Effects of human β-defensin-3 on biofilm formation-regulating genes *dltB* and *icaA* in *Staphylococcus aureus*

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Abstract. An understanding of the regulatory mechanisms that drive *Staphylococcus aureus* biofilm formation may lead to the development of an effective strategy to control the increasing number of refractory clinical infections it causes. The present study examined the effects of the antimicrobial agent human β-defensin 3 (hBD-3) and the antibiotics vancomycin and clindamycin on the expression of the *S. aureus* biofilm formation-regulating genes, *icaA* and *dltB*, during bacterial adhesion and biofilm formation. Transcription (mRNA) levels of *dlt* and *ica* genes were measured using quantitative polymerase chain reaction, and slimes of *S. aureus* biofilm were examined with confocal scanning laser microscopy during *S. aureus* adhesion and biofilm formation. Although hBD-3, vancomycin and clindamycin led to significantly attenuated biofilm formation, their treatment-associated effects on the mRNA expression of *dlt* and *ica* were not identical. Vancomycin and clindamycin induced sustained expression of the *dlt* and *ica* genes, which may be harnessed to induce biofilm formation. However, hBD-3 did not have a significant affect on the transcription level of *dltB* during either bacterial adhesion or biofilm formation. Therefore, the mechanism of hBD-3 that regulated the suppression of biofilm formation appears to differ from the mechanisms of vancomycin and clindamycin.

Introduction

Numerous bacteria attach to the surfaces of organisms or medical implants to secrete an extracellular matrix, also known as a biofilm, that forms a highly structured and complex community. These bacteria carry a specific infectious phenotype different from that of planktonic bacteria, which may include degrees of antibiotic resistance. Infections due to bacterial biofilms may be characterized by repeated refractory episodes with no effective cure (1). In recent years, departments of trauma surgery worldwide have reported a dramatic increase in the incidence of *Staphylococcus (S.) aureus* biofilm infections associated with the use of medical implants (2), and have also been detected in 93.5% of chronic wounds (3).

S. aureus bacteria embedded within the biofilm may have a resistance to antibiotics that is 10-1,000X stronger than their free-floating counterparts (4). A number of antibiotics, including aminoglycoside antibiotics, may even induce bacterial biofilm formation (4). Therefore there is an urgent clinical requirement to identify a novel effective measure to treat *S. aureus* biofilm infections. Insights into the mechanism of action of *S. aureus* biofilm infections and methods to intervene in biofilm formation may be an effective way to control *S. aureus* biofilm infections. The *dltABCD* operon of *S. aureus* is responsible for D-alanine activation and synthesis into teichoic acid (5-7). *S. aureus* bacteria that are deficient in the *dlt* operon are unable to attach to the surfaces of polyethylene and glass, and therefore are not able to form biofilms (8). The *ica* operon (including *icaA*, *icaB*, *icaC* and *icaD*) encodes the synthesis of polysaccharide intercellular adhesin (PIA) (9-14), which mediates biofilm formation. The location and products of the *ica* operon and polysaccharide produced by Ica protein have been extensively studied *in vitro*. Biofilm formation depends on *ica* gene expression and PIA synthesis (15-20). Therefore, an understanding of the effects of antibiotics on the expression of biofilm formation-related genes, such as *dlt* and *ica*, are of notable importance in the control of *S. aureus* infections.

Human β-defensin 3 (hBD-3) is a 45-amino acid peptide that is considered the most promising of its class in the prevention and treatment of implantation-associated infections (21). It has a strong lethal effect on *S. aureus* compared with vancomycin and other antibiotics at low concentrations and can have a strong bactericidal effect (22). The majority of studies of the effects of hBD-3 on the *dlt* and *ica* operons have been limited to planktonic *S. aureus*, while the effect of hBD-3 on these genes in *S. aureus* biofilms has not been well investigated. The present study examined the effects of hBD-3, vancomycin and clindamycin on the biofilm formation-regulating genes, *icaA* and *dltB*, during *S. aureus* adhesion and biofilm formation.

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Materials and methods

Stock solutions. Stock solutions of hBD-3 (Sigma, St. Louis, MO, USA) were reconstituted in 10 mM acetic acid to a concentration of 1.0 mg/ml. Stock solutions of vancomycin (K.K, Seishin Laboratories, Eli Lilly, Kobe, Japan) and clindamycin (Hainan Shuangcheng Pharmaceuticals Co., Ltd., Hainan, China) were dissolved in distilled water to a concentration of 10 mg/ml.

S. aureus cultures. *S. aureus* ATCC 25923 standard strain, obtained from Daping Hospital, the Third Military Medical University (Chongqing, China), were grown in tryptone soya broth (TSB) at 37˚C under vigorous shaking. The minimum inhibitory concentrations for this strain are 8 mg/l for hBD-3 (23-26), 0.5 mg/l for vancomycin and 0.25 mg/l for clindamycin (27).

Biofilm formation. Biofilm formation of *S. aureus* was conducted in 96-well polyvinyl chloride (PVC) plates as previously described (28). Briefly, bacteria from overnight cultures were diluted 1:1,000, and 5 μl of these bacterial suspensions were added to each well containing 100 μl of the biofilm medium. The biofilm medium consisted of 0.5 ml TSB supplemented with 0.2% (w/v) glucose, with or without hBD-3 (8 mg/l), vancomycin (0.5 mg/l) or clindamycin (0.25 mg/l). As hBD-3 degrades gradually (29), hBD-3 was added again after 3 h.

Evaluation of extracellular polymeric substance (EPS) via confocal scanning laser microscopy. Calcofluor white, a polysaccharide binding dye, has been used to stain the extracellular matrix of biofilms formed by bacteria (30). Therefore, to determine whether the adhered structures of *S. aureus* were encased in EPS, the biofilm was stained with 50 mM calcofluor white (Sigma). The staining was performed in duplicate for 15 min in the dark at room temperature, and slime production was then observed using confocal scanning laser microscopy (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany).

Quantitative polymerase chain reaction (qPCR) detection of the changes in dltB and icaA transcription levels. To prepare the samples of total RNA, single colonies of *S. aureus* standard strain ATCC 25923 were inoculated in 5 ml TSB medium, into which 8 μg/ml hBD-3, 1 μg/ml vancomycin or 0.25 μg/ml clindamycin were added. *S. aureus* bacteria, which were adhered to the surface of the plate at 6 h and encased in a biofilm at 24 h, were collected and centrifuged at 14,000 g for 10 min. The bacteria were then resuspended in TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA), and subjected to high-speed shaking following the addition of special abrasive.

The subsequent procedures of RNA extraction were conducted in accordance with the manufacturer's instructions (Invitrogen Life Technologies). The total RNA was examined on agarose gel, which demonstrated that the total RNA extracted from different phases treated with hBD-3, vancomycin and clindamycin were of high quality.

The mRNA levels of *dlt* and *ica* genes were measured using qPCR. The extracted RNAs were retro-transcribed to cDNAs in the presence of random primers (Table I) using reverse transcriptase AMV in accordance with the manufacturer's instructions (Takara, Kyoto, Japan). L-lactate dehydrogenase (Ldh) was used as an endogenous control. qPCR was performed in triplicate using SYBR Green Master mix (Takara) on an ABI 9700 system (Invitrogen Life Technologies). The PCR conditions were as follows: 95˚C for 15 sec, and 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec. The values were normalized to the expression of the test gene using the $2^{-\Delta\Delta}$CT method (31). The threshold cycles (CTs) were recorded for all of the samples for the target gene and the endogenous control *Ldh*. A melting curve analysis was performed for each run. The relative gene expression of the target gene was calculated as ΔCT, determined by subtracting the CT of the *Ldh* gene from the CT of the target gene. Differential expression of the target gene is demonstrated as -ΔΔCT, determined by subtracting the ΔCT (mean value) of the test samples from that of the control samples.

Table I. Base sequences and predicted sizes of polymerase chain reaction products for *dltB*, *icaA* and *Ldh* specific oligonucleotide primers used in the present study.

Target gene	Oligonucleotide sequence (5'-3')	Product size (bp)
dltB	F: GTGGACATCAGATTCACTTCC R: ATAGAACCATCACGAATTTCC	118
icaA	F: GGCTGCGGTAACTGGCAATCC R: CTTGCCAGTTAAAGATTGGGC	121
Ldh	F: TTGGTGACGCAATGGACT R: AGTTTCGCCAGGCTTTCT	137

Ldh, L-lactate dehydrogenase.

Image and statistical analyses. Biofilm images were captured using Image-Pro Plus Version 6.0 (Media Cybernetics, Bethesda, MD, USA). The slime-stained area and the integrated optical density were measured. The data are expressed as the mean ± standard deviation. The χ^2 test and t-test were performed with SPSS 17.0 software (SPSS, Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of hBD-3, vancomycin and clindamycin on S. aureus biofilm formation. As indicated from the areas of slime generated from single-cell colonies determined via Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA) processing, it was identified that following 6 h of treatment, hBD-3, vancomycin and clindamycin were associated with significant increases in the secretion of slime by *S. aureus*, and the area of each experimental group was larger and notably different from that of the control group ($P<0.05$; Fig. 1 and 2). A total of 24 h following incubation with hBD-3, vancomycin or clindamycin, the areas of *S. aureus* biofilms in the three experimental groups decreased significantly relative to that of the control group ($P<0.05$; Fig. 2).

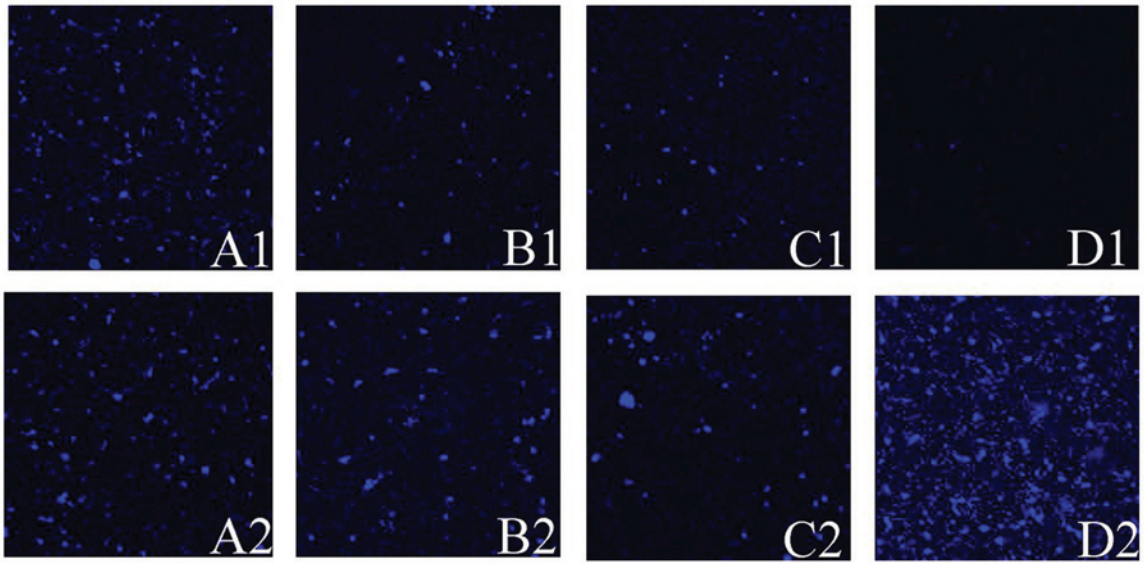

Figure 1. *S. aureus* biofilm formation following (1) 6 h and (2) 24 h incubation. The blue fluorescent stain is *S. aureus* secretion of bacterial biofilm polysaccharide protein complexes. (A) Biofilm formation under human β-defensin 3 treatment; (B) biofilm formation under vancomycin; (C) biofilm formation under clindamycin; (D) biofilm formation of the control group. Calcofluor white stain. Scale bar=40 μm. *S. aureus*; *Staphylococcus aureus*.

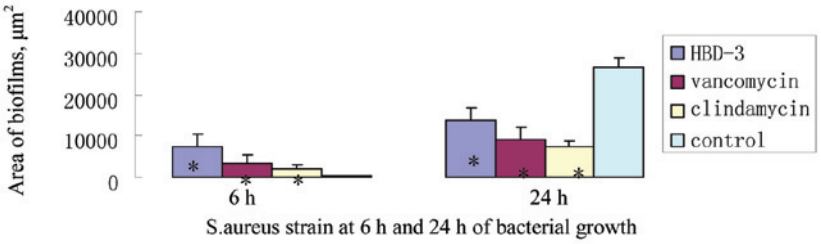

Figure 2. Effects of hBD-3, vancomycin and clindamycin on the stained areas of biofilms. Following 6 h of treatment, hBD-3, vancomycin and clindamycin were all able to significantly stimulate the secretion of slime by *S. aureus*. At 24 h following treatment, the areas of *S. aureus* biofilms in the three experimental groups decreased significantly compared with that of the control group. Data are expressed as mean ± standard deviation. *P<0.05, compared with control. hBD-3, human β-defensin 3; *S. aureus*; *Staphylococcus aureus*.

Effects of hBD-3, vancomycin and clindamycin on transcription levels of dltB and icaA. qPCR was performed to detect the effects of hBD-3, vancomycin or clindamycin on the transcription levels of the *dltB* gene in *S. aureus* strain ATCC 25923, which adhered to a surface at 6 h and were encased in a biofilm at 24 h. The total RNA was examined on an agarose gel, which demonstrated that the total RNA extracted from different phages treated with hBD-3, vancomycin and clindamycin was of a high quality (Fig. 3).

The melting and qPCR amplification curves were used to verify the quality of qPCR and the expression levels of *dltB* and *Ldh* (Fig. 4A and B). The results demonstrated that compared with the control group, incubation with hBD-3 caused no significant change in the transcription level of the *dltB* gene in biofilms at 6 and 24 h of bacterial growth. The transcription levels of the *dltB* gene in the bacterial biofilms incubated with either vancomycin or clindamycin were significantly elevated at 24 h (P<0.05; Fig. 4C).

Since the *icaADBC* genes share a common promoter, the present study aimed to detect the transcription of *icaA* to represent the transcription level of the *ica* operon in *S. aureus* biofilms. The melting and qPCR amplification curves indicated the quality of the qPCR and the expression levels of *icaA* and

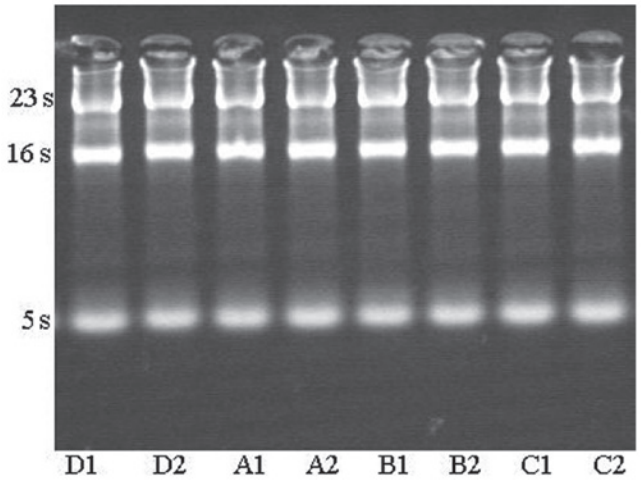

Figure 3. Gel electrophoresis results of total RNA extracted from different phases treated with (A) hBD-3, (B) vancomycin and (C) clindamycin following (1) 6 h and (2) 24 h. (D) Control. hBD-3, human β-defensin 3.

Ldh (Fig. 5A and B). The surface-adherent bacteria incubated with hBD-3 for 6 h had a higher *icaA* transcription level than the control group (P<0.05; Fig. 5C). This effect lasted, as the

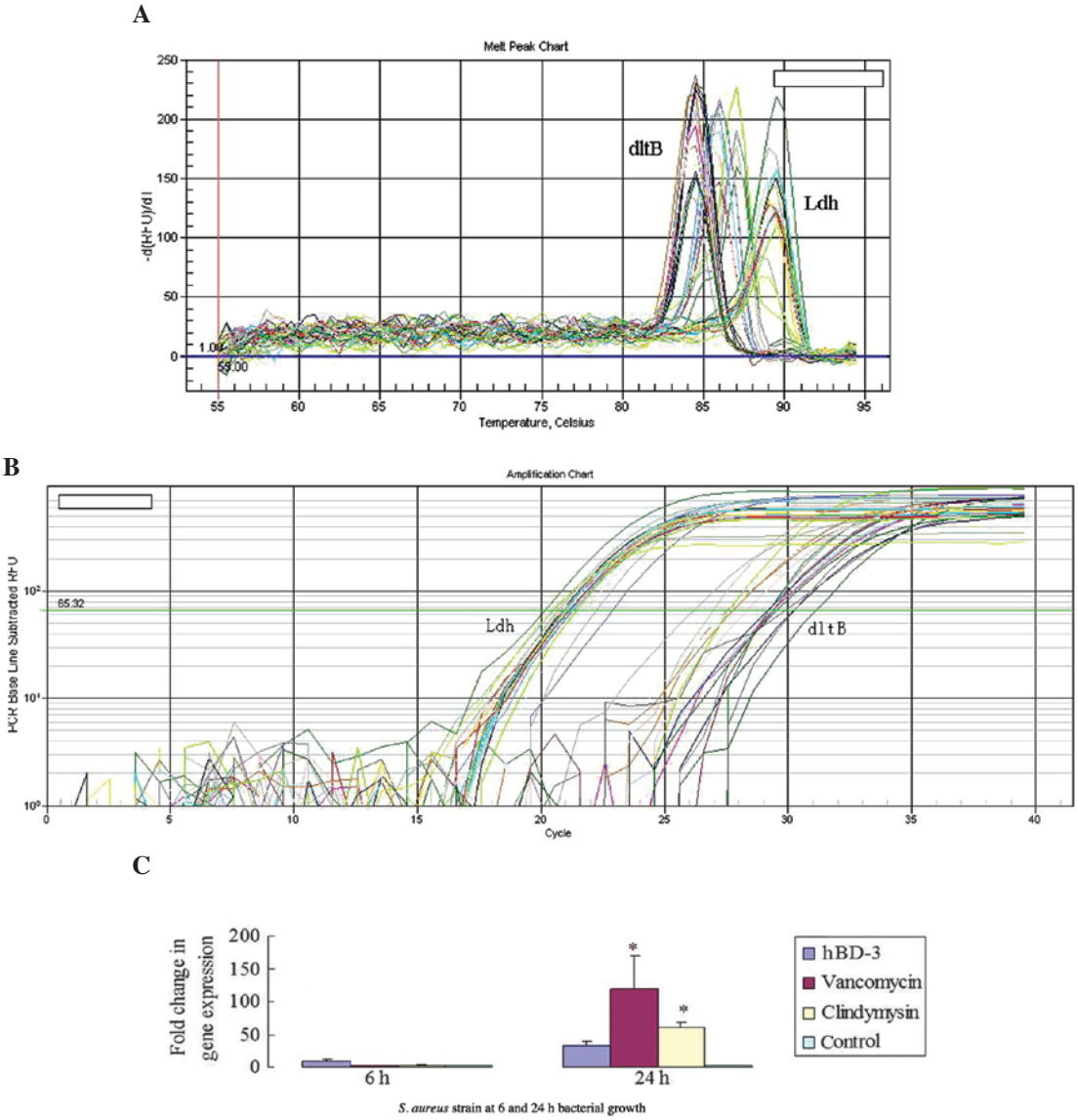

Figure 4. Effects of hBD-3, vancomycin and clindamycin on the transcription levels of *dltB* in *S. aureus*. (A) Melting curves for *dltB* and *Ldh* transcription levels following treatment with hBD-3, vancomycin and clindamycin. (B) The amplification curves of qPCR for *dltB* and *Ldh* transcription levels following treatment with hBD-3, vancomycin and clindamycin. (C) qPCR results for the effects of hBD-3, vancomycin, and clindamycin on transcriptional levels of *dltB* in *S. aureus*. *P<0.05 compared with control. hBD-3, human β-defensin 3; *S. aureus*; *Staphylococcus aureus*; qPCR, quantitative polymerase chain reaction; *Ldh*, L-lactate dehydrogenase.

ica transcription levels remained elevated significantly at 24 h (P<0.05). The *icaA* transcription levels marginally increased in the surface-adherent bacteria incubated with vancomycin and clindamycin at 6 h (P>0.05) and enhanced significantly at 24 h (P<0.05; Fig. 5C).

Discussion

In the present study, the antimicrobials hBD-3, vancomycin and clindamycin were selected to examine their effects on *S. aureus* biofilm formation. The progression from the initial adhesion of bacteria to a surface to the formation of biofilms is a dynamic process (32). The results revealed that all of the antimicrobials promoted the secretion of EPS by the bacteria during the initial adhesion stage, each led to significantly attenuated biofilm formation in the biofilm formation stage. However, the data revealed that the underlying regulatory mechanisms of hBD-3, vancomycin and clindamycin on the attenuation of biofilm formation are not the same. Vancomycin and clindamycin induced a moderate increase in *icaA* transcription during bacterial adhesion, and such induction was significantly more pronounced during biofilm formation compared with the untreated control. By contrast, hBD-3 stimulated *icaA* upregulation throughout the entire process, which suggests a complex regulatory function for hBD-3 in biofilm formation.

The *dltABCD* operon is the predominant functional gene cluster that regulates *S. aureus* adhesion, and is capable of markedly modifying surface charges on the teichoic acid molecules that are attached to the cell wall of the bacteria (33). These modifications allow the bacteria to bind to a bare polymer surface through hydrophobic interactions and initiate the process of biofilm formation. The *dlt* operon of *S. aureus* may be regulated by cations (34) or respond to cationic anti-

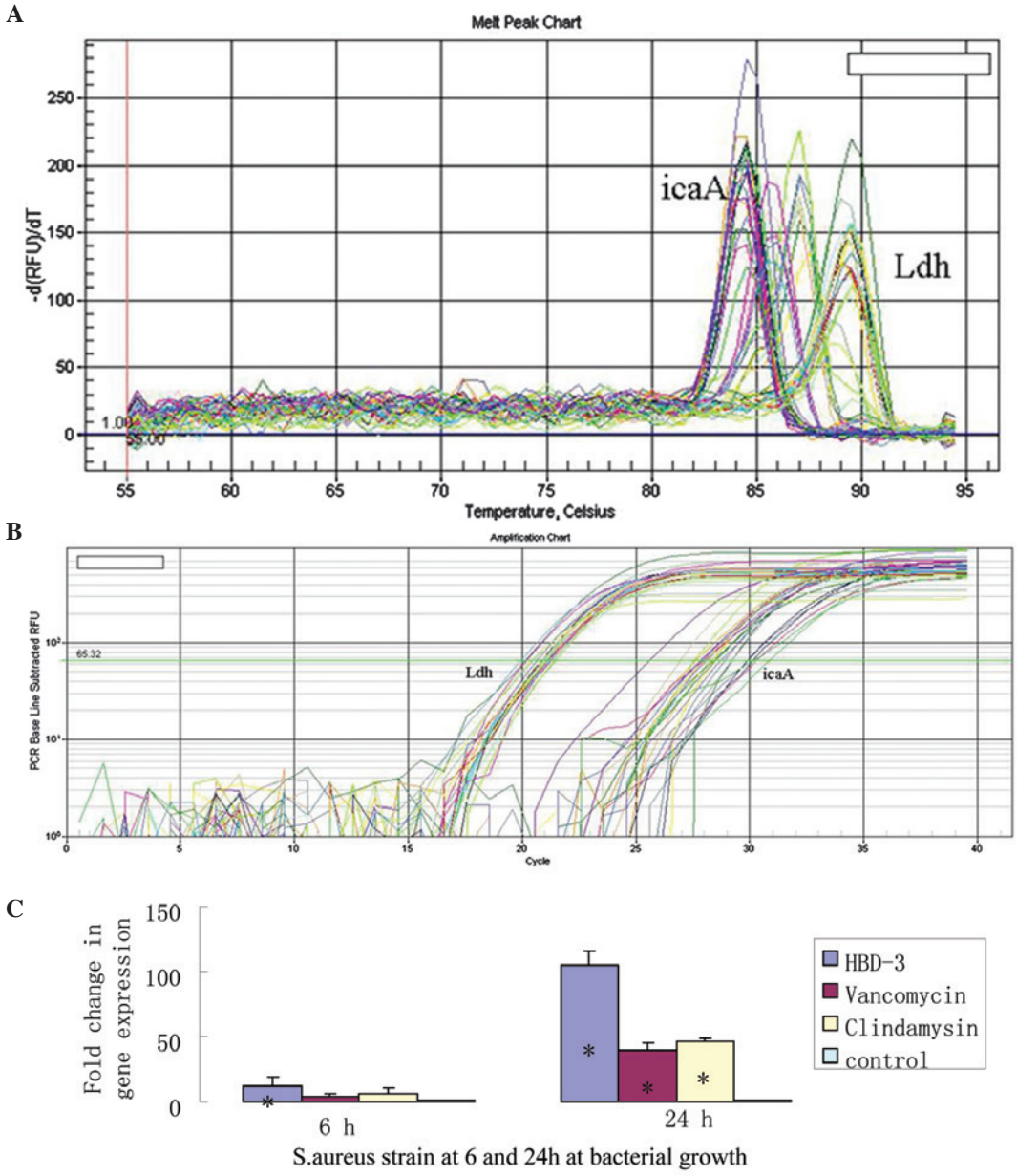

Figure 5. Effects of hBD-3, vancomycin and clindamycin on transcription levels of *icaA* in *S. aureus*. (A) Melting curves for *icaA* and *Ldh* transcription levels following treatment with hBD-3, vancomycin and clindamycin. (B) The amplification curves of qPCR for *icaA* gene and *Ldh* gene for effects of hBD-3, vancomycin and clindamycin. (C) qPCR results for the effects of hBD-3, vancomycin and clindamycin on transcription levels of *icaA* in *S. aureus*. $^{*}P<0.05$, compared with control. hBD-3, human β-defensin 3; *S. aureus*; *Staphylococcus aureus*; qPCR, quantitative PCR; Ldh, L-lactate dehydrogenase.

microbial peptides through the graRS regulatory system, and has a key role in bacterial resistance to cationic antimicrobial peptides (29,35-37). The present study demonstrated that vancomycin and clindamycin significantly induced the upregulation of *dltB* transcription in biofilms. However, unlike these antibiotics and other cationic antimicrobial peptides, hBD-3 did not have a significant affect on the transcription level of the *dltB* gene during either bacterial adhesion or biofilm formation. Previous studies have reported similar findings concerning the effects of hBD-3 on planktonic *S. aureus* (36,38), however to the best of our knowledge, the present study was the first to demonstrate the role of hBD-3 on the *S. aureus dlt* operon in biofilm formation, which is the phenotype that causes the majority of clinically refractory infections. Further studies are required to elucidate the underlying differences in the inhibitory mechanisms among hBD-3, vancomycin and clindamycin on biofilm formation.

The formation of the *S. aureus* biofilm is a complex process, and external factors differ in their effects on signal transduction mechanisms. In the present study, vancomycin and clindamycin induced sustained expression of the *dlt* and *ica* genes, which have key roles in biofilm formation. Consequently, vancomycin and clindamycin may be harnessed to induce biofilm formation. Attenuated biofilm formation in bacteria treated with vancomycin or clindamycin may be attributable to their bactericidal action that may have led to an absolute reduction in the number of bacteria and consequential decline in the area of biofilms. By contrast, hBD-3 exhibited

notably more complicated effects on the target biofilm-related genes. It had no affect on the *dlt* operon, despite a significant upregulation of the *ica* operon in the adhesion and biofilm formation stages. This result provides genetic evidence that hBD-3 has a different role in *S. aureus* biofilm formation from that of vancomycin and clindamycin. Biofilm formation is an important mechanism for antibiotic resistance of *S. aureus*, and *dlt* genes have also been implicated in the resistance of *S. aureus* (39,40). Therefore, the present study may also provide clinically useful information for understanding and thus controlling antibiotic resistance of *S. aureus*.

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