European Review for Medical and Pharmacological Sciences 2018; 22: 4033-4044

The regulation of *DLTA* gene in bacterial growth and biofilm formation by *Parvimonas micra*

K. LIU, B.-X. HOU

Department of Endodontics and Operative Dentistry, School of Stomatology, Capital Medical University, Dongcheng District, Beijing, China

Abstract. – OBJECTIVE: To evaluate the effect of *dltA*-deficient mutant on the bacterial growth and biofilm formation by *P. micra* ATCC 33270. *Parvimonas micra* contributes to many human polymicrobial infections and is common in dental plaque biofilms of patients with periodontal and endodontic conditions. Lipoteichoic acid (LTA) performs several functions in gram-positive bacteria, including maintenance of cationic homeostasis and modulation of autolytic activities. The activation of *dltA* gene expression protects LTA expressing gram-positive bacteria from innate immune anti-microbial defense.

MATERIALS AND METHODS: Deficient mutant of the *dltA* gene was created from *P. micra* ATCC 33270 by homologous recombination. Colony-forming units (CFUs) and turbidity helped estimate the growth of *P. micra*. Crystal violet staining, Confocal Scanning Laser Microscopy (CSLM) and Scanning Electron Microscopy (SEM) evaluated biofilm mass and structure.

RESULTS: *P. micra* ATCC 33270 with *dltA*-deficient mutant was successfully established. CFUs of the wild-type strains were significantly higher than that of the *dltA*-deficient mutant strains after 24 h, 48 h, 72 h and 7 d culture (all $p < 0.05$). The growth rate of *dltA*-deficient mutant strains was significantly lower than their wild-type counterparts. Furthermore, crystal violet staining showed that the *dltA* mutant formed significantly less biofilm as compared to wild-type strains. The *dlt*A-deficient mutant synthesized a thin and incomplete biofilm after incubation for 48 h. With increasing incubation time, all biofilm units were seen to shrink, and this structure almost disappeared after 7 days of culture as observed by CSLM and SEM.

CONCLUSIONS: The *dltA* gene is associated with bacterial growth and biofilm formation by *P. micra* ATCC 33270.

Key Words:

Parvimonas micra, Lipoteichoic acid, Biofilm formation, Homologous recombination, *dltA* gene.

Abbreviations

BHI: brain heart infusion; CFUs: Colony-forming units; CSLM: Confocal Scanning Laser Microscopy; LTA: Lipoteichoic acid; SEM: Scanning Electron Microscopy.

Introduction

Parvimonas micra (also referred to as *Peptostreptococcus micros* and *Micromonas micros*), was previously reported to have both smooth and rough morphotypes in subgingival plaques, and is an important anaerobic Gram-positive oral cavity colonizer in human subjects[1-5]. Previous researches[6-8] indicated that *P. micra* is one of the most frequently isolated bacteria in dento-alveolar infections of endodontic origin; it is closely associated with additional oral polymicrobial anaerobic infections, and primarily as soft-tissue abscesses[2]. Moreover, in the setting of acute apical abscesses, known to be serious endodontic diseases that emanate from pulpal infection with opportunistic oral microorganisms, previous studies[9,10] confirmed that infection with *P. micra* was one of the most common opportunistic infections isolated from infected patients. Thus, there is urgent clinical research need to study the pathogenicity of *P. micra* and its related characteristics.

The cell wall of Gram-positive bacteria consists of different types of anionic molecules and other essential units that contribute to biofilm formation on periodontal surfaces. Biofilms are highly structured microbial communities, which form a complex biological substrate of self-produced extracellular matrix comprised of exopolysaccharides and lipoteichoic acid (LTA), which is a major constituent of biofilms[11,12]. The formation of the biofilm provides mechanical stability and cohesiveness to promote the development of a highly acidic microenvironment, which is critical for the pathogenesis of dental caries and other

Corresponding Author: Benxiang Hou, MD; e-mail: houbenxiang@gmail.com

periodontal conditions including gingivitis and periodontitis and subsequent cardiovascular disease development and progression in susceptible subjects[11-13].

LTA is also considered a virulence factor displaying an important role in infections and in post-infectious sequelae caused by Gram-positive bacteria[14,15]. LTA is an amphiphilic polymer consisting of polyglycerol phosphate and a glycolipid anchor inserted into the cell membrane that is mostly replaced with D-alanine at the C-2 position of glycerol[14,15]. Based on its derivation and chemical structures, LTA was divided into five different types: Type I, II, III, IV and V. The exact function of each type remains largely unclear[16,17]. Previous studies found that mutants of the LTA gene would lead to significant morphological and physiological defects and attenuated virulence[18,19]. In addition, resistance to cationic anti-microbial peptides by Gram-positive cocci was enhanced by structural modification of the LTA backbone[20-22]. Thus, it is important to explore the precise functions of LTA to identify improved solutions to the clinical eradication of pathogenic oral microorganisms.

In addition to the functions of LTA, Reichmann et al[23] showed that LTA polymers are modified with D-alanine residues and under condition where D-alanine is lacking, this can lead to increased susceptibility to cationic anti-microbial peptides. Four proteins, referred to as DltA through Dlt-D, are essential for the incorporation of D-alanine into cell wall polymers like LTA. It has been established that DltA transfers D-alanine residues in the cytoplasm of the cell onto the carrier protein DltC. Proteins required for d-alanine incorporation into LTA and other teichoic acids are encoded in the dlt operon[23]. The activation of the dlt operon and specifically activation of *dltA* gene expression affords LTA expressing gram positive bacteria protection from anti-microbial peptide attack and innate immune anti-microbial defenses such as neutrophil extracellular traps[24]. Thus, in the present research, we established a *dltA*-deficient mutant of *P. micra* by homologous recombination to analyze the contribution of *dltA* to its growth, biofilm mass, and structure.

Materials and Methods

Bacterial Strains and Growth Conditions

P. micra ATCC 33270 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and cultivated in brain heart infusion (BHI) broth (Oxoid Ltd., Basingstoke, Hampshire, England, UK) without agitation at 37°C at GeneScience AG300 anaerobic incubator (N_2: 80%; CO_2: 10%; H_2:10%) (GeneScience Pharmaceuticals Co., Ltd., Changchun, China). *Escherichia coli* DH5 and TOP10 (Invitrogen, Carlsbad, CA, USA) were grown aerobically in Luria-Bertani broth (Oxoid Ltd., Basingstoke, Hampshire, England, UK) at 37°C.

Construction of the dltA-Deficient Mutant

The inactivation of the *dltA* gene (GenBank accession No. NZ_DS483518.1; PEPMIC_RS05520 in the *P. micra* genome) was confirmed by the method of Cieslewicz et al[25] with the following modifications. Genomic DNA from *P. micra* clone ATCC 33270 was prepared by DNeasy Tissue kit (Qiagen, Hilden, Germany). The upstream and downstream arms of the *dltA* gene were amplified from the *P. micra* ATCC33270 genomic DNA with primers Δ*dltA* – Forward (F)1- *Kpn* I and Δ*dltA* – Reverse (R)1- *Pst* I and *Sma* I, and primers Δ*dltA* -F2-*Pst* I and *Sma* I and Δ*dltA* – R2- *Hind* III, respectively. According to the method of Higuchi et al[26], overlap extension PCR was used to create a PCR product lacking a portion of *dltA*, and this product was then cloned into the pUC18 cloning vector (TaKaRa, Otsu, Shiga, Japan) by digestion and ligation, referring to the method of Schäfer et al[27]. The digestion mixtures (100 µl) were mixed using 30 µl of the *dltA* gene fragment (or pUC18 cloning vector), 5 µl *Hind* III and 5 µl *Kpn* I, 10 µl of 10 × buffer, and 50 µl ddH_20. This mixture was incubated for 2 h 30 min at 37°C. The ligation mixtures were set up in a 10 µl reaction volume containing 7 µl of the digested *dltA* gene fragment, 1 µl of digested pUC18 cloning vector, 1 µl of T4 DNA ligase (TaKaRa, Otsu, Shiga, Japan); 1 µl of 10 × buffer was kept overnight at 16°C. The resulting plasmid, pUC18-Δ*dltA*, was transformed into the *E. coli* DH5α strain and screened with Ampicillin (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 100 µg/ml, and verified by Colony PCR and sequenced with the Primer Δ*dltA* -F1-*Kpn* I and Δ*dltA*-R2-*Hind* III. The *Gm* cassette, amplified from pBBR1MCS-5 with primer GM-F-*Pst* I and GM-R-*Sma* I, was digested with *Pst* I and *Sma* I, and ligated with pUC18-Δ*dltA*, which was cleaved by the same restriction enzymes via T4 DNA ligase (reaction

volume as mentioned above). The resulting plasmid, pUC18-Δ*dltA*-*Gm*, was transformed into *E. coli* TOP10 strain and screened with ampicillin at the same concentration above. The flowchart for the derivation of the plasmid pUC18-Δ*dltA*-*Gm* is shown in Figure 1.

The constructed strain was then verified by colony PCR and sequenced with the primer Δ*dltA*-F1-*Kpn* I and Δ*dltA*-R2-*Hind* III. The extracted plasmid, pUC18-Δ*dltA*-*Gm*, was transformed into *P. micra* ATCC 33270 by electroporation. According to the electroporation experiment of Fiedler and Wirth[28], electrocompetent *P. micra* was prepared. Electroporation was performed in a Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA), while the parameters were set at 2.5 kV and 5.7 ms. The pulsed cells were transferred to the BHI medium with no antibiotics and incubated anaerobically without shaking at 37°C overnight. The overnight culture was then diluted with sterile-water and spread on an agar plate of BHI with Garamycin (Sigma-Aldrich 50 μg/ml, St. Louis, MO, USA) to select the transformants. Single colonies were picked up and cultured with non-antibiotic liquid BHI medium. In order to differentiate the true deletion mutants and wild-type revertants, chromosomal DNA of previous single colonies was also isolated for running the conformation PCR and sequencing with the primer Δ*dltA* -F1-*Kpn* I and Δ*dltA* -R2-*Hind* III. The *dltA* deletion mutant was designated *P. micra* 33270 Δ*dltA*. For the construction of the pUC18-Δ*dltA*-*Gm* suicide plasmid, restriction enzyme sites, such as *Kpn* I, *Pst* I, *Sma* I and *Hind* III, were considered while designing the primers, and all the restriction enzymes were purchased from TaKaRa (Otsu, Shiga, Japan).

Growth Assays of P. micra

In order to obtain the growth difference of the wild-type strains and dltA-deficient mutant strains, over-night growth cultures of both

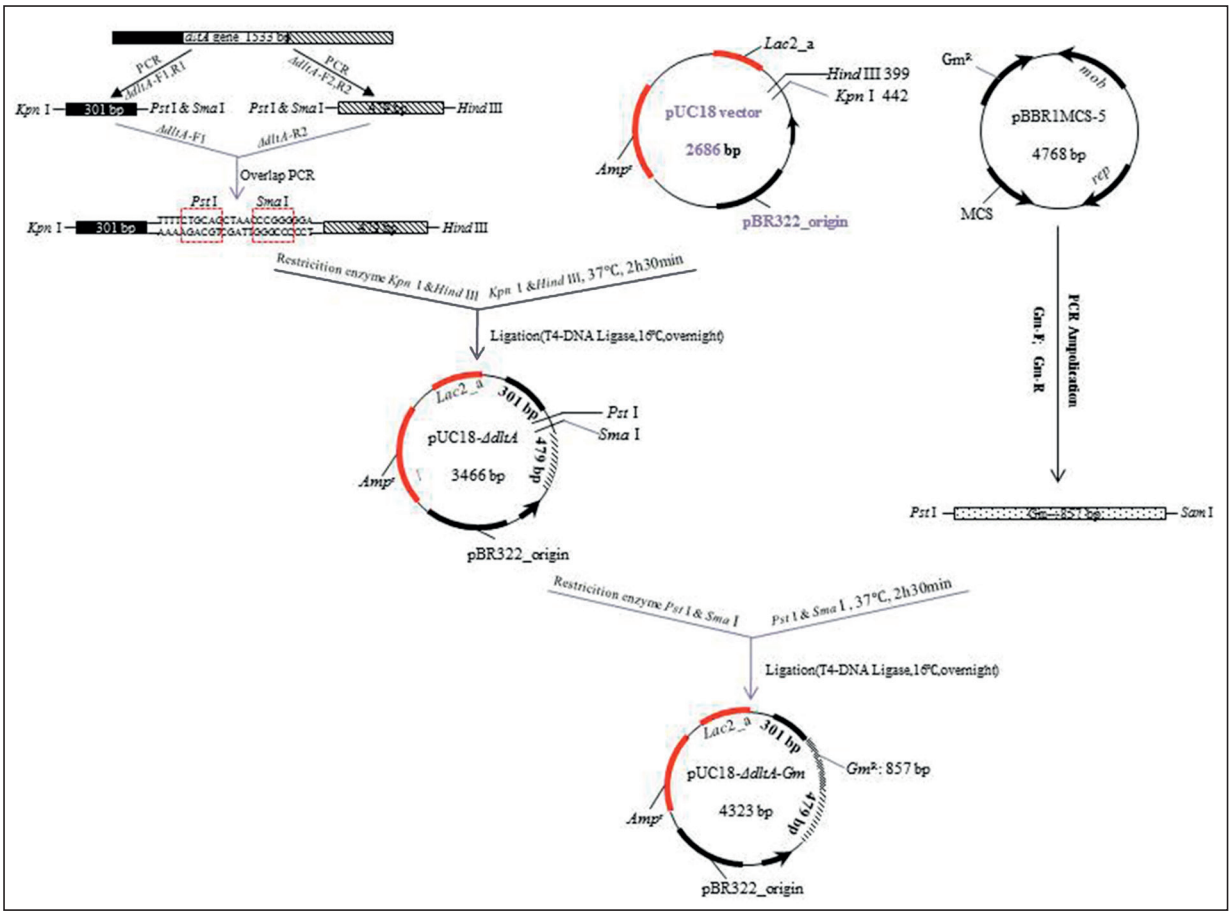

Figure 1. Construction of the pUC18-Δ*dltA*-Gm suicide plasmid.

strains were used to adjust the bacterial suspension to 0.05 at OD_{660nm} in BHI medium. We collected samples from both the wild-type and mutant cultures at every 6 hours from 0 to 72 hours. At each time point, 50 µl of the cultures were sampled, to determine the growth condition of each strain using the Bioscreen™ automated turbidImeter (LabSystems Diagnostics Oy, Helsinki, Finland)[29]. In order to determine the bacterial number by colony forming unit (CFU)/ml, viable bacteria were determined using BHI agar plates.

Biofilm Formation Assays

Quantification of the Mass of Biofilm by Crystal Violet Staining

Overnight cultures of *P. micra* ATCC 33270 and its mutant were diluted with sterilized BHI broth to an OD_{660} of 0.5 respectively, 200 µl aliquots of which were added into each well of 96 well cell culture plates (Costar 3599, Corning, NY, USA), and incubated for 24 h, 48 h, 72 h and 7 days, respectively. The mass of each biofilm, isolated form different incubation time, was qualified by Crystal violet staining as previously described[30].

Analysis of Biofilm Structure via Confocal Scanning Laser Microscopy (CSLM) and Scanning Electron Microscopy (SEM)

The CSLM experiment was conducted as follows: 200 µl diluted strain cultures (*P. micra* and its mutant, OD_{660} = 0.5) and sterile cover slips were transformed into each well of 6 well cell culture plates, and incubated for 24, 48, and 72 h and for 7 days, respectively. Cover glasses, sampled from different culture periods, were gently washed in 1 ml phosphate buffered saline (PBS) solution. According to the operation manual, cover slips were stained with 10 µL SYTO® 9 and propidium iodide (PI) (mix with 1:1) dye mixture at room temperature in the dark for 15 minutes, according to the LIVE/DEAD® BacLight™ Bacterial Viability Kits (Invitrogen, Carlsbad, CA, USA) instructions. The biofilms were examined under a ZEISS LSM 700 microscope (Carl Zeiss MicroImaging, Gottingen, Germany). The height of the biofilm was analyzed with ISA-2 software that was developed by the Biofilm Structure and Function Research Group at the Center for Biofilm Engineering at Montana State University as previously described[31,32].

Biofilms of *P. micra* and its mutant, formed at different incubation periods (48 h, and 72 h and then at 7 days), were also analyzed by SEM as follows: strains were cultured with sterilized cover slips as mentioned above in each well of 96 well cell culture plates. The biofilms formed on the cover slips were then washed with distilled PBS solution gently and fixed with 5% glutaraldehyde and 3% formaldehyde solution for 4 hours. After being dehydrated through a graded ethanol series (80%, 90% and 95%) for 3 hours, the cover slips were kept at a vacuum dryer overnight; after that, sputter coated with gold via ESC-101 SEM sample coating system (Elionix, Tokyo, Japan). The biofilms were then examined and photographed at × 500 and ×2500 magnification under an electron microscope (S4800, Hitachi, Tokyo, Japan).

Statistical Analysis

All the statistical tests were performed in SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Data are shown as mean±standard deviation from at least three independent experiments performed in triplicate. Statistical significance was evaluated by the Student's *t*-test or one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test. p-value < 0.05 was considered statistically significant.

Results

Successful Construction of dltA-Deficient Mutant Strains

The 301 bp *dltA* gene left arm and 479 bp right arm fragments were successfully amplified from chromosome DNA of *P. micra* ATCC 33270 with primers Δ*dltA*-F1-*Kpn* I and Δ*dltA*-R1-*Pst* I and *Sma* I, and primers Δ*dltA*-F2-*Hind* III and Δ*dltA*-R2-*Pst* I and *Sma* I, respectively (Figure 2A). These two DNA fragments were then connected by the PCR-based overlap extension method (Figure 2B), and the resultant 780 bp fragment was cloned into plasmid pUC18 (about 2686 bp) via enzyme digestion (restriction enzyme *Kpn* I and *Hind* III) and ligation (T4 DNA ligase) to generate a pUC18-*ΔdltA* vector. Colony PCR and enzyme digestion verification (digested with *Kpn* I and *Hind* III) were done for the screening of positive clone, (Figure 2C). Based on this vector, a constitutively expressed gentamycin resistant gene (amplified from plasmid pBBR1MCS-5, about 857 bp) was inserted into the sequence of *dltA* gene in pUC18-*ΔdltA* vector to generate the

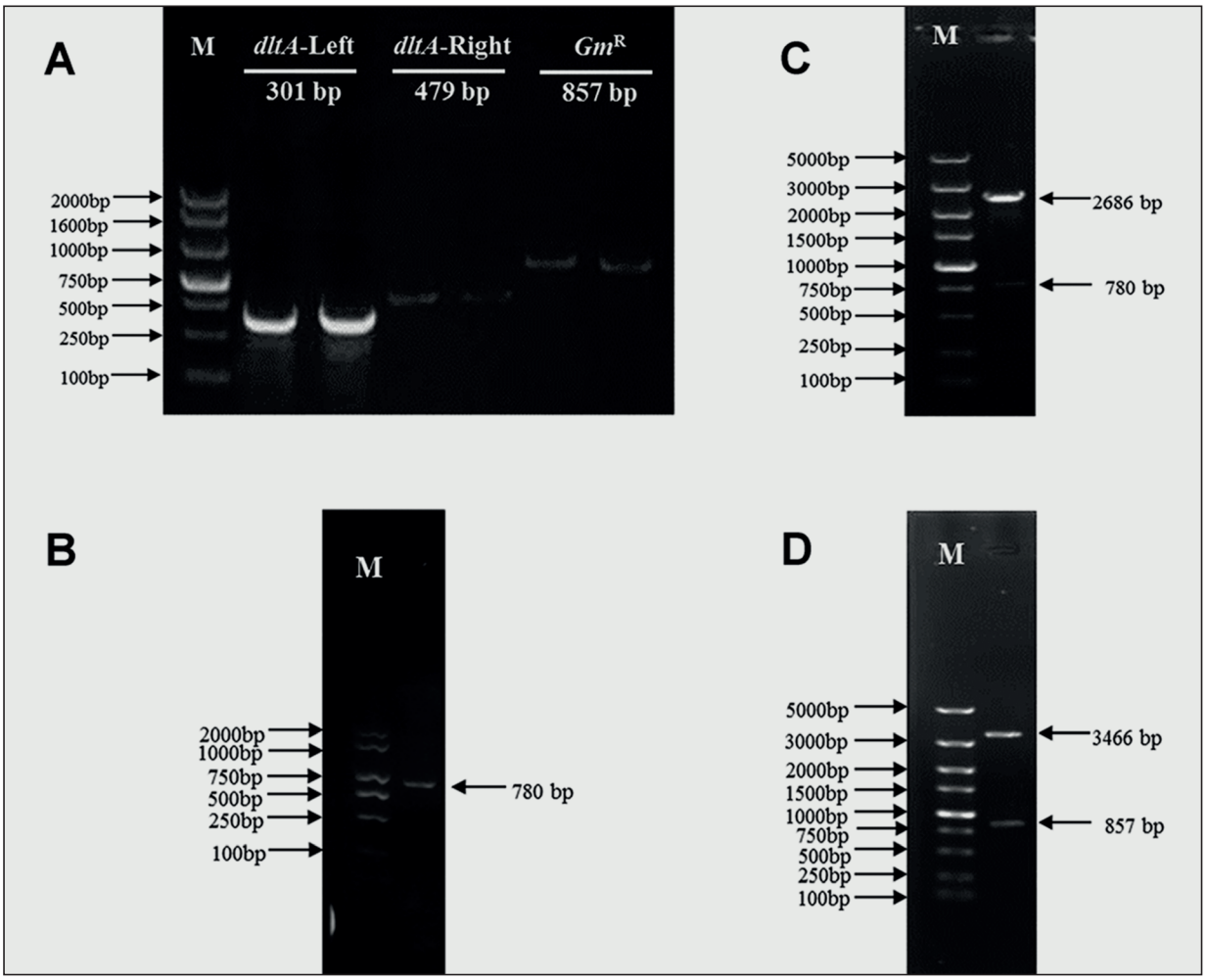

Figure 2. Construction of the pUC18-*ΔdltA*-Gm suicide plasmid. ***(A)*** The amplification results of the *dltA*-left arm (310bp), the *dltA*-right arm (479 bp) and the *Gm* gene fragment (857 bp) from genomic DNA of wild-type *Parvimonas micra* and pBBR1MCS-5 plasmid DNA, respectively; ***(B)*** The *dltA*-left arm and *dltA*-right arm fragments were connected by the PCR-based overlap extension method. ***(C)*** Construction of pUC18-*ΔdltA* plasmid. The resultant 780 bp fragment (Figure 2B) was cloned into plasmid pUC18 (about 2686 bp). ***(D)*** Construction of the pUC18-Δ*dltA*-*Gm* suicide plasmid. A constitutively expressed gentamycin resistant gene (amplified from plasmid pBBR1MCS-5, about 857 bp) inserted into the sequence of *dltA* gene in pUC18-*ΔdltA* vector (about 3466) to generate the suicide plasmid pUC18-Δ*dltA*-*Gm*.

suicide plasmid pUC18-Δ*dltA*-*Gm* for the following homologous recombination. The successfully constructed pUC18-Δ*dltA*-*Gm* plasmid was confirmed by colony PCR. Five out of 10 selected colonies showed the desired *dltA*-*Gm* gene fragment by DNA gel electrophoresis analysis. Enzyme digestion verification was also conducted with *Pst* I and *Sma* I (Figure 2D). Additionally, the pUC18-Δ*dltA*-*Gm* plasmid was finally confirmed until the DNA sequencing analysis was finished.

In order to make *dltA-deficient mutant* of *P. micra* ATCC 33270, the suicide plasmid pUC18-Δ*dltA*-*Gm* was transformed into the wild type strain by electroporation. Incubated for 5 days on gentamycin selective plate, about 58 positive colonies were obtained. These may be the mutants, because the wild type stain is gentamycin sensitive and the suicide plasmid cannot replicate in *P. micra*. The verification work was conducted as follows: chromosomal DNA was extracted from 5 of these colonies and 6 wild type strains for PCR amplification of *dltA*-*Gm* and *dltA* fragments with primer *dltA*-F and *dltA*-R, respectively (Figure 3A). According to the agarose gel electrophoresis results (Figure 3A), *dltA*-deficient mutants have a strong band between 1500 bp and 2000 bp (*dltA*-*Gm* fragment is 1637 bp), while the wild type strains'

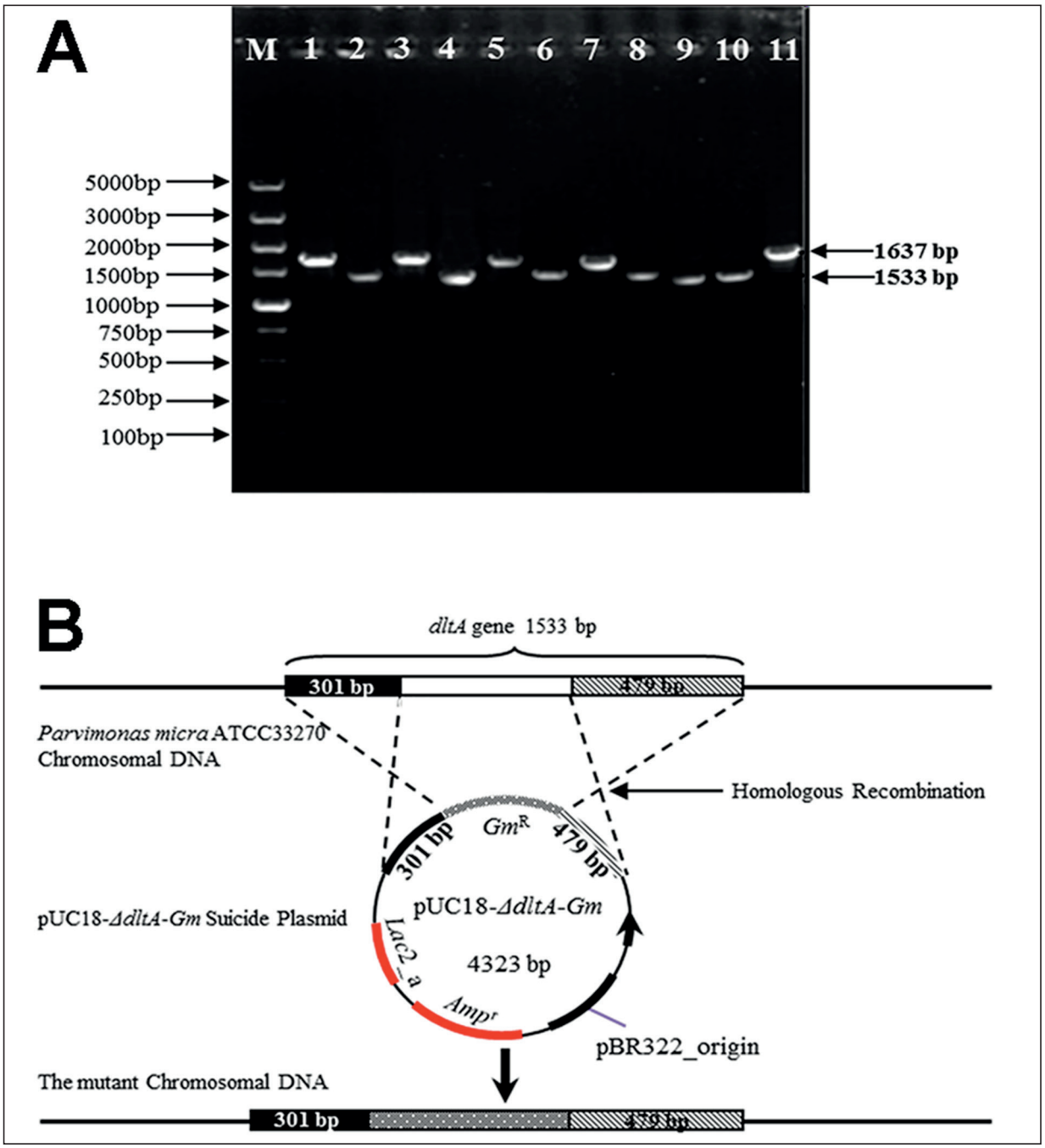

Figure 3. Successful establishment of *P. micra* (ATCC 33270) with *dltA*-deficient mutant. ***(A)*** Agarose gel electrophoresis results showed that *dltA*-deficient mutants have a strong band between 1500 bp and 2000 bp (*dltA-Gm* fragment is 1637 bp), while bands in the wild-type strains are around 1500 bp (*dltA* gene fragment is 1533 bp). The mutant strains are shown in wells 1, 3, 5, 7, and 11 and the wild-type strains in wells 2 ,4, 6, 8, 9, and 10. ***(B)*** Homologous recombination occurred between *dltA* homolog-flanking sequences in the pUC18-*ΔdltA-Gm* suicide plasmid and chromosomal DNA of *P. micra*.

bands are around 1500 bp (*dltA* gene fragment is 1533 bp). This suggests that, as for the mutant, the original *dltA* gene was successfully replaced with the constructed gene combination *dltA-Gm* via homologous recombination as shown in Figure 3B. *DltA-deficient mutant* strains were finally confirmed until the DNA sequencing analysis was finished.

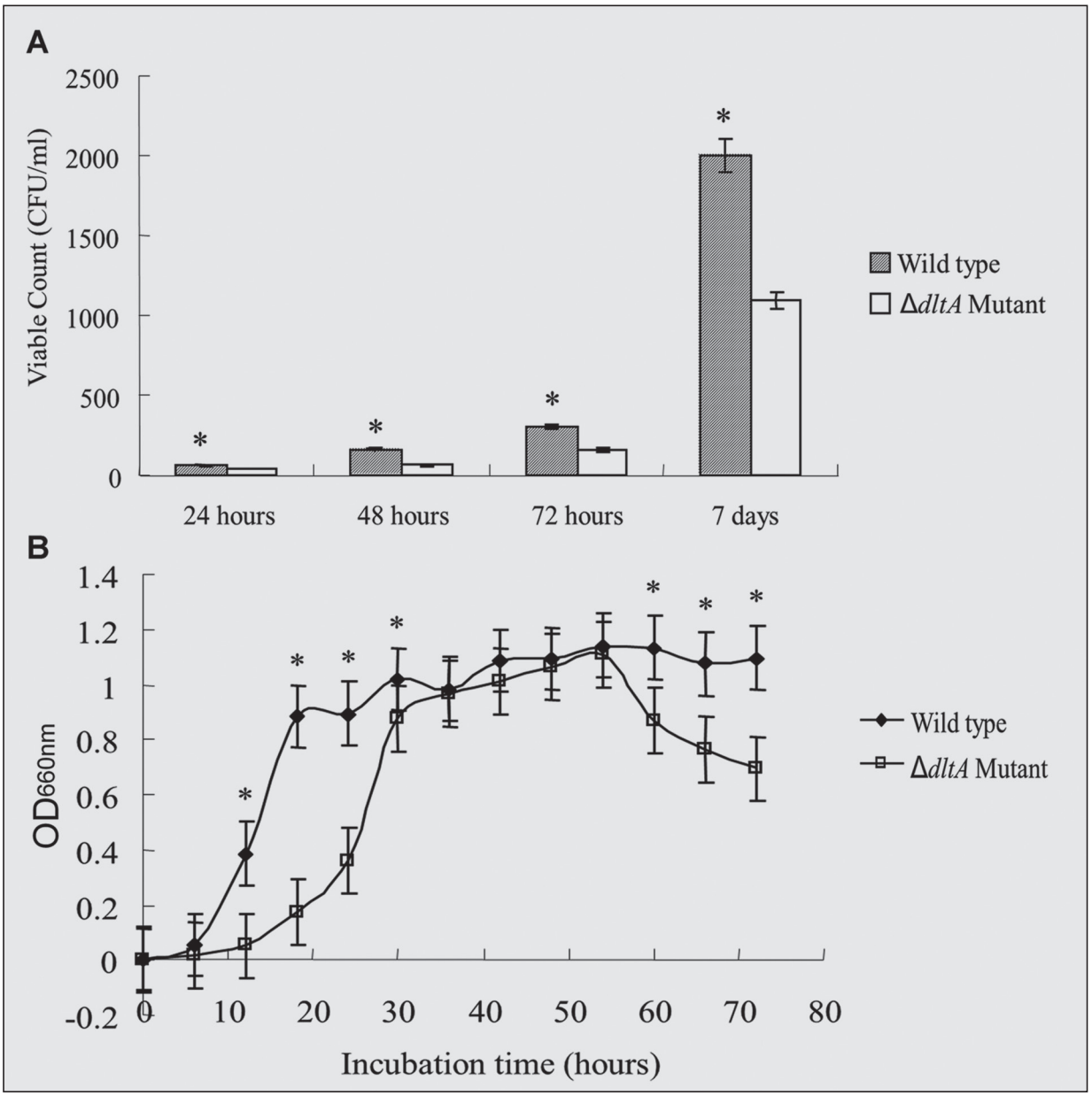

Figure 4. Effect of the *dltA*-deficient mutant on the growth of *P. micra* ATCC 33270. ***(A)*** Flat colony counting results of the *P. micr*a wild-type and the dltA mutant; ***(B)*** Growth curve test of the *P. micra* wild-type and the *dltA* mutant at different incubation times. Data are shown as mean±standard deviation (SD) from at least three independent experiments performed in triplicate; *$p < 0.05$ *vs.* mutant.

Effect of dltA-Deficient Mutant on the Growth of P. micra ATCC 33270

The effect of *dltA*-deficient mutant on the growth of *P. micra* ATCC 33270 was studied by flat colony counting method. The result suggests that the CFUs of the wild-type strains are significantly higher than that of *dltA*-deficient mutant strains at four different sampling periods (Figure 4A) (all $p < 0.05$), which means the growth of *P. micra* will be affected apparently by the deficient of *dltA* gene in the biofilms. According to the growth curve (Figure 4B) the growth rates of the wild-type strains are significantly higher than the *dltA*-deficient ones.

Effect of dltA-Deficient Mutant on the Mass of Biofilm of P. micra ATCC 33270

The mass of each biofilm was measured by crystal violet staining to evaluate the effect of

dltA-deficient mutant on biofilm formation of *P. micra* ATCC 33270. The results suggested that the mass of biofilms produced by *P. micra* wild type was similar to the mutant's at 24 h, but significantly larger than the latter at 48 h, 72 h and 7 days, respectively (Figure 5), confirming that *dltA* gene deficient can significantly affect the biofilm formation ability of *P. micra* to some extent.

Effect of dltA-Deficient Mutant on Biofilm Architecture of P. micra ATCC 33270

The differences of biofilm architectures between the wild type and *dltA*-deficient mutant of *P. micra* ATCC 33270 were measured by CSLM and SEM. According to the CSLM analysis results, biofilm height of wild type was significantly higher than that of the *dltA*-deficient mutant at each sampling time (all $p < 0.05$; Figure 6).

In addition, the SEM analysis reflects that the *dltA*-deficient mutant cannot form complete biofilm at any detection time. The wild type strain can form the thick and completed biofilm after 48 hours of incubation (Figure 7). With the incubation time increasing, all the biofilm units will combine together into a whole networked structure at 72 hours, and this structure becomes even rigorous and complexed when the culture time reached 7 days. However, for the *dltA*-deficient mutant, the strain can form a thin and incomplete biofilm after incubation for 48 hours. With the incubation time increasing, all the biofilm units didn't show the trend of combination but actually they were shrinking. This kind of structure almost disappeared after 7 days of culture.

Discussion

Previous studies[33-35] showed that DltA protein, significantly encoded by *dltA* gene, is a D-alanine-D-alanyl carrier protein ligase, which belongs to the adenylate-forming family and is assumed to undergo major structural rearrangements during the LTA production reaction cycle, that are driven by small changes in electrostatic interactions introduced by substrate binding and product formation. The deficient of this gene may directly affect the production of LTA, while strains either lacking LTA or with defects in LTA backbone synthesis have severe growth defects, according to the result of Garufi et al[36]. This may be the reason why the growth rates of *dltA*-deficient mutant strains were significantly lower than the wild type counterpart, observed in the present work.

As Gross et al[37] mentioned before, the deficient of *dltA* gene will cause the lacing of D-alanine esters of the strain, which will, in turn, affect the pattern of surface-bound proteins. The al-

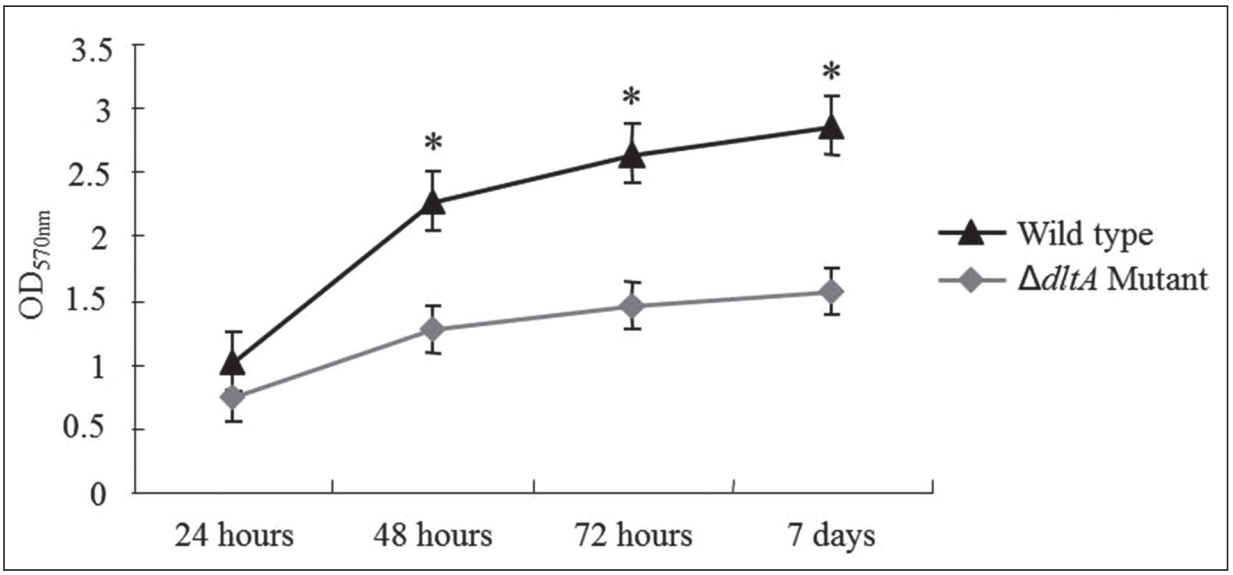

Figure 5. Effect of the *dltA*-deficient mutant on biofilm mass of *P. micra*. Quantification of the biofilm mass was determined by crystal violet staining at 24, 48, and 72 h and at 7 d. Data are shown as mean± SD from at least three independent experiments performed in triplicate; *$p < 0.05$ *vs.* mutant.

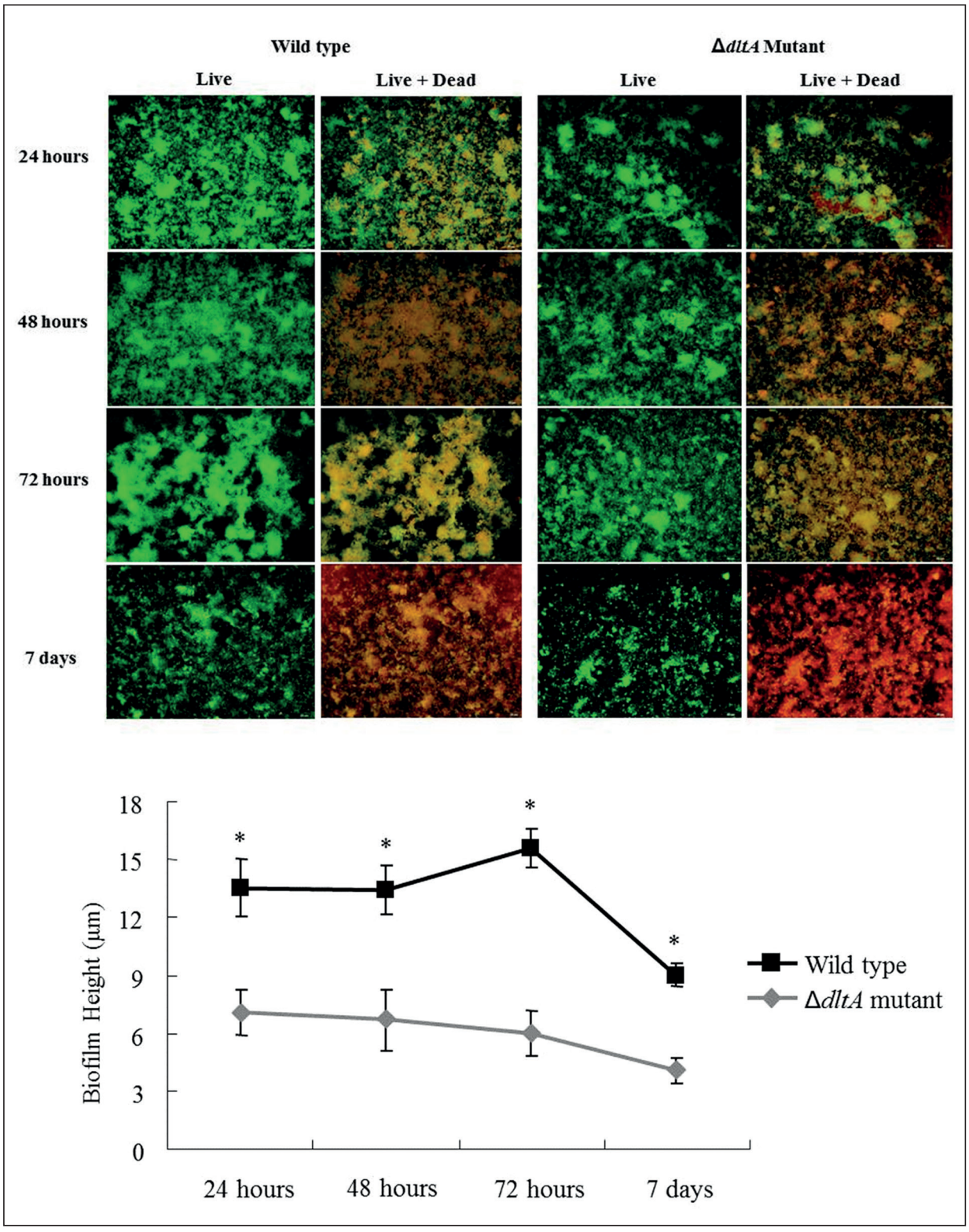

Figure 6. Effect of the *dltA*-deficient mutant on biofilm height of *P. micra* ATCC 33270. The biofilm height was determined by confocal scanning laser microscopy at 24, 48, and 72 h and at 7 d. CLSM images were acquired with a 60-oil immersion objective. Data are shown as mean ± SD from at least three independent experiments performed in triplicate. *$p<0.05$ *vs.* mutant.

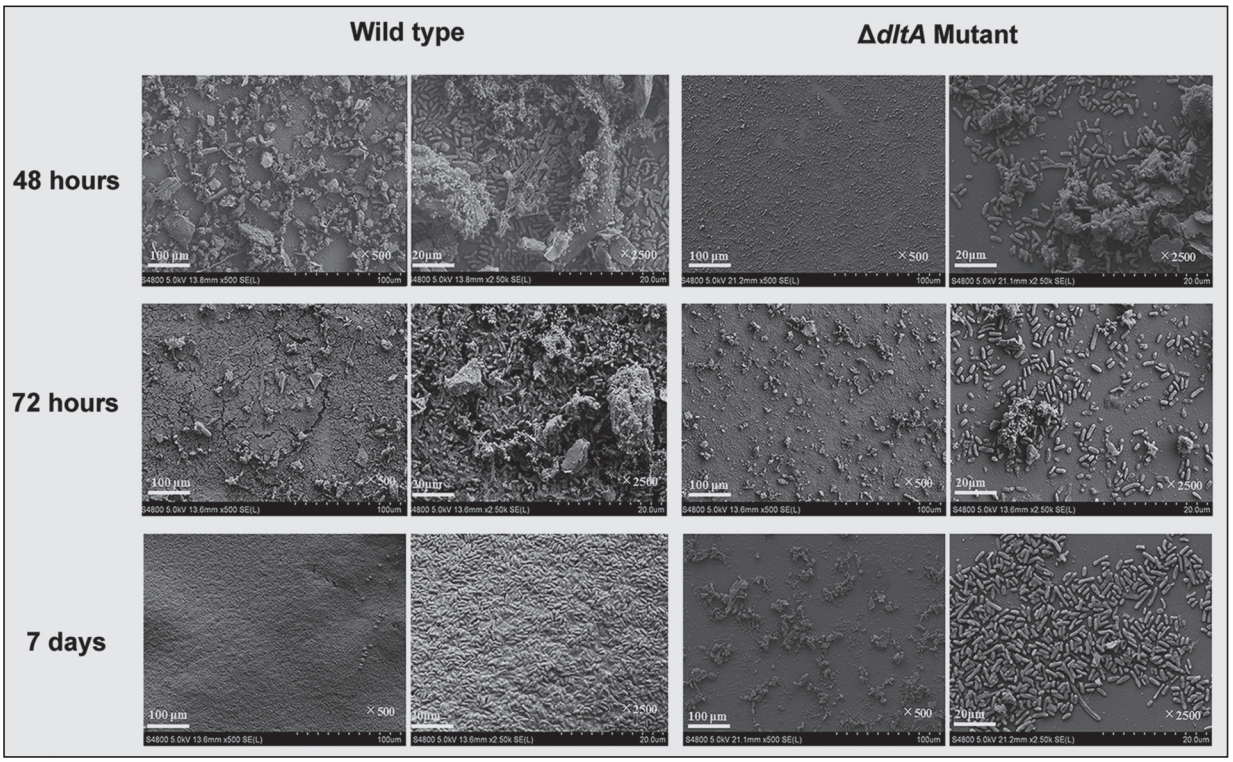

Figure 7. Effect of the *dltA*-deficient mutant on biofilm structure derived from *P. micra* ATCC 33270**.** The *P. micra* wild-type and the *dltA* mutant were cultured anaerobically on cover slips in 96-well plates for 48, and 72 h and for 7 d. Biofilm structures were observed by scanning electron microscopy.

tered surface-bound proteins, especially autolytic enzymes, may affect the initial binding ability of the *P. micra* to polystyrene plates and thus a decrease in the biofilm formation after adherence, which contributed to the attenuation of the biofilm production by the mutant together.

These characteristics resemble those previously reported in *dlt*-inactivated strains of oral bacteria. According to the previous work of Fabretti et al[38] on the biofilm formation related *dlt* operon of *Enterococcus faecalis* 12030, *dlt* deletion mutant produced about 50% less biofilm than the wild type. The *dltA* gene is one of the important genes of *dlt* operon, which encodes the D-alanine and ligates it to the D-alanyl carrier protein (Dcp). Thus, disruption of *dltA* gene will definitely eliminate D-alanylation of LTA[31], which affects the net surface charge of the bacteria, thereby modulating electrostatic interactions between cells and inanimate surface. This might partly explain the reason for observed differences in the biofilm architecture between *P. micra* wild-type and the *dltA*-deficient mutant.

Meanwhile, according to prior study by Gross et al[37] on the net charge of teichoic acid in *Staphylococcus aureus*, the charge of teichoic acids plays a vital role in the interaction between bacteria and surfaces, which in turn affects the initial step of biofilm formation and biofilm structure of *P. micra*. The strong negative charge of the mutant, acquired from the deficient of the *dltA* gene in present study, leads to the pronounced increase in the repulsive forces, thus greatly affecting the adherence of *P. micra* ATCC 33270 and its biofilm formation ability. Because of the attenuation of the bacterial growth and initial adherence, difference of net surface charge and change of surface-associated proteins patterns, the biofilm formation capability of *P. micra* ATCC 33270 is significantly decreased by the *dltA* deficient mutant.

Conclusions

P. micra ATCC 33270 with *dltA*-deficient mutant were successfully constructed by homologous recombination. In addition, *dltA*-deficient mutant attenuated the bacterial growth and biofilm formation by *P. micra* ATCC 33270.

Acknowledgements
The authors thank Professor Haluk Beyenal for the CLSM data that were analyzed by ISA software and the Gene and the Linda Voiland School of Chemical Engineering and Bioengineering, Washington State University, USA, for assisting with the statistical analysis.

Funding
This work was supported by the National Natural Science Foundation of China (No. 81170952) from the National Natural Science Foundation Committee (83, Shuangqing Road, Haidian District, Beijing, China), and the Beijing Municipal Natural Science Foundation (No. 7132111) from the Beijing Natural Science Fund Committee (16, Xizhimen South Street, Xicheng District, Beijing, China).

Conflict of Interest
The Authors declare that they have no conflict of interests.

References

1) Peters LB, Wesselink PR, van Winkelhoff AJ. Combinations of bacterial species in endodontic infections. Int Endod J 2002; 35: 698-702.
2) Gomes BP, Lilley JD, Drucker DB. Associations of endodontic symptoms and signs with particular combinations of specific bacteria. Int Endod J 1996; 29: 69-75.
3) Sundqvist G. Associations between microbial species in dental root canal infections. Oral Microbiol Immunol 1992; 7: 257-262.
4) van Dalen PJ, van Steenbergen TJ, Cowan MM, Busscher HJ, de Graaff J. Description of two morphotypes of Peptostreptococcus micros. Int J Syst Bacteriol 1993; 43: 787-793.
5) van Dalen PJ, van Winkelhoff AJ, van Steenbergen TJ. Prevalence of peptostreptococcus micros morphotypes in patients with adult periodontitis. Oral Microbiol Immunol 1998; 13: 62-64.
6) Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. Periodontol 2000 1994; 5: 78-111.
7) Murdoch DA. Gram-positive anaerobic cocci. Clin Microbiol Rev 1998; 11: 81-120.
8) Rams TE, Feik D, Listgarten MA, Slots J. Peptostreptococcus micros in human periodontitis. Oral Microbiol Immunol 1992; 7: 1-6.
9) George N, Flamiatos E, Kawasaki K, Kim N, Carriere C, Phan B, Joseph R, Strauss S, Kohli R, Choi D, Baumgartner JC, Sedgley C, Maier T, Machida CA. Oral microbiota species in acute apical endodontic abscesses. J Oral Microbiol 2016; 8: 30989.
10) Qi Z, Cao H, Jiang H, Zhao J, Tang Z. Combinations of bacterial species associated with symptomatic endodontic infections in a Chinese population. Int Endod J 2015; 49: 17-25.
11) Klein MI, Hwang G, Santos PH, Campanella OH, Koo H. Streptococcus mutans-derived extracellular matrix in cariogenic oral biofilms. Front Cell Infect Microbiol 2015; 5: 10.
12) Noble S. Clinical Textbook of Dental Hygiene and Therapy (2nd ed.). West Sussex 2012 Wiley-Blackwell; pp. 430.
13) Nguyen CM, Kim JW, Quan VH, Nguyen BH, Tran SD. Periodontal associations in cardiovascular diseases: the latest evidence and understanding. J Oral Biol Craniofac Res 2015; 5: 203-206.
14) Ginsburg I. Role of lipoteichoic acid in infection and inflammation. Lancet Infect Dis 2002; 2: 171-179.
15) Neuhaus FC, Baddiley J. A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. Microbiol Mol Biol Rev 2003; 67: 686-723.
16) Mohamadzadeh M, Pfeiler EA, Brown JB, Zadeh M, Gramarossa M, Managlia E, Bere P, Sarraj B, Khan MW, Pakanati KC, Ansari MJ, O'Flaherty S, Barrett T, Klaenhammer TR. Regulation of induced colonic inflammation by Lactobacillus acidophilus deficient in lipoteichoic acid. Proc Natl Acad Sci U S A 2011; 108: 4623-4630.
17) Percy MG, Grundling, A. Lipoteichoic acid synthesis and function in gram-positive bacteria. Annu Rev Microbiol 2014; 68: 81-100.
18) Abachin E, Poyart C, Pellegrini E, Milohanic E, Fiedler F, Berche P, Trieu-Cuot P. Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of listeria monocytogenes. Mol Microbiol 2002; 43: 1-14.
19) Collins LV, Kristian SA, Weidenmaier C, Faigle M, Van Kessel KP, Van Strijp JA, Götz F, Neumeister B, Peschel A. Staphylococcus aureus strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice. J Infect Dis 2002; 186: 214-219.
20) Dmjanovic M, Kharat AS, Eberhardt A, Tomasz A, Vollmer W. The essential tacF gene is responsible for the choline-dependent growth phenotype of Streptococcus pneumoniae. J Bacteriol 2007; 189: 7105-7111.
21) Hermoso JA, Lagartera L, González A, Stelter M, García P, Martínez-Ripoll M, García JL, Menéndez M. Insights into pneumococcal pathogenesis from the crystal structure of the modular teichoic acid phosphorylcholine esterase Pce. Nat Struct Mol Biol 2005; 12: 533-538.
22) Vollmer W, Tomasz A. Identification of the teichoic acid phosphorylcholine esterase in streptococcus pneumoniae. Mol Microbiol 2001; 39: 1610-1622.
23) Reichmann NT, Cassona CP, Gründling A. Revised mechanism of D-alanine incorporation into cell wall polymers in Gram-positive bacteria. Microbiology 2013; 159: 1868-1877.
24) Wartha F, Beiter K, Albiger B, Fernebro J, Zychlinsky A, Normark S, Henriques-Normark B. Capsule and D-alanylated lipoteichoic acids protect Strep-

tococcus pneumoniae against neutrophil extracellular traps. Cell Microbiol 2007; 9: 1162-1171.

25) Cieslewicz MJ, Kasper DL, Wang Y, Wessels MR. Functional analysis in type Ia group B Streptococcus of a cluster of genes involved in extracellular polysaccharide production by diverse species of streptococci. J Biol Chem 2001; 276: 139-146.

26) Higuchi R, Krummel B, Saiki RK. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res 1988; 16: 7351-7367.

27) Schäfer A, Tauch A, Jäger W, Kalinowski J, Thierbach G, Pühler A. Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of corynebacterium glutamicum. Gene 1994; 145: 69-73.

28) Fiedler S, Wirth R. Transformation of enterococcus faecalis and enterococcus faecium by electroporation. In G. M. Dunny, P. P. Cleary & L. L. McKay (Eds.), Genetics and molecular biology of streptococci, lactococci, and enterococci (pp. 301) 1991; Washington, D.C.: American Society for Microbiology.

29) Charteris WP, Kelly PM, Morelli L, Collins JK. Antibacterial activity associated with Lactobacillus gasseri ATCC 9857 from the human female genitourinary tract. World J Microbiol Biotechnol 2001; 17: 615-625.

30) O'Toole GA. Microtiter dish biofilm formation assay. J Vis Exp 2011; 30: pii: 2437.

31) Beyenal H, Donovan C, Lewandowski Z, Harkin G. Three-dimensional biofilm structure quantification. J Microbiol Methods 2004; 59: 395-413.

32) Beyenal H, Lewandowski Z, Harkin G. Quantifying biofilm structure: facts and fiction. Biofouling 2004; 20: 1-23.

33) McBride SM, Sonenshein AL. The dlt operon confers resistance to cationic antimicrobial peptides in Clostridium difficile. Microbiology 2011; 157: 1457-1465.

34) McCormick NE, Halperin SA, Lee SF. Regulation of D-alanylation of lipoteichoic acid in Streptococcus gordonii. Microbiology 2011; 157: 2248-2256.

35) Schirner K, Marles-Wright J, Lewis RJ, Errington J. Distinct and essential morphogenic functions for wall- and lipo-teichoic acids in Bacillus subtilis. EMBO J 2009; 28: 830-842.

36) Garufi G, Hendrickx AP, Beeri K, Kern JW, Sharma A, Richter SG, Schneewind O, Missiakas D. Synthesis of lipoteichoic acids in Bacillus anthracis. J Bacteriol 2012; 194: 4312-4321.

37) Gross M, Cramton SE, Götz F, Peschel A. (2001). Key role of teichoic acid net charge in Staphylococcus aureus colonization of artificial surfaces. Infect Immun 2001; 69: 3423-3426.

38) Bibbò S, Ianiro G, Giorgio V, Scaldaferri F, Masucci L, Gasbarrini A, Cammarota G. The role of diet on gut microbiota composition.Eur Rev Med Pharmacol Sci 2016; 20: 4742-4749.