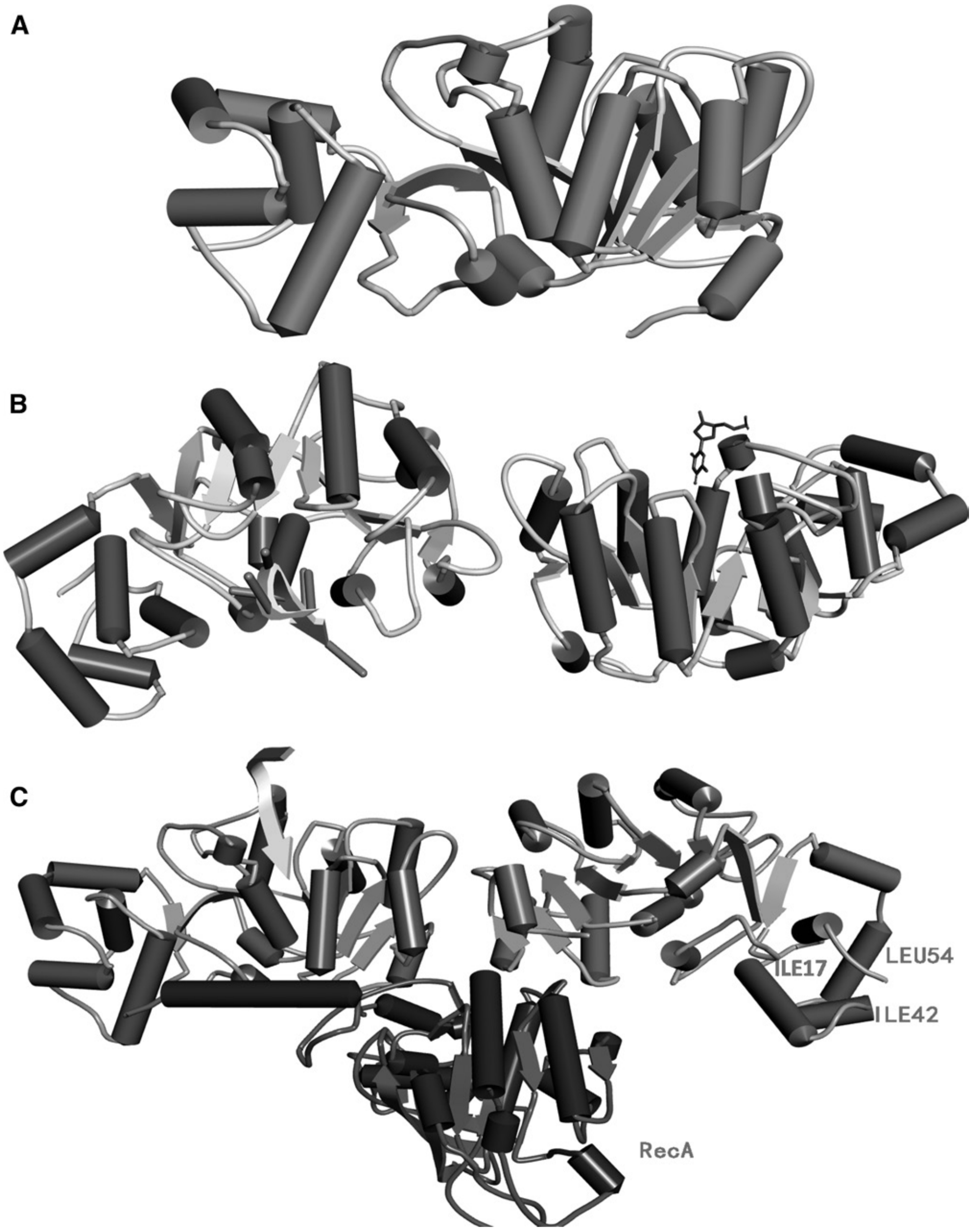

Fig. 3. The 3D simulated structures (see color online). A) The 3D simulated structure of the mutant DprA monomer. B) The 3D simulated structure of the mutant DprA dimer (the small molecule present in panel 4B shows a ssDNA binding on the DprA). C) The optimal docking mode of the mutant DprA dimer and RecA.

Three colonies were selected randomly from every group (VREF, VREF258, VREF90, LCT-EF90, and LCT-EF258). The strains were grown in TH medium at 37°C to the exponential growth phase. The total RNA was isolated using Trizol (Invitrogen). cDNA was synthesized using M-MLV (TaKaRa Bio Inc.) reverse transcriptase and random primer N6. The primers specific for vanA and 16SrRNA were designed based on the nucleotide sequences published in the GenBank database (Accession nos. M97297 and AJ301830). Transcripts were quantified by real-time fluorescent quantitative PCR (Roche LightCycler® 480II, Roche, Mannheim, Germany) with Evagreen® (Biotium, Fremont, CA). The reactions were performed under the following conditions: 5 min at 95°C, 10 s at 95°C, 10 s at 60°C, 10 s at 72°C for 40 cycles, and 3 min at 72°C. The mRNA level of the vanA gene was normalized to the 16SrRNA level. The $-\Delta\Delta CT$ value was used to calculate the relative quantity. **Table II** lists the primer sequences.

The 3D structure of the mutant DprA was simulated using Discovery Studio^{TM} software (Biovia Co., Ltd.). Templates homologous to DprA were identified using a BLAST search (NCBI) and the structure of the homologous template 3UZE_A was downloaded. The sequence was then aligned using Align Sequence Templates. The tertiary structure of DprA was built according to the Build Homology Model protocol.

Previous studies have shown that DprA protein dimerization is crucial for binding ssDNA and loading the recombinase RecA onto ssDNA during transformation.[15,23] The structural information for the DprA dimer of *Helicobacter pylori* is known;[30] its PDB ID is 4LJR. Using the superposition method, the two mutant $_{Ef.}$DprA monomers could be superimposed with the $_{Hp.}$DprA dimer subunits A and B, after which the A and B chains of 4LJR could be removed to achieve the mutant DprA dimer structure. In addition, the ssDNA molecules C and D of 4LJR were retained and renamed $_{Ef.}$DprA-DNA. Optimized results were achieved using the molecular mechanics method and in situ ligand minimization to construct a model of the mutant DprA dimer structure.

In the mutant DprA monomer, the amino acid SER17 mutated into ILE17. The Build Mutants function was used to build S17I mutation in the hydrophobic core of the mutant DprA monomer and compare the stability of the hydrophobic core.

The Calculate Mutation Energy (Stability) function was used to calculate the effects of the S17I mutation on the stability of the DprA dimer. The Calculate Binding Energies function was used to calculate the combined free energy of the DprA dimer in combination with the ssDNA DT35.

DprA also interacts with RecA and promotes the loading of RecA on ssDNA.[15] RecA uses the PDB ID 1G18 protein crystal structure and forms a molecular dock with the DprA dimer via

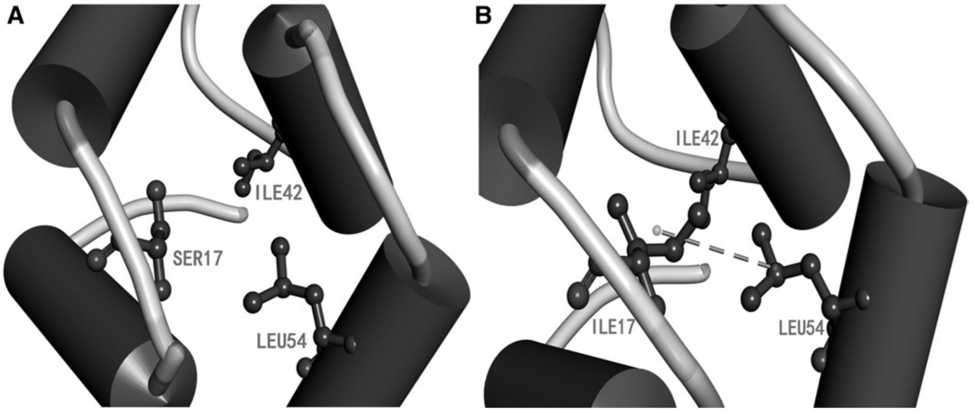

Fig. 4. ILE17 and LEU54 generate a hydrophobic interaction (see color online). A) In the wild DprA monomer, the amino acid SER17 and LEU54 cannot generate a hydrophobic interaction. B) In the mutant DprA monomer, the amino acid SER17 mutated into ILE17. ILE17 and LEU54 can generate a hydrophobic interaction. The grey (violet-colored online) dotted line is meant to represent the new hydrophobic interaction.

docking proteins (ZDock). The docking position with the minimum ZRank value is the optimal docking position. The ZRank score considers the Fan Dehua force, electrostatic energy, and dissolution energy between the molecules. Using the Calculate Mutation Energy (Binding) function, we calculated the interaction affinity between the mutant DprA dimer and RecA.

Statistical Analysis

Comparisons between the two groups were performed with an independent samples *t*-test using SPSS 17.0 statistical software (SPSS China Co., Ltd., Shanghai, China). The differences were considered significant at $P < 0.01$.

RESULTS

The result of amplified, sequenced, and aligned results showed that only dprA had mutated in LCT-EF258. The mutation of dprA in LCT-EF258 was a single nucleotide C changed to A (**Fig. D**, section A; online at https://doi.org/10.3357/amhp.5120sd.2018), and after the strains were passaged 10 times on the ground, this mutation did not reverse. The amino acid coded by the mutant nucleotide changed from S to I (Fig. D, section B; online at https://doi.org/10.3357/amhp.5120sd.2018); thus, this mutation was a nonsynonymous substitution.

The bacterial growth curves of the two strains were generally consistent and showed that the logarithmic growth phases were at 2–6 h (**Fig. 1**). The number of 100-μl competent cells was 7.6×10^{15}. Then the numbers of VREF258 that grew on the TH agar plates containing 2 $\mu g \cdot ml^{-1}$ vancomycin after electroporation were 269, 301, and 213. The numbers of VREF90 that grew on the TH agar plates containing 2 $\mu g \cdot ml^{-1}$ vancomycin after electroporation were 580, 500, and 589. The difference between the VREF258 group and the VREF90 group was considered significant at $P = 0.002$ ($P < 0.01$), given that the conditions were identical in terms of the number of cells, plasmid dose, voltage, time and medium.

We also electroporated both strains without pMD18T-P23-vanA-Homo DNA to determine whether there were any spontaneous mutations conferring resistance to vancomycin, but no clones grew on plates with vancomycin. We compared the vanA mRNA levels between the VREF90 group and VREF258 group. The results from real-time fluorescent qPCR showed that the vanA mRNA level of the VREF258 group was lower than that of the VREF90 group [$P = 0.002$ ($P < 0.01$)]. The relative quantities of vanA mRNA are shown in **Fig. 2**.

The 3D simulated structure of the mutant DprA monomer is shown in **Fig. 3A**. The 3D simulated structure of the mutant DprA dimer is shown in **Fig. 3B**. The optimal docking mode of the mutant DprA dimer and RecA is shown in **Fig. 3C**.

In the mutant DprA monomer, the amino acid SER17 mutated to ILE17. ILE17 and LEU54 can generate a hydrophobic bond, which makes the structure of the hydrophobic core more stabilized (**Fig. 4**). The results showed that this mutation caused a 2.89 $kcal \cdot mol^{-1}$ decrease in energy and increased the stability of the entire DprA dimer structure.

The results showed that the combined free energy of the wild-type DprA with the ssDNA DT35 was -457.0988 $kcal \cdot mol^{-1}$, where as that of the mutant DprA was -438.1288 $kcal \cdot mol^{-1}$. Therefore, this mutation caused an 18.97 $kcal \cdot mol^{-1}$ increase in the combined free energy and a decrease in the binding affinity between DprA and ssDNA.

DprA also interacts with RecA and promotes the loading of RecA on ssDNA.[11] The results showed that the free energy of the mutant DprA combined with RecA was 0.01 $kcal \cdot mol^{-1}$ greater than that of the wild-type DprA. However, this change in the free energy was too small to affect the interaction affinity between the mutant DprA dimer and RecA.

DISCUSSION

DprA plays a prominent role in the exogenous horizontal gene transformation of *S. pneumoniae, H. pylori, B. subtilis,* and *Rhodobacter capsulatus*. However, no study has confirmed the function of DprA in *E. faecium*. In this study, the result showed a reduction of the exogenous horizontal gene transfer in dprA mutated *E. faecium*.

In horizontal gene transfer, one of the functions of DprA is to bind exogenous ssDNA and protect it from nucleases.[18] Through the 3D structural simulation of the mutant DprA, we found that the structure of the hydrophobic core of the mutant DprA was more stabilized and the binding affinity of ssDNA to the mutant DprA decreased. The other function of DprA is to interact with RecA and promotes the loading of RecA on ssDNA.[15] The results showed that the change in the free energy was too small to affect the interaction affinity between the mutant DprA dimer and RecA.

The software used in this study, Discovery Studio™, is an applied software that is based on the molecular dynamics theory, which can dynamically describe molecular movement and is widely used in the field of theoretical bioscience research to investigate protein folding mechanisms, enzyme catalytic reaction mechanisms, protein movement, and biomacromolecular conformational changes.[8,22,29] Our study is an attempt to apply the molecular dynamics theory to the study of microorganisms in space.

DprA is a conserved protein in bacteria.[3,27] Under environmental stress, some conserved protein-encoding genes will mutate, allowing organisms to adapt to the environment in order to survive.[2,9] In this study, the dprA mutation is stable and did not reverse even when the strain LCT-EF258 was passaged 10 times after returning to the ground.

Vancomycin-resistant *E. faecium* represents a growing threat in hospital-acquired infections.[4,19] *E. faecium* can acquire vancomycin resistance through horizontal gene transfer.[12] In this study, the dprA mutant could affect the ability of *E. faecium* to acquire exogenous resistance gene vanA, which offered us an interesting path to block the dissemination of resistance genes between strains.

Space has given us new insights into bacteria. Future research will be performed at China's space station to study the bacteria in the space environment, to design approaches to prevent the antibiotic resistance of bacteria, and to ensure the health of the astronauts.

ACKNOWLEDGMENTS

This work was supported by the National Basic Research Program of China (973 program) (No. 2014CB744400), the Program of China Manned Spaceflight (No. 040203), the National Significant Science Foundation of China (No. 2015ZX09J15102-003), and Major Project of Military Medicine of China (No. BWS17C030).

Yi Yu and De Chang contributed equally to this work.

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

The authors have declared that no competing interests exist.

Authors and affiliations: Yi Yu, M.D., Ph.D., De Chang, M.D., Ph.D., Junfeng Wang, M.D., Ph.D., and Changting Liu, M.S., M.M., Nanlou Respiratory Diseases Department, Chinese PLA General Hospital, Beijing, China, and Qiang Guo, M.D., Ph.D., Laboratory of Vaccine and Antibody Engineering, Beijing Institute of Biotechnology, Beijing, China.

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