The Two-Component Signal Transduction System ArlRS Regulates *Staphylococcus epidermidis* Biofilm Formation in an ica-Dependent Manner

Yang Wu¹, Jiaxue Wang¹, Tao Xu¹, Jingshan Liu¹, Wendi Yu², Qiang Lou¹, Tao Zhu¹, Nianan He³, Haijing Ben¹, Jian Hu¹, Friedrich Götz², Di Qu*¹

¹ Key Laboratory of Medical Molecular Virology of the Ministry of Education and Ministry of Public Health, Institute of Medical Microbiology and Institute of Biomedical Sciences, Shanghai Medical College of Fudan University, Shanghai, P.R. China, ² Microbial Genetics, University of Tübingen, Tübingen, Germany, ³ Department of Ultrasound, Zhongshan Hospital of Fudan University, Shanghai, China

Abstract

Due to its ability to form biofilms on medical devices, *Staphylococcus epidermidis* has emerged as a major pathogen of nosocomial infections. In this study, we investigated the role of the two-component signal transduction system ArlRS in regulating *S. epidermidis* biofilm formation. An ArlRS-deficient mutant, WW06, was constructed using *S. epidermidis* strain 1457 as a parental strain. Although the growth curve of WW06 was similar to that of SE1457, the mutant strain was unable to form biofilms in vitro. In a rabbit subcutaneous infection model, sterile disks made of polymeric materials were implanted subcutaneously following with inoculation of WW06 or SE1457. The viable bacteria cells of WW06 recovered from biofilms on the embedded disks were much lower than that of SE1457. Complementation of ArlRS genes expression from plasmid in WW06 restored biofilm-forming phenotype both in vivo and in vitro. WW06 maintained its ability to adhere to initial attachment. Transcription levels of several genes involved in biofilm formation, including *icaADBC*, sigB, and sarA, were decreased in WW06, compared to SE1457; and *icaR* expression was increased in WW06, detected by real-time reverse-transcription PCR. The biofilm-forming phenotype was restored by overexpressing *icaADBC* in WW06 but not by overexpressing *sigB*, indicating that ArlRS regulates biofilm formation through the regulation of *icaADBC*. Gel shift assay showed that ArlR can bind to the promoter region of the *ica* operon. In conclusion, ArlRS regulates *S. epidermidis* biofilm formation in an ica-dependent manner, distinct from its role in *S. aureus*.

Introduction

*Staphylococcus epidermidis* is an opportunistic pathogen that normally colonizes human skin and mucosal surfaces. Over the past two decades, *S. epidermidis* has emerged as a major pathogen of nosocomial infections, particularly infections involving indwelling medical device [1–3]. *S. epidermidis* pathogenesis is associated with its ability to colonize polymer surfaces to form multilayered biofilms, which impair the efficacy of antibiotic treatments and serve to protect the bacteria from the host immune system [4,5]. Staphylococcal biofilm formation is a complicated process that is regulated by multiple regulatory factors including SigB, agr, SarA, and two-component signal transduction systems (TCSs) [6–10]. TCSs mediate a diverse range of adaptive responses to environmental stresses and play a vital role in bacterial pathogenesis [11–14]. In *S. epidermidis*, whereas the TCSs Agr, LytSR and SacRS are known to be involved in biofilm formation [15–17], the role of the ArlRS TCS remains unclear.

The ArlRS TCS was first identified in *Staphylococcus aureus* [18,19]. ArlRS strikingly modifies the extracellular proteolytic activity of *S. aureus* and is a regulator of virulence gene expression. Mutations in either arlR or arlS increase the production of Protein A, α-toxin, β-hemolysin, lipase, coagulase and serine protease. It was suggested that ArlRS may interact with both agr and sarA regulatory loci to modulate the virulence regulation network. A recent study found ArlRS positively regulate *S. aureus* capsule formation in a sigma B dependent manner [20]. Transcriptional profile analysis showed that in *S. aureus*, ArlRS positively regulated virulence factor genes such as *sdrC*, *sdrD* and *sdrE*, and functioned as a repressor of several toxin genes including *lukD*, *lukE*, *phlC* and *hlgC* [21]. Furthermore, ArlRS was found to be involved in *S. aureus* biofilm formation. An *arlS* gene transposition mutant of *S. aureus* clinical strain MT23142 exhibited an increased ability to form biofilms on polymer surfaces, resulting from altered bacterial autolysis and peptidoglycan hydrolyase activity [18]. Deletion of the *arlRS* locus in *S. aureus* clinical strain 15961 enhanced initial cell
Table 1. Plasmids and bacterial strains.

| Plasmids    | Description                                                                 |
|-------------|-----------------------------------------------------------------------------|
| pBT2        | temperature-sensitive E.coli-Staphylococcus shuttle vector                   |
| pTXicaADBC  | icaADBC cloned in pTX15, a xylose inducible plasmid                          |
| pTXsigB     | sigB cloned in pTX15                                                        |
| pCNarS      | ars cloned in pCN51, a Cd²⁺ inducible plasmid                               |
| pCNarRS     | arlRS cloned in pCN51                                                       |

| Bacterial strains | Description                                      |
|-------------------|--------------------------------------------------|
| RP62A             | a standard strain of S. epidermidis, biofilm positive |
| ATCC12228         | a standard strain of S. epidermidis, biofilm negative   |
| SE1457            | a clinical strain of S. epidermidis, biofilm positive    |
| WW06              | a arlS gene deletion mutant of SE1457, in which ArlR cannot be translated |
| ParSRS            | WW06 complemented with the plasmid pCNarS |
| pPsigB            | WW06 complemented with the plasmid pTXsigB |
| picaADBC          | WW06 complemented with the plasmid pTXicaADBC |
| ΔicaC             | ΔicaC gene deletion mutant of SE1457 |
| ΔatlE             | ΔatlE gene deletion mutant of SE1457 |
| icacPrS           | ΔicaC complemented with the plasmid pCNarRS |

doi:10.1371/journal.pone.0040041.t001

attachment and biofilm formation when cultured in Hussain-Hastings-White-modified medium (HHWm) [22].

Staphylococcal biofilm formation occurs in two steps: bacterial cells attach to the material surface; intercellular adhesion between bacterial cells forms multi-layered structures. In the second step, poly-N-acetylglucosamine (PNAG) in S. aureus or polysaccharide intercellular adhesin (PIA) in S. epidermidis synthesized by ica operon-encoded enzymes play a key role [5,23].

Toledo-Arana’s study demonstrated that although the accumulation of PNAG was increased in the icaADBC mutant of SE1457, ica-independent manner [22].

Here, we demonstrate for the first time that the ArlRS plays an important role in the regulation of S. epidermidis biofilm formation, and acts in an ica-independent manner distinct from the role of ArlRS in S. aureus biofilm formation.

Materials and Methods

Ethics Statement

All procedures performed on rabbits were conducted according to relevant national and international guidelines (the Regulations for the Administration of Affairs Concerning Experimental Animals, China, and the NIH Guide for the Care and Use of Laboratory Animals) and were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Medical College of Fudan University (IACUC Animal Project Number: 201109307-066).

Bacterial Strains, Plasmids and Growth Media

Bacterial strains and plasmids used in this study are listed in Table 1. B-Medium and Tryptic soy broth (TSB, Oxoid, Cambridge, UK) were used for S. epidermidis cultivation and biofilm formation. Media were supplemented with erythromycin (10 µg/ml), ampicillin (100 µg/ml), tetracycline (10 µg/ml) or chloramphenicol (10 µg/ml) when appropriate for purposes of selection.

Construction of arlS Gene Knockout Mutant and Complementation Strains

The arlS gene in S. epidermidis SE1457 was deleted using the temperature-sensitive vector pBT2 [24]. Briefly, an erythromycin-resistance cassette (ermB) was inserted into the pBT2 plasmid. Then the regions flanking arlS gene were amplified by PCR and inserted into pBT2-ermB. Primers for PCR were designed according to the genomic sequence of S. epidermidis RP62A (GenBank accession number CP000029). Primer sequences are listed in Table 2. The recombinant plasmid, designated pBT2-arlS, was transformed by electroporation into S. aureus strain RN4220 then into SE1457. A procedure for allelic displacement of the arlS gene was performed as previously described [24,25]. The mutant, designated WW06, was verified by PCR, RT-PCR and direct sequencing. Complementation studies were performed using a vector pCN51 [26] with a shine-dalgarno sequence plus either the arlS gene alone or arlRS genes. The resulting plasmids, pCN-arlS and pCN-arlRS, were transformed by electroporation into WW06, forming two complementary strains, ParS and ParRS, respectively.

Expression of Recombinant ArlR (rArlR) and Preparation of the anti-ArlR Antisera

The arlR gene was amplified from the genomic DNA of SE1457 and inserted into the vector pET-28a(+) to obtain the recombinant plasmid pET-arlR. The recombinant plasmid was then transformed into Escherichia coli BL21 (DE3). The expressed His-tagged ArlR protein was purified using the ProBond™ Purification System (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions.
Table 2. Primers used in this study.

| Name   | Primers                                                                 | Applications                  |
|--------|--------------------------------------------------------------------------|-------------------------------|
| ICAP-S | 5'-CTTCCAAATCTAATCCCTCCCTTT-3'                                          | icaADBC promoter              |
| ICAP-AS| 5'-TTTTTCCTACCTACTTTGCCTTGTTA-3'                                        | icaADBC promoter              |
| ICAA-S | 5'-GAGGGAAATCAACAAGCA-3'                                                | icaA fragment                 |
| ICAA-AS| 5'-AGGCACTAACAATCCAGCA-3'                                               | icaA fragment                 |
| U-S    | 5'-CCGAAATTCCTACGATGACAC-3'                                             | arlR (upstream)              |
| U-A    | 5'-CGGCGATCGGAGGTATGGGATGGTG-3'                                         | arlR (upstream)              |
| D-S    | 5'-AAGCTGAGGAATACACATACCCATACG-3'                                       | arlR (downstream)            |
| D-A    | 5'-CTGCTAGCTGTATGTTGAGGGGAAT-3'                                         | arlR (downstream)            |
| U'-S   | 5'-CGCTAAAGAATACCTGTT-3'                                                | arlR (upstream)              |
| U'-A   | 5'-GGCGTAGGGACCTTTTA-3'                                                 | arlR (upstream)              |
| D'-S   | 5'-CTATGGTAGCATTTAGGG-3'                                                | arlR (downstream)            |
| D'-A   | 5'-AAGTGTCAAAGGCTTACTG-3'                                               | arlR (downstream)            |
| erm-S  | 5'-CTATTGGATGATTTAGG-3'                                                 | erm8 cassette                 |
| erm-A  | 5'-GGGAGCCGGACCTTTTA-3'                                                 | erm8 cassette                 |
| arlS-S'| 5'-TCAACCTGAATCATTACACC-3'                                             | arlS amplification            |
| arlS-A'| 5'-GATGCTTATTAGGACGCTCAT-3'                                            | arlS amplification            |
| sarA-S | 5'-TTCAAATCAAAGTACGCT-3'                                                | sarA amplification            |
| sarA-AS| 5'-TCTCTCTCTTTATTTCTAC-3'                                               | sarA amplification            |
| sigB-S | 5'-TACCTGAAACAAATTACCAATG-3'                                            | sigB amplification            |
| sigB-AS| 5'-CACCTATTAGACACCAACATACC-3'                                           | sigB amplification            |
| arlS-S | 5'-ATTATTCAAGGTCACTCACA-3'                                              | arlS amplification            |
| arlS-A | 5'-ATCTCGGTATTTATGCTCACA-3'                                             | arlS amplification            |
| icaA-S | 5'-TTATGGTGTTATGCAAGGACGTGTT-3'                                         | icaA amplification Real-time PCR |
| icaA-AS| 5'-TCTCTCAGATTCATTGACTCATGC-3'                                         | icaA amplification Real-time PCR |
| luxS-S | 5'-GAGCAGATGATTAATGGT-3'                                                | luxS amplification            |
| luxS-AS| 5'-TGTGAGATTCATGTGTTT-3'                                                | luxS amplification            |
| rsbU-S | 5'-TCTCTCMTACATGTAC-3'                                                  | rsbU amplification            |
| rsbU-AS| 5'-ATAGGTCAAGGTTACATCCACA-3'                                            | rsbU amplification            |
| icaR-S | 5'-GGAGCAGCTGATTGGAATGACAT-3'                                           | icaR amplification            |
| icaR-AS| 5'-TCTTCAGACTATTTACCAACTGAC-3'                                         | icaR amplification            |
| gyrB-S | 5'-TATGATGGAGGGCTACATTGACA-3'                                           | gyrB amplification            |
| gyrB-AS| 5'-TATCAGCCACTTCCAAC-3'                                                 | gyrB amplification            |

Detection of ArlR Expression in SE1457 and WW06 by Western Blot

Overnight cultures of *S. epidermidis* strains SE1457 and WW06 were inoculated in 100 ml TSB and incubated at 37°C for 4 h. After centrifugation, pellets were washed three times with distilled water and resuspended in 2 ml phosphate buffered saline. After incubation with 100 μg lysostaphin at 37°C for 1 h, cells were ultrasonicated and centrifuged. Supernatants were assayed for total protein concentration by Bradford method. One microgram of bacterial cell extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes (GE-Whatman, Shanghai, China); purified rArlR (200 ng) was used as a control. Blots were probed with anti-rArlR antiserum (diluted 1:1000) followed by horseradish peroxidase-labeled goat anti-mouse IgG (diluted 1:2000) (Sangon, Shanghai, China). HRP activity was visualized via chemiluminescence detection using CSPD (Roche, Mannheim, Germany).

Bacterial Initial Attachment Assay

The initial attachment ability of *S. epidermidis* cells was tested as described by Heilmann et al. [27].

Semi-quantitative Detection of Biofilms

*S. epidermidis* biofilm formation was detected as previously described [28]. Overnight bacterial cultures grown in TSB were diluted 1:200, then transferred to 96-well polystyrene microtiter plates (200 μl per well). After incubation at 37°C for 24 h, wells were washed gently three times with PBS and stained with 2% crystal violet for 5 min. Next, the plate was rinsed under running water and resuspended in 2 ml phosphate buffered saline. After centrifugation, pellets were washed three times with distilled water and resuspended in 2 ml phosphate buffered saline. After incubation with 100 μg lysostaphin at 37°C for 1 h, cells were ultrasonicated and centrifuged. Supernatants were assayed for total protein concentration by Bradford method. One microgram of bacterial cell extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes (GE-Whatman, Shanghai, China); purified rArlR (200 ng) was used as a control. Blots were probed with anti-rArlR antiserum (diluted 1:1000) followed by horseradish peroxidase-labeled goat anti-mouse IgG (diluted 1:2000) (Sangon, Shanghai, China). HRP activity was visualized via chemiluminescence detection using CSPD (Roche, Mannheim, Germany).
**Figure 1. Effect of ArlRS deficiency on S. epidermidis growth and biofilm formation in vitro.** (A) Semi-quantitative detection of biofilms formed by SE1457, WW06, ParlS and ParlRS over a 24 h period in 96-well polystyrene microtiter plates: The biofilms were stained with 2% crystal violet. (B) The crystal violet was dissolved in ethanol and the absorbance was determined at 570 nm. The mean value ± standard deviation for each strain is shown. (C) Growth curves of *S. epidermidis* strains: Overnight cultures of SE1457, WW06, ParlS and ParlRS were diluted to OD595 = 0.08 in 150 ml fresh TSB and incubated at 37°C with shaking. The value of OD595 was monitored hourly. The curves represent the means of three independent experiments with standard deviation (SD). *Staphylococcus epidermidis* strain ATCC12228, a non-biofilm forming control; RP62A, a biofilm positive control. doi:10.1371/journal.pone.0040041.g001

tap water, air-dried, and ethanol was used to dissolve the crystal violet; finally, absorbance was determined at 570 nm.

**Scanning Electron Microscope Observations of Biofilms**

Overnight cultures of *S. epidermidis* strains were diluted in 1:200 in TSB and added in 96-well plates containing fragments of a central vein catheter (ABLE, Guangdong, China). After incubation at 37°C for 24 h, catheter fragments were washed and fixed with 2.5% glutaric dialdehyde (pH 7.4), followed by a secondary fixation with 1% osmium tetroxide. Catheter fragments were then washed twice with distilled water, dehydrated in 70%, 95% and 100% ethanol, dried, fixed on cylindrical metal plates and gilded with an ion sputter (HCP-2, Hitachi, Tokyo, Japan). Catheter fragments were observed for biofilm formation using a scanning electron microscope (XL-30, Philips, Eindhoven, Netherlands). SEM micrographs were taken at x3000 and x10000 magnifications.

**Biofilm Formation of the arlRS Mutant in vivo**

Three New Zealand White female rabbits (2.0–2.5 kg) were anesthetized by intravenous injection with sodium pentobarbital (35 mg/kg of body weight). After the back of rabbit was denuded of fur, sterile polyethylene disks (1.0 cm diameter, 0.1 cm thickness, with 2 mm chimb) were implanted subcutaneously at four sites bilateral to the spine, as reported previously [29–31], three disks per site. About 10^9 *S. epidermidis* cells from overnight cultures of SE1457, WW06 and ParlRS were collected, resuspended in TSB and injected subcutaneously into different implantation site respectively, using fresh TSB as a negative control. Three days after, the rabbits were euthanized and the implants were removed. The biofilms were scraped from the disks and the viable bacteria (colony forming units, CFUs) were determined. Differences in log_{10} CFU counts among groups were assessed with one-way analysis of variance (ANOVA) tests using software SPSS version 11.5 (SPSS Inc., Chicago, IL, USA).

**Semi-quantitative Detection of Polysaccharide Intercellular Adhesin (PIA)**

PIA detection was performed as previously described [32] using wheat germ agglutinin (WGA) as a surrogate for anti-PIA antibody, using an icaC deletion mutant of SE1457 as a PIA negative control, and strain RP62A as a PIA positive control. Overnight cultures of SE1457, WW06, RP62A and ΔicaC in TSB supplemented with 0.5% glucose were diluted to an OD595 of 0.07 with fresh media, added into sterile 6-well cell cultures plates (6 ml/well) (Nunc, Roskilde, Denmark), and incubated at 37°C under static conditions. After 24 h incubation, cultures and the attached biofilms were transferred into 2 ml tubes and centrifuged. To prepare cell surface extracts for PIA detection, pellets were resuspended in 0.5 M ethylenediaminetetraacetic acid, pH 8.0 (3 ml per gram wet weight), followed by heating at 100°C for 5 min. After centrifugation, 40 μl of the supernatant was mixed with 10 μl of proteinase K (20 mg/ml) and incubated at 37°C for 3 h, followed by heating at 100°C for 10 min. Different dilutions of the extracts were transferred to a nitrocellulose membrane, using a 96-well dot blot system (Biotometra, Goettingen, Germany). Membranes were blocked with 3% bovine serum albumin and incubated with 2 μg/ml HRP-labeled wheat germ agglutinin (WGA-HRP conjugate, Lectinotest Laboratory, Ukraine) for 1 h. HRP activity was visualized by chromogenic detection using 4-Chloro-1-Naphthol.

**Analysis of Transcriptional Levels of Biofilm-related Genes in WW06**

To define the arlRS regulon, transcriptional levels of biofilm-related genes, icaADBC, icaR, rshU, sigB, luxS and sarA in the arlRS-deficient strain WW06 and the wild-type parent strain SE1457 were analyzed by RT-qPCR analysis using the ABI 7500 real-time PCR system. Gene-specific primers (Table 2) were designed to yield approximately 100 bp specific products; the housekeeping gene gyrB was used as an endogenous control. All samples were analyzed in triplicate and normalized against gyrB gene expression.

**Electrophoretic Mobility Shift Assay (EMSA)**

Interaction of the recombinant ArlR and the icaADBC promoter was analyzed by EMSA using the DIG Gel Shift Kit (Roche). rArlR was phosphorylated prior to gel shift reaction by incubating rArlR with 50 mM acetyl phosphate for 1 h. The DNA fragment upstream icaADBC (ICAP, 216 bp) was amplified and linked with digoxin-labeled dd-UTP. The resulting DNA fragment, Dig-ICAP, was used for EMSA in native PAGE. Lanes 1–2 were loaded with 0.8 ng Dig-ICAP alone and 0.8 ng Dig-ICAP mixed with 2 μg rArlR, respectively. In lanes 3–5, increasing amounts of unlabeled ICAP (12.5, 62.5, 125 fold increase in the amount of Dig-ICAP in lane 2, respectively) was added as a specific competitor to the mixture of Dig-ICAP and rArlR. In lane 6, 100 ng of a 405 bp icaA fragment was added as a nonspecific competitor. Primers used for to amplify partial icaA gene and ICAP are listed in Table 2. The DNA fragments were transferred to positively charged nylon membranes (Roche) by electro-blotting and visualized by an enzyme immunoassay following the manufacturer’s instruction.

**Cell Autolysis Assay**

Autolysis assays for *S. epidermidis* strains were performed as previously described [33]. Strains SE1457, WW06, ParlRS and ΔatlE were cultured in TSB containing 1 M NaCl at 37°C to exponential phase (OD_{600} = 0.7). Additionally, WW06 and ParlRS were cultured in TSB containing 1 M NaCl and 2 μM GdCl_{2}. Bacterial cells in 50 ml cultures were harvested by centrifugation, washed twice with ice-cold water and resuspended in 50 ml of 0.05 M Tris/HCl (pH 7.2) containing 0.05% (v/v) Triton X-100. Cells were then incubated at 30°C with shaking (200 rpm), and OD_{500} was measured at 30 min intervals.
Role of ArlRS in SE Biofilm Formation
Results

Construction of an arlRS Gene-deficient Mutant Strain of S. epidermidis

In S. epidermidis strain SE1457, the TCS arlRS consists of two genes, arlS and arlR, which share the same promoter and overlap by 4 bp. To investigate the function of arlRS in S. epidermidis biofilm formation, an ArlRS deficient mutant was constructed by replacing the arlS gene with an erythromycin-resistant cassette in the biofilm-forming SE1457 strain. The mutant was designated WW06. Western blot analysis of the whole cell extracts from WW06 using antiserum against the recombinant ArlR (rArlR, Figure S1) showed that no ArlR was expressed in WW06 (Figure S2). Thus, WW06 was an ArlRS-deficient mutant.

ArlRS Deficiency in S. epidermidis Abolishing Biofilm Formation in vitro

The impact of ArlRS mutation on biofilm formation was investigated using both polystyrene microtiter plates and intravenous catheters. In the microtiter plates, biofilm formation in WW06 was dramatically decreased (OD570 = 0.41 ± 0.04), compared to its wild-type counterpart (OD570 = 3.06 ± 0.47) (Figure 1 A, B). To rule out the possibility of a polar effect, two complementation strains, ParlS and ParlRS, were constructed using plasmids expressing arlS gene and arlRS genes respectively. While ParlRS partially restored biofilm-forming ability (OD570 = 1.84 ± 0.00), the biofilm-forming ability of ParlS (OD570 = 0.45 ± 0.02) was similar to that of WW06.

To further investigate the ability of S. epidermidis strains WW06, ParlS, ParlRS, and SE1457 to form biofilms, these strains were incubated with catheter fragments in 96-well plates at 37 °C for 24 h and biofilm formation morphology was assessed by SEM. The wild-type strain SE1457 generated a compact, thick biofilm on the catheter surface, whereas the mutant WW06 formed only a few bacterial cell clusters. ParlRS formed a flat biofilm on the catheter, whereas ParlS formed only a small number of microcolonies similar to WW06 (Figure 2).

In terms of bacterial growth, the growth curves of SE1457, WW06, ParlS and ParlRS were similar (Figure 1C). Additionally, in an assay measuring initial adherence, the numbers of attached cells of SE1457 and WW06 were 3.64 × 10^4/cm^2 and 3.58 × 10^4/cm^2, respectively, which showed that mutation of arlRS exhibited little effect on bacterial growth and primary attachment.

Influence of ArlRS Deficiency on S. epidermidis Biofilm Formation in vivo

To investigate the effect of ArlRS deficiency on S. epidermidis biofilm formation in vivo, a biofilm infected rabbit model was established. The biofilm formation of SE1457, WW06 and ParlRS on the implanted disks in vivo was observed under an optical microscope and evaluated by determination of viable cells recovered from biofilms. SE1457 formed much thicker biofilms than WW06 on the implanted disks. The log_{10}CFU/disk number of viable cells recovered from the biofilms of the arlRS mutant WW06 was significantly lower than that of SE1457 (3.08 ± 0.42 vs 4.97 ± 0.11, P<0.001) (Figure 3). The log_{10} number of viable CFU in biofilm of the complementation strain ParlRS was partially restored, which is higher than that of WW06 (4.02 ± 0.25 vs 3.08 ± 0.42, P<0.001).

Figure 2. Biofilms formed on catheter fragments surfaces observed by scanning electron microscope (SEM). S. epidermidis strains WW06, ParlS, ParlRS and SE1457 were incubated with central vein catheter fragments in 96-well plates. After incubation at 37 °C for 24 h, biofilm formed on the surface of the catheter fragments were observed under a transmission electron microscope (XL-30, Philips). Images were obtained at different magnifications (x3000, x10000) for biofilms formed by SE1457 (a, b), WW06 (c, d), ParlS (e, f) and ParlRS(g, h).
doi:10.1371/journal.pone.0040041.g002

Figure 3. Influence of ArlRS deficiency on viable S. epidermidis cells recovery from the implanted disks in the rabbit model. Biofilms of SE1457, WW06 and ParlRS were formed in vivo on sterile polyethylene disks that were implanted subcutaneously in three New Zealand White female rabbits. Three days after infection, the implants were removed. Biofilms were scraped from the disks and CFUs of the viable bacteria recovered from the biofilms were determined and expressed as mean ± standard deviation. Asterisks denote statistically significant difference, P<0.001.
doi:10.1371/journal.pone.0040041.g003
Triton X-100 induced autolysis of the strains was determined by measuring the change in the value of OD595 at 30 min intervals; results are expressed as lysis percentages. Percent lysis was calculated as follows: 
\[
[\text{OD}_{0} - \text{OD}_{\text{at time of interest}}]/\text{OD}_{0} \times 100\%.
\]
Experiments were carried out three times independently. doi:10.1371/journal.pone.0040041.g004

ArI RS Mutation Affecting Bacterial Autolysis
To investigate the effect of ArI RS on bacterial autolysis, SE1457, WW06 and ParI RS were incubated with 0.1% Triton X-100 for 2.5 h and the percent autolysis was calculated. The lytic percentage of WW06 reached 97.83%, which was significantly higher than the 55.36% lytic percentage for SE1457. The autolysis rate of ParI RS was similar to that of WW06 when cultured in media without cadmium chloride (CdCl2); however, when cultured with 2 mM CdCl2 to induce arI RS expression, the lytic percentage of ParI RS was similar to wild-type (Figure 4).

Transcriptional Analysis of Biofilm-related Genes in WW06
To define the arI RS regulon and the role of ArI RS in S. epidermidis biofilm formation, total RNAs from the arI RS-deficient strain WW06 and the wild-type parent strain SE1457 in mid-log growth phase (4 h) were extracted, and transcriptional levels of several biofilm-related genes, rsbU, sigB, sarA, icaR, luxS and icaA, were analyzed by RT-qPCR, using the housekeeping gene gyrB as an internal control. The time point was selected because arI RS transcription in SE1457 reached peak levels at 4 h [34].

Transcription of the biofilm-related genes rsbU, sigB, sarA and icaA in WW06 were found to be down-regulated 14.5 fold, 8.4 fold, 4.3 fold and 7.5 fold, as compared to SE1457, whereas icaC transcription was upregulated 2.4 fold and expression of the luxS gene was similar (Figure 5A). As ArI RS is a DNA binding protein, we hypothesized that ArI RS may bind directly to the promoter region of icaADBC to modulate transcription of the operon. Thus, EMSA were performed with digoxin-labeled DNA directly upstream of icaADBC (ICAP, 216 bp) and recombinant ArI R (rArI R). As shown in Figure 7, rArI R bound to Dig-ICAP and formed a DNA-protein complex, shifting Dig-ICAP behind (lane 2) compared to Dig-ICAP alone (lane 1). When increasing amounts of unlabeled ICAP was added as a specific competitor, more free Dig-ICAP was observed (lanes 3–5), while when a nonspecific competitor, icaC fragments, were added, no Dig-ICAP was shifted back (lane 6). It indicated a potential mechanism for ArI RS regulation of ica expression.

To further investigate the role of IcaR in regulating WW06 biofilm formation, effect of ethanol or sodium chloride on biofilm production of WW06 and SE1457 was examined. Addition of 4% NaCl to BM media increased SE1457 biofilm formation.

Figure 4. Effect of arI RS mutation on S. epidermidis Triton X-100-induced autolysis. SE1457, WW06, ParI RS and icaD were cultured in TSB containing 1 M NaCl at 37 °C to exponential phase (OD590 = 0.7). WW06 and ParI RS were also cultured in TSB containing 1 M NaCl and 2 μM CdCl2. Triton X-100 induced autolysis of the strains was determined by measuring the change in the value of OD595 at 30 min intervals; results are expressed as lysis percentages. Percent lysis was calculated as follows: [OD595-OD0]/OD0 X 100%. Experiments were carried out three times independently.
(approximately 3.1 fold), while had no effect on that of WW06, shown in Figure 8. SE1457 showed enhanced biofilm production in BM media supplemented with either 2% or 4% ethanol (1.8 fold or 2.3 fold, respectively), whereas ethanol showed very limited effect on WW06 biofilm formation (1.3 fold 1.4 fold increase).

Discussion

The two-component signal transduction system ArlRS is a global regulator of virulence genes in S. aureus [19], modulating the extracellular proteolytic activity, capsule formation, biofilm formation and production of Protein A, α-toxin, β-hemolysin, lipase, coagulase and serine protease. However, the function of ArlRS in S. epidermidis was unclear. Thus, we investigated the role of ArlRS in S. epidermidis, an opportunistic pathogen that lacks many virulence genes [35] but can form biofilms as a major pathogenic factor. First, an ArlRS deficient mutant WW06 was constructed. WW06 showed dramatically decreased biofilms formation in vitro and in vivo. No obvious difference was found in either growth curves or initial attachment of WW06 and SE1457. Extracellular DNA has been reported to play an important role in biofilm formation [36–38]. In this study, however, the quantity of cDNA in the unwashed biofilms of the two strains was similar (data not shown), although the arlRS mutant strain WW06 exhibited a significantly higher autolysis rate than SE1457. Thus, we hypothesize that ArlRS modulates S. epidermidis biofilm formation by regulating the expression of the biofilm-related genes involved in the step of intercellular adhesion.

Many factors are involved in the second step of biofilm formation, e.g. polysaccharide intercellular adhesin (PIA) [39], accumulation associated protein (Aap) [40,41]. Extracellular
matrix binding protein (Embp) [42], etc. The ica (intercellular adhesin) operon plays a crucial role by synthesizing PIA that is a main component of the extracellular polymeric substances (EPS). The ica operon is composed of the icaR (regulatory) gene and icaADBC (biosynthesis) genes. The icaR gene is located upstream of the icaADBC and is transcribed divergently, encoding a transcriptional repressor that negatively regulates icaADBC transcription in both S. aureus and S. epidermidis. Staphylococcal biofilms formation involves ica-dependent and ica-independent pathways [43]. In the present study, we have demonstrated that ArlRS activates icaADBC transcription, leading to PIA production and S. epidermidis biofilm formation. First, RT-qPCR analysis showed that icaADBC expression was significantly reduced in the ArlRS-deficient mutant WW06. Secondly, whereas semi-quantitative PIA detection revealed strong PIA production in the wild-type strain SE1457, little or no PIA expression was detected in WW06 mutant. Furthermore, complementation of icaADBC in WW06 restored its ability to form biofilms, whereas overexpression of arlRS in the icaC deletion mutant of SE1457 (ΔicaC) did not recover biofilm formation. Together, these results indicate that icaADBC is the downstream biofilm-related effector in the ArlRS regulation pathway. Moreover, EMSA demonstrated that recombinant ArlR binds to the 216-bp region located upstream of icaADBC. Overall,
it reveals that ArlRS regulates *S. epidermidis* biofilm formation in an ica-dependent manner.

Previous studies have showed that ArlRS promotes biofilm formation in *S. aureus* strain ISP794 and strain 15981 which is capable of producing strong biofilms in TSB while unable to produce a biofilm in HHWm [18,22]. Fournier, B. et, al. have found that an *arlS* gene transposition mutant (BF16) of *S. aureus* clinical strain ISP794 exhibited an increased ability to form biofilms on polymer surfaces, resulting from enhanced bacterial autolysis and altered peptidoglycan hydrolase activity. In the present study, *arlRS* mutation in *S. epidermidis* resulted in increased Triton X-100 induced bacterial autolysis, but decreased biofilm formation. The difference of altered biofilm formation between *arlS* mutant of *S. aureus* (BF16) and *arlRS* mutant of *S. epidermidis* (WW06) could be explained that due to different methods used for gene mutation (transposon insertion in the former and gene knock out in the latter), ArlR is absent in WW06 while present and may have function in BF16. Toledo-Arana, A. et, al. have reported that deletion of the *arlRS* locus in *S. aureus* clinical strain 15981 enhanced initial cell attachment and provoked accumulation of PNAG and biofilm formation when cultured in Hussain-Hastings-White-modified medium (HHWm). However, in HHWm the biofilm formation of the *arlRS* and *icaADBC* operon double mutant increased at the same level as the *arlRS* mutant, suggesting ArlRS is involved in early stage of strain 15981’s biofilm development in HHWm in an ica-independent manner. Interestingly, mutation of *arlRS* in *S. epidermidis* resulted in decreased biofilm formation in TSB in an ica-dependent manner. Bioinformatics analysis showed

---

**Figure 7. Binding of ArlR to the icaADBC promoter.** Lanes were loaded as follows: lane 1, 0.8 ng Dig-ICAP alone; lane 2, 0.8 ng Dig-ICAP and 2 μg rArlR; lanes 3–5, Dig-ICAP, rArlR and increasing amounts of unlabeled ICAP (12.5, 62.5, 125 fold increase in Dig-ICAP, respectively); and lane 6, Dig-ICAP, rArlR and 100 ng of a 405 bp *icaA* fragment. The DIG-labeled DNA fragments were transferred to positively charged nylon membranes and visualized by an enzyme immunoassay using anti-Digoxigenin-AP, Fab-fragments and the chemiluminescent substrate CSPD. Chemiluminescent signals were recorded on X-ray film. DIG-ICAP, digoxin-labeled icaADBC promoter region; ICAP, unlabeled icaADBC promoter region; *icaA*, icaA gene fragment.

**Figure 8. Effect of ethanol or sodium chloride on biofilm formation of SE1457 and WW06.** (A) Semi-quantitative detection of biofilms formed by SE1457, WW06 in BM supplemented with 4% NaCl, 2% EtOH or 4% EtOH in 96-well polystyrene microtiter plates: The biofilms were stained with 2% crystal violet. (B) The crystal violet was dissolved in ethanol and the absorbance was determined at 570 nm. The mean value ± standard deviation for each strain is shown.

---

**Role of ArlRS in SE Biofilm Formation**

**Figure 7. Binding of ArlR to the icaADBC promoter.** Lanes were loaded as follows: lane 1, 0.8 ng Dig-ICAP alone; lane 2, 0.8 ng Dig-ICAP and 2 μg rArlR; lanes 3–5, Dig-ICAP, rArlR and increasing amounts of unlabeled ICAP (12.5, 62.5, 125 fold increase in Dig-ICAP, respectively); and lane 6, Dig-ICAP, rArlR and 100 ng of a 405 bp *icaA* fragment. The DIG-labeled DNA fragments were transferred to positively charged nylon membranes and visualized by an enzyme immunoassay using anti-Digoxigenin-AP, Fab-fragments and the chemiluminescent substrate CSPD. Chemiluminescent signals were recorded on X-ray film. DIG-ICAP, digoxin-labeled icaADBC promoter region; ICAP, unlabeled icaADBC promoter region; *icaA*, icaA gene fragment.

**Figure 8. Effect of ethanol or sodium chloride on biofilm formation of SE1457 and WW06.** (A) Semi-quantitative detection of biofilms formed by SE1457, WW06 in BM supplemented with 4% NaCl, 2% EtOH or 4% EtOH in 96-well polystyrene microtiter plates: The biofilms were stained with 2% crystal violet. (B) The crystal violet was dissolved in ethanol and the absorbance was determined at 570 nm. The mean value ± standard deviation for each strain is shown.

---

**Figure 7. Binding of ArlR to the icaADBC promoter.** Lanes were loaded as follows: lane 1, 0.8 ng Dig-ICAP alone; lane 2, 0.8 ng Dig-ICAP and 2 μg rArlR; lanes 3–5, Dig-ICAP, rArlR and increasing amounts of unlabeled ICAP (12.5, 62.5, 125 fold increase in Dig-ICAP, respectively); and lane 6, Dig-ICAP, rArlR and 100 ng of a 405 bp *icaA* fragment. The DIG-labeled DNA fragments were transferred to positively charged nylon membranes and visualized by an enzyme immunoassay using anti-Digoxigenin-AP, Fab-fragments and the chemiluminescent substrate CSPD. Chemiluminescent signals were recorded on X-ray film. DIG-ICAP, digoxin-labeled icaADBC promoter region; ICAP, unlabeled icaADBC promoter region; *icaA*, icaA gene fragment.

**Figure 8. Effect of ethanol or sodium chloride on biofilm formation of SE1457 and WW06.** (A) Semi-quantitative detection of biofilms formed by SE1457, WW06 in BM supplemented with 4% NaCl, 2% EtOH or 4% EtOH in 96-well polystyrene microtiter plates: The biofilms were stained with 2% crystal violet. (B) The crystal violet was dissolved in ethanol and the absorbance was determined at 570 nm. The mean value ± standard deviation for each strain is shown.
that arlR and ars genes are conserved in *S. epidermidis* (over 95% nucleotide sequence identities among strains RP62A, SE1457 and ATCC12228) while *S. epidermidis* strain RP62A and *S. aureus* strain COL [44] share relatively high nucleotide sequence identities of *arlR* and *ars* genes (74.8% and 70%, respectively). It appears ArlRS is involved in different mechanisms that regulate biofilm formation in the closely related species.

Current findings indicate that *icaADBC* expression in *S. epidermidis* is regulated by multiple regulatory factors including RsbU, SigB, IcaR, SarA and LuxS[8,45–48]. Knobloch, J.K. et, al. reported that inactivation of *rsbU* gene (a positive regulator of *sigB*) in *S. epidermidis* was associated with both a reduction in PIA levels and biofilm formation, and the regulation of biofilm formation by *rsbU* is SigB dependent. Handke, L.D. et, al. reported that *ica* transcription is down-regulated in the *sigB* mutant [45]. The regulation of *ica* transcription by SigB was found to be mediated through the upregulation of IcaR expression. IcaR is a transcriptional repressor of *ica* [49]. The *icaR* gene deletion in *S. epidermidis* strain O-47 [50] led to increased PIA synthesis. In the present study, decreased transcription of *rsbU*, *sarA* and *icaADBC*, and increased *ica* expression are observed in the *arlRS* mutant WW06, compared with SE1457. The results are consistent with the previous findings, indicating a potential mechanism that the regulation of *ica* expression by ArlRS may be mediated by IcaR. The global transcriptional regulator SarA plays an important role in staphylococcal biofilm formation [9,51–54]. Transcription of *alcA* was decreased by about 4.3 fold in the WW06 mutant, which is consistent with previous findings that SarA positively regulates *ica* operon expression in an IcaR-independent manner; its role in ArlRS regulation pathway requires further investigation. As a quorum sensing system, LuxS repressed *S. epidermidis* biofilm formation through a cell-cell signaling mechanism based on autoinducer 2 secretions. LuxS affects biofilm formation by altering production of PIA via transcriptional regulation of the *ica* operon. In the present study, transcriptional levels of *luxS* in the SE1457 and WW06 were similar, indicating *luxS* may not play a role in the ArlRS regulation pathway in *S. epidermidis*.

Taken together, it reveals that in *S. epidermidis*, the ArlRS two-component signal transduction system is involved in the modulation of bacterial autolysis and in the regulation of biofilm formation an *ica*-dependent manner, which is distinct from the role of ArlRS in *S. aureus* and deserves further study.

**Supporting Information**

**Figure S1** Purification of the recombinant ArlR by affinity chromatography. The arlR gene was cloned in the expression vector pET28a(+) to form pET-arlR, which was transformed in the *E. coli* BL21(DE3+). After induction with 0.4 mM IPTG for 12 h, the recombinant ArlR was purified by affinity chromatography. M: Protein molecular weight marker; rArlR: the purified recombinant ArlR (about 27 kDa). (TIF)

**Figure S2** Detection of ArlR expression in SE1457 and WW06 by Western blot. The lanes were loaded with 200 ng purified recombinant ArlR (rArlR), 1 µg bacterial cells extract of WW06, and 1 µg bacterial cells extract of SE1457, respectively. Antiserum from the mouse immunized with 5 µg recombinant ArlR was diluted by 1:1000. An unspecific protein band with a lower molecular mass was present in each lane. (TIF)

**Acknowledgments**

We thank Prof. J.M. van Dijl (University of Groningen, Netherlands) for the gift of the plasmid pCN51.

**Author Contributions**

Conceived and designed the experiments: YW FG DQ. Performed the experiments: YW TX JXW JRL. Analyzed the data: YW TX JRL. Wrote the paper: YW DQ. Provided critical technical support: WQY QL TZ NAH HJB JH.

**References**

1. Otto M (2009) *Staphylococcus epidermidis*—the ‘accidental’ pathogen. Nat Rev Microbiol 7: 555–567.
2. Ziebuhr W, Henning S, Erkart M, Kranzler H, Batzilla C, et al. (2006) Nosocomial infections by *Staphylococcus epidermidis*: how a commensal bacterium turns into a pathogen. Int J Antimicrob Agents 28 Suppl 1: S14–20.
3. von Eiff C, Proctor RA, Peters G (2001) Coagulase-negative staphylococci. J Infect Dis 184: 1187–1203.
4. Vuong C, Otto M (2002) *Staphylococcus epidermidis* infections. Microbes Infect 4: 481–489.
5. Otto M (2008) Staphylococcal biofilms. Curr Top Microbiol Immunol 322: 207–226.
6. Knobloch JK, Jager S, Hensekotte MA, Rohde H, Mack D (2004) RsbU-dependent regulation of *Staphylococcus epidermidis* biofilm formation is mediated via the alternative sigma factor sigmaB by repression of the negative regulator gene *is242*. Infect Immun 72: 3828–3834.
7. Lauderdale KJ, Bolis BR, Cheung AL, Horswill AR (2009) Interconnections between Sigma B, agr, and proteolytic activity in *Staphylococcus aureus* biofilm maturation. Infect Immun 77: 1623–1635.
8. Tommo MA, Marit M, Valle J, Mannua GC, Cheung AL, et al. (2003) SarA is an essential positive regulator of *Staphylococcus epidermidis* biofilm development. J Bacteriol 185: 2348–2356.
9. Sharma-Kuinikel BK, Mann EE, Ahn JS, Kuechenmeister LJ, Dunman PM, et al. (2009) The *Staphylococcus aureus* LytSR two-component regulatory system affects biofilm 191–4775.
10. Dubhar S, Benea GC, Poupel O, Maudel T (2007) New insights into the WallA/WalR (YveG/YveF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in *Staphylococcus aureus*. J Bacteriol 189: 8257–8269.
11. Skerker JM, Prasad MS, Perchuk BS, Biondi EG, Laub MT (2005) Two-component signal transduction pathways regulating growth and cell cycle progression in a bacterium: a system-level analysis. PLoS Biol 3: e334.
12. Bader MW, Sanowar S, Daley ME, Schneider AR, Cho U, et al. (2005) Recognition of antimicrobial peptides by a bacterial sensor kinase. Cell 122: 461–472.
13. Stock AM, Robinson VL, Goudreau PN (2000) Two-component signal transduction. Annu Rev Biochem 69: 183–215.
14. Hoch JA (2000) Two-component and phosphorelay signal transduction. Curr Opin Microbiol 3: 165–170.
15. Zhu T, Lou Q, Wu Y, Hu J, Yu F, et al. (2010) Impact of the *Staphylococcus epidermidis* LytSR two-component regulatory system on murine hyaluronidase activity, pyruvate utilization and global transcriptional profile. BMC Microbiol 10: 287.
16. Vuong C, Gerke C, Somerville GA, Fischer ER, Otto M (2003) Quorum-sensing control of biofilm factors in *Staphylococcus epidermidis*. J Infect Dis 188: 706–718.
17. Lou Q, Zhu T, Hu J, Ben H, Yang J, et al. (2011) Role of the SarRS two-component regulatory system in *Staphylococcus epidermidis* autoinduction and biofilm formation. BMC Microbiol 11: 146.
18. Fournier B, Hooper DC (2000) A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic activity of *Staphylococcus aureus*. J Bacteriol 182: 3953–3964.
19. Fournier B, Kler A, Rapoport G (2001) The two-component system ArlRS-ArlR is a regulator of virulence gene expression in *Staphylococcus aureus*. Mol Microbiol 41: 247–261.
20. Meier S, Goerke C, Wolz C, Stahl K, Homereva D, et al. (2007) sigmaB and the sigmaB-dependent arlRS and yabJ-yabG loci affect capsule formation in *Staphylococcus aureus*. Infect Immun 75: 4562–4571.
21. Liang X, Zheng L, Lianweir C, Lamford D, Holmes D, et al. (2005) Global regulation of gene expression by ArlRS, a two-component signal transduction regulatory system of *Staphylococcus aureus*. J Bacteriol 187: 5496–5492.
22. Toledo-Arana A, Merino N, Vergara-Irigaray M, Debarbouille M, Penades JR, et al. (2000) *Staphylococcus aureus* develops an alternative, σ~independent~ biofilm in the absence of the arlRS two-component system. J Bacteriol 187: 5318–5329.
23. Crumpton SE, Gerke C, Schnell NF, Nichols WV, Gotz F (1999) The intercellular adhesion (ica) locus is present in Staphylococcus aureus and is required for biofilm formation. Infect Immun 67: 5427–5433.

24. Bruckner R (1997) Gene replacement in Staphylococcus carnosus and Staphylococcus xylosus. FEMS Microbiol Lett 151: 1–8.

25. Vuong C, Gotz F, Otto M (2000) Construction and characterization of an agr deletion mutant of Staphylococcus epidermidis. Infect Immun 68: 1048–1053.

26. Charpentier E, Anton AI, Barry F, Alfonso B, Fang Y, et al. (2004) Novel cassette-based shuttle vector system for gram-positive bacteria. Appl Environ Microbiol 70: 6076–6085.

27. Heilmann C, Hussain M, Peters G, Gotz F (1997) Evidence for autolysin-mediated primary attachment of Staphylococcus epidermidis to a polystyrene surface. Mol Microbiol 24: 1013–1024.

28. Christensen GD, Simpson WA, Younger J, Baddour LM, Barrett FF, et al. (1985) Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol 22: 996–1006.

29. Carmen JC, Roeder BL, Nelson JL, Beckstead BL, Runyan CM, et al. (2004) Ultrasonically enhanced vancomycin activity against Staphylococcus epidermidis biofilms in vivo. J Biomater Appl 18: 237–245.

30. Rediske AM, Roeder BL, Nelson JL, Robinson RL, Schaalje GB, et al. (1999) Pulsed ultrasound enhances the killing of Escherichia coli biofilms by aminoglycoside antibiotics in vivo. Antimicrob Agents Chemother 44: 771–772.

31. Rediske AM, Roeder BL, Brown MK, Nelson JL, Robinson RL, et al. (1999) Ultrasonic enhancement of antibiotic action on Escherichia coli biofilms: an in vivo model. Antimicrob Agents Chemother 43: 1211–1214.

32. Gerke C, Kraft A, Sussmuth R, Schweitzer O, Gotz F (1998) Characterization of the N-acetylglucosaminyltransferase activity involved in the biosynthesis of the Staphylococcus epidermidis polysaccharide intercellular adhesin. J Biol Chem 273: 18586–18593.

33. BrunsKILL EW, Bayles KW (1996) Identification and molecular characterization of a putative regulatory locus that affects autolysis in Staphylococcus aureus. J Bacteriol 178: 611–618.

34. Liu JR, Sun ZP, Xu T, Wu Y, Li DW, et al. (2009) Detection of ArlR expression in different growth phases of Staphylococcus epidermidis. Journal of Microbes and Infection 4: 92–96.

35. Zhang YQ, Ren SX, Li HL, Wang XY, Fu G, et al. (2003) Genome-based analysis of virulence genes in a non-biofilm-forming Staphylococcus epidermidis strain (ATCC 12220). Mol Microbiol 49: 1577–1593.

36. Qin Z, Ou Y, Yang L, Zhu Y, Tolker-Nielsen T, et al. (2007) Role of autolysin-mediated DNA release in biofilm formation of Staphylococcus epidermidis. Microbiology 153: 2083–2092.

37. Allesen-Holm M, Barlen KB, Yang L, Klausen M, Webb JS, et al. (2006) A characterization of DNA release in Pseudomonas aeruginosa cultures and biofilms. Mol Microbiol 59: 1114–1128.

38. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS (2002) Extracellular DNA required for bacterial biofilm formation. Science 295: 1487.

39. Mack D, Fischer W, Krotscheck A, Leopold K, Hartmann R, et al. (1996) The intercellular adhesin involved in biofilm accumulation of Staphylococcus epidermidis is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. J Bacteriol 178: 175–183.

40. Hussain M, Herrmann M, von Eiff C, Perdreau-Remington F, Peters G (1997) A 140-kilodalton extracellular protein is essential for the accumulation of Staphylococcus epidermidis strains on surfaces. Infect Immun 65: 518–524.

41. Rohde H, Burdelski C, Bartsch K, Hussain M, Buck F, et al. (2005) Induction of Staphylococcus epidermidis biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. Mol Microbiol 55: 1883–1895.

42. Christner M, Franke GC, Schommer NW, Wendi U, Wegert K, et al. (2010) The giant extracellular matrix-binding protein of Staphylococcus epidermidis mediates biofilm accumulation and attachment to fibronectin. Mol Microbiol 75: 187–207.

43. O’Gara JP (2007) ica and beyond: biofilm mechanisms and regulation in Staphylococcus epidermidis and Staphylococcus aureus. FEMS Microbiol Lett 270: 179–188.

44. Gill SR, Feuts DE, Archer GL, Mongolin EF, Deboy RT, et al. (2005) Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant Staphylococcus aureus strain and a biofilm-producing methicillin-resistant Staphylococcus epidermidis strain. J Bacteriol 187: 2426–2438.

45. Haufler LD, Slater SR, Conlon KM, O’Donnell ST, Olson ME, et al. (2007) SigmaB and SarA independently regulate polysaccharide intercellular adhesin production in Staphylococcus epidermidis. Can J Microbiol 53: 82–91.

46. Conlon KM, Humphreys H, O’Gara JP (2002) icaR encodes a transcriptional repressor involved in environmental regulation of ica operon expression and biofilm formation in Staphylococcus epidermidis. J Bacteriol 184: 4400–4408.

47. Xu L, Li H, Vuong C, Vadyvaloo V, Wang J, et al. (2006) Role of the luxS quorum-sensing system in biofilm formation and virulence of Staphylococcus epidermidis. Infect Immun 74: 488–496.

48. Krobloch JK, Bartsch K, Sabotke A, Rohde H, Feucht HH, et al. (2001) Biofilm formation by Staphylococcus epidermidis depends on functional RsbU, an activator of the sigH operon: differential activation mechanisms due to ethanol and salt stress. J Bacteriol 183: 2624–2633.

49. Jeun WY, Ko TP, Liu CL, Gao KY, Lin CL, et al. (2008) Crystal structure of EcaR, a repressor of the TreR family implicated in biofilm formation in Staphylococcus epidermidis. Nucleic Acids Res 36: 1567–1577.

50. Gotz F (2002) Staphylococcus and biofilms. Mol Microbiol 43: 1367–1378.

51. Beeken KE, Mraz LN, Griffin LM, Zielinska AK, Shaw LN, et al. (2010) Epistatic relationships between sarA and agr in Staphylococcus aureus biofilm formation. PLoS One 5: e10790.

52. Tretonda MP, Manns AC, Cheung AL, Lasa I, Penades JR (2005) SarA positively controls bap-dependent biofilm formation in Staphylococcus aureus. J Bacteriol 187: 5790–5798.

53. Valle J, Toledo-Arana A, Berasain C, Ghigo JM, Amorena B, et al. (2003) SarA and not sigD is essential for biofilm development by Staphylococcus aureus. Mol Microbiol 48: 1073–1087.

54. Beeken KE, Blevins JS, Smeltzer MS (2003) Mutation of sarA in Staphylococcus aureus limits biofilm formation. Infect Immun 71: 4206–4211.