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Giant Extracellular Matrix Binding Protein Expression in *Staphylococcus epidermidis* is Regulated by Biofilm Formation and Osmotic Pressure

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Abstract

Staphylococcus epidermidis is an opportunistic bacterium that thrives as a commensal cutaneous organism and as a vascular pathogen. The *S. epidermidis* extracellular matrix binding protein (Embp) has been reported to be a virulence factor involved in colonization of medical device implants and subsequent biofilm formation. Here, we characterize the expression patterns of Embp in planktonic and biofilm cultures, as well as under high osmotic stresses that typify the commensal environment of the skin. *Embp* expression without osmotic stress was similar for planktonic and adherent cultures. Addition of osmotic stress via NaCl caused slight increases in *embp* expression in planktonic cultures. However, in adherent cultures a 100-fold increase in *embp* expression with NaCl versus controls occurred and coincided with altered biofilm morphology. Results suggest that the central role of Embp lies in commensal skin colonization, stabilizing the cell wall against osmotic stresses, rather than as a virulence factor promoting adhesion.

Introduction

Over half of hospital-acquired infections are associated with implanted devices with the case-to-fatality ratio of these infections as high as 40 % [1]. Even innocuous skin bacteria, such as *Staphylococcus epidermidis*, can adhere to implanted medical devices in the vasculature via specific and non-specific adhesion mechanisms. Biofilm colonies formed by adherent *S. epidermidis* enable the bacterium to evade both antibiotics and the host immune system, leading to infection, sepsis, and potentially death. Biofilms are microbial cell-produced mucilaginous matrices of extra-cellular DNA [2], proteins [3, 4], and polysaccharides [5]. The danger of biofilms arises from their secretion of virulence factors, their relatively large size within the vasculature, and the propensity for fragments to detach from the biofilm and occlude blood vessels downstream [6].

While expression of the *icaADBC* operon and its associated production of polysaccharide intracellular adhesin (PIA) is the most studied mechanism of *S. epidermidis* biofilm formation [7], a number of *S. epidermidis* strains lacking the *icaADBC* operon still possess the ability to form biofilms through alternative mechanisms. These alternative matrix

molecules include proteins, such as the extracellular matrix binding protein (Embp) and the accumulation associated protein (Aap), and extracellular DNA [2–4].

Embp is a giant 1,100-kDa protein localized to the cell wall of *S. epidermidis* with potential functional similarity to large homologous proteins produced in other pathogenic bacteria such as *Staphylococcus aureus* and *Abiotrophia defectiva*. In *A. defectiva*, the Emb protein has been identified as a major fibronectin (FN) adhesion protein [8]. In *S. aureus*, however, while the 1,000-kDa extracellular matrix binding homolog (Ebh) is able to bind weakly to FN, its major function is proposed to be structural support of the cell wall [9, 10]. *S. aureus* exhibits strong binding to FN through other protein receptors, FnbpA and FnbpB, while Ebh-negative *S. aureus* mutants are unable to withstand hyper-osmotic pressure shifts.

The *S. epidermidis* Embp has been reported to be involved in both specific binding to FN-coated surfaces and in subsequent biofilm formation by Christner et al. [4]. However, significant specific receptor:ligand-mediated adhesion of *S. epidermidis* to FN adsorbed on a variety of such surfaces has been contradicted by other works [11, 12]. Embp-mediated cell:cell adhesion properties elucidated by the Christner et al. work could serve other beneficial roles in biofilm producing strains of *S. epidermidis*. Consequently, there is a need to further characterize the expression and purpose of Embp in both suspended and adherent biofilm cultures.

Materials and Methods

Cell Culture and Sample Collection

Staphylococcus epidermidis strain 1457, isolated from a patient with catheter-related sepsis, and strain CV2 (an isogenic *agr*- deletion mutant derived from 1457) were generously provided by Dr. Michael Otto (NIH, Bethesda MD) [13, 14]. The biofilm-negative strain *S. epidermidis* 12228 was purchased from ATCC. *S. epidermidis* strains SCH1.2, SCH1.3, and SCH1.5, cultured from intravascular catheter infections, were provided by Dr. Xuan Qin (Seattle Children's Hospital) and further genotyped and characterized biochemically. All strains were kept in glycerol at -80°C until use, then cultured on 10 g/L tryptic soy broth (TSB) agar plates. A single colony was then transferred into TSB medium and grown overnight at 37°C . From this cell suspension, samples were diluted 1/50 into new medium for growth at 37°C either as batch planktonic cell cultures under shaking conditions (200 rpm waterbath shaker), or as batch biofilm cultures under static fluid (minimal mixing) conditions.

Planktonic cell growth was quantified by optical density at 600 nm (OD_{600}) and calibrated to approximate cell number (OD_{600} of 1.0 $\approx 10^9$ cells). Adherent biofilm cells were removed from substrata using a microultrasonic cell disruptor with ASI tip (Kontes Co., Vineland, NJ) operated at mild sonication (30 % maximum setting for 15 s) before measuring the optical density of the resultant cell suspension. For mRNA and protein expression studies, $\sim 10^9$ cells were collected from either planktonic or adherent cultures and immediately added to an equal volume of a 1:1 mixture of ice cold acetone and ethanol and stored at -80°C for at least twenty minutes or until further use.

$\Delta embp$ *S. epidermidis* 1457 Mutation

Creation of an *embp* deletion mutant ($\Delta embp$) was attempted by four separate methods. Homologous recombination using the pKOR1 shuttle vector was first attempted to remove the entire *embp* gene. Insertional inactivation was performed to interrupt the expression of *embp* by replacing the first 1,000 bp with an antibiotic cassette. A suicide vector (pCIV2) was used to prevent transmission of non-recombined plasmid to daughter cells. Finally, a temperature sensitive vector (pBT2) containing erythromycin resistance was used to prevent

embp expression. Summary of results related to these attempts is provided in the Supplementary Information.

Western Blotting

Proteins, expressed by planktonic or adherent cells, were collected by centrifuging 10^9 cells, rinsing twice in PBS (pH 7.4), followed by re-suspending cells in 0.5 ml Laemmli sample buffer and boiling at 95 °C for 5 min. Proteins were then separated by SDS-PAGE and analyzed via Western blot. Because of its large size, Embp migrates only into the first 2 mm of a 4–15 % acrylamide gel (BioRad, Carlsbad, CA) and must be transferred to the PVDF membrane without methanol while using high voltage (60 V max for 16 h). The membranes were blocked with 1 % BSA in TBS overnight and then probed for two hours with a 1/5,000 dilution of anti-rEmbp2588 antiserum donated by Dr. Holger Rodhe (Universitätsklinikum Hamburg-Eppendorf, Germany). Blots were rinsed and then incubated with a secondary alkaline phosphatase-conjugated goat anti-rabbit antibody (Pierce, Rockford, IL) at a dilution of 1/10,000, rinsed again, and detected by incubating with BCIP/NBT (MP Bio, Solon, OH).

Cell Lysis and mRNA Purification

Frozen cell suspensions of either planktonic or adherent cells were thawed on ice, centrifuged at $10,000\times g$ for 10 min at 4 °C, and the supernatant removed. Cell pellets were dried for five minutes on ice and then re-suspended in 500 μ l Tris-EDTA buffer (pH 7.5). The cell suspension was transferred to a Bio101 FastProtein™ Blue tube with Lysing Matrix B (1- μ m silica beads) and processed in a Mini-Beadbeater-16 for one minute, then cooled on ice for one minute. The process was repeated for a total of four homogenization cycles. Suspensions were centrifuged and the supernatant was removed to a new tube for RNA purification. RNA purification was performed using an RNeasy™ Mini Kit (QIAGEN) following the manufacturers protocol including the optional on-column RNase-free DNase I treatment. RNA purity was assessed on a UV spectrophotometer and samples with an OD_{280}/OD_{260} ratio between 1.8 and 2.2 were used for further analysis.

Two-Step Reverse Transcription Quantitative PCR

QIAGEN's QuantiTect™ RT Kit was used to synthesize cDNA for qRT-PCR according to the manufacturer's instructions with products stored at -20 °C until use (Table 1). qPCR was performed on cDNA using Quanti-Tect™ SYBR™ Green PCR Kit. Samples were assayed in triplicate and qPCR was performed on a Rotor-Gene 3000™ Real Time Thermal Cycler (Corbett Research/QIAGEN) followed by a melt curve of PCR products. Genomic DNA standards were amplified with each reaction to calculate the relative copy number of each gene of interest. A housekeeping gene encoding gunaylate kinase, *gmk*, was used as a reference control [15].

qRT-PCR Analysis

Initial analysis was performed on the Rotor-Gene™ RG-3000 (Corbett Life Sciences/QIAGEN, Valencia, CA) to insure that melt curves showed only one peak per primer set. Samples that failed to amplify were excluded from further analysis. $2^{-\Delta\Delta C_t}$ analysis was performed according to the method of Livak and Schmittgen [16]. Relative gene copy expression analysis was performed to determine the differences in expression between multiple genes being analyzed to compare across multiple times/conditions (as described in Supplementary Information).

Biofilm Staining and Confocal Microscopy

An overnight culture of *S. epidermidis* 1457 cells was rinsed, adjusted to a concentration of 5×10^4 cells/ml in PBS, and added to wells of a sterile 6-well polystyrene plate. The plate was then incubated at 37 °C for 1 h to allow cells to attach. The supernatant was then replaced with TSB or TSB + 1.5 M NaCl and plates were incubated for an additional 48 h. Biofilm samples were then removed with a cell scraper and washed with PBS. Non-specific binding was blocked by incubating the cell suspension in goat serum before antibody binding. Cell suspensions were then incubated in a 1:250 dilution of rabbit anti-rEmbp2588 antibody before being re-suspended in a 1:200× dilution of DyLight 488™-conjugated goat anti-rabbit secondary antibody solution (Pierce, Rockford, IL). SYTO 60 red fluorescent nucleic acid stain (Invitrogen, Carlsbad, CA) was used as counterstain. Cell suspensions were then filtered onto a 0.22-µm Cyclopore™ membrane (Whatman, Piscataway, NJ). Five random fields of each membrane were imaged using a 100× oil immersion lens on an epifluorescent microscope (Carl Zeiss Microimaging, Thornwood, NY). These images were analyzed by Image J software (NIH, Bethesda, MD) to determine % of cells staining for Embp from the total number of cells. Cells incubated with isotype matched rabbit anti-*Pseudomonas aeruginosa* as a negative control were also analyzed for each sample type to confirm specific binding of anti-rEmbp2588 to Embp.

Cultivation of biofilms under varying conditions of osmotic pressures was performed as above, but used 4-well Lab-Tek™ chamber slides (Thermo Scientific) instead of 6-well plates. After 48-h incubation, the supernatant was aspirated without disturbing the biofilms and the samples were incubated with Live/Dead BacLight™ bacterial stain (Invitrogen) for visualization. The medium chamber was then removed and biofilms samples were covered with coverslip for microscopy. All microscopic image acquisitions were carried out by Confocal Laser Scanning Microscopy (Nikon A1R-Si Confocal/RIRF imaging system, Melville, NY) with a 100× oil objective.

Biofilm Quantitation

COMSTAT software (Center for System Microbiology, Arne Heydorn, Oxford, England) [17], a powerful tool for classifying biofilm 3-D structure, was used to quantify the architecture of biofilms (defined by different COMSTAT-generated features of the three-dimensional biofilms). Biofilm thickness, roughness, and surface area to volume ratio were calculated for each of the biofilm conditions tested.

To determine the roughness coefficient, R , Eq. 1 was used:

$$R = \frac{1}{N} \sum_{i=1}^N \frac{|L_{fi} - \bar{L}_f|}{\bar{L}_f} \quad (1)$$

where L_{fi} is the i th individual biofilm thickness measurement, \bar{L}_f is the mean biofilm thickness, and N is the number of thickness measurements. Surface area-to-volume ratio is equivalent to the biofilm's surface area divided by the volume of the biofilm. Three independent experiments and three image stacks for each condition were analyzed to acquire mean values for each condition.

Statistical Analysis

Unpaired Student's T -tests were performed to determine significance between controls and sample types, as well as between osmotic conditions with and without NaCl supplementation.

Results and Discussion

Low Expression of *embp* Gene During Planktonic and Biofilm Cultures

We hypothesized that *embp* expression levels would be increased during events (either adhesion or biofilm formation) that required Embp. Thus, we quantified *embp* expression in *S. epidermidis* over time in both planktonic and biofilm cultures and compared this to *icaA* expression, a gene known to be involved in biofilm formation but not in initial adhesion. The *embp* gene exhibited only low levels of expression throughout both planktonic and biofilm cultures (Fig. 1a). This was in stark contrast to the expression patterns of the *icaA* gene (Fig. 1b), which exhibited low levels of expression throughout planktonic growth but were dramatically increased in biofilm cultures.

At these later stages in biofilm cultures, quorum sensing and environmental stress responses would have been activated, as cell density had peaked and cells would have exhausted nutrient sources or dissolved oxygen. *icaA* gene expression is known to be regulated by both quorum sensing (*LuxS*) and stress response mechanisms (*RsbU* and σ^B) [5, 18]. Since expression of *icaA*, but not *embp*, is significantly different under biofilm forming conditions, it would appear that *embp* is not under the same control as *icaA*. Indeed, Yao et al. [19] reported no difference between *embp* expression in 24-hr planktonic cultures as compared to 24-hr adherent cultures in their genome wide analysis of *S. epidermidis* 1457.

Embp Protein Expression

Western blots confirmed low level synthesis and production of Embp in cell wall extracts of various *S. epidermidis* strains (Fig. 2a). Biofilm-forming *S. epidermidis* (strain 1457), a non-biofilm forming cutaneous strain of *S. epidermidis* (ATCC 12228), and four separate clinical *S. epidermidis* strains isolated from catheter associated infections each produced Embp in TSB planktonic cultures although the clinical strains showed more variation in production. In addition, we confirmed that expression of Embp in *S. epidermidis* 1457 cells was not different over time compared to the CV2 strain (an isogenic *agr*- mutant) for planktonic or adherent biofilm cells at either 4 h (Fig. 2b) or 16 h (Fig. 2c) of culture. Samples of the only high MW (> 500 kDa) protein band visible in silver stained SDS-PAGE gels were sequenced at the University of Washington's Mass Spectrometry Center and verified that the giant protein detected in Western Blots was Embp. Because *agr* plays an important role in cell survival during stationary growth, we would have expected a difference in Embp expression in any one or more of these culture conditions (early vs. late, planktonic vs. static biofilm growth) had Embp been controlled by the accessory regulator gene [20]. However, this was not the case, indicating that Embp is likely to be regulated by unrelated mechanisms.

Osmotic Pressure Effects on *embp* Gene Expression

Since few changes in *embp* expression were seen comparing biofilm and planktonic cells, we hypothesized that *S. epidermidis* gene expression of *embp*, rather than being influenced by adhesion and/or biofilm formation would, instead, be altered under osmotic stresses. To determine the optimal concentration of NaCl for supplementation, a preliminary series of batch planktonic cultures were carried out at NaCl concentrations from 0 to 4 M NaCl (up to 23.2 % w/v NaCl). Figure 3a shows that *S. epidermidis* culture growth rates and extent were unaffected by NaCl concentrations < 1 M (5.8 % w/v NaCl), but growth rate and extent were severely inhibited at 2 M NaCl and cells did not grow > 4 M NaCl. Thus, subsequent osmotic stress experiments were carried out at 1.5 M NaCl to induce stress without severe growth inhibition.

To compare the gene expression of *embp* in the presence of osmotic stresses, cells were grown in planktonic and biofilm cultures using TSB supplemented with 1.5 M NaCl or TSB

alone. As seen in Fig. 3b, planktonic cultures of *S. epidermidis* had similar *embp* expression in TSB and in TSB + 1.5 M NaCl. However, in biofilm cultures, the effect of NaCl on *embp* expression is pronounced with *embp* gene expression causing up-regulation throughout the course of the biofilm culture. The expression of *embp* is 3-times higher in 1.5 M NaCl supplemented TSB at 4 h growth, continues to increase to 6-times higher at 12 h, and reaches 100-times greater expression in osmotically stressed conditions at 24 h growth versus TSB alone. These experiments show that *embp* expression is induced by *S. epidermidis* cell stress under high salt concentrations, but that the effect is limited to adherent cells only.

Osmotic Pressure Effects on Embp Expression and Biofilm Formation

Because the addition of 1.5 M NaCl led to an upregulated gene expression in biofilm cultures, we then quantified the protein expression and morphological characteristics of the cells and biofilm. The addition of 1.5 M NaCl to TSB resulted in significantly increased cell wall-localized Embp as determined by immunofluorescence staining of adherent growth (Fig. 4a). This osmotic stress yielded 30 and 50 % increases in Embp protein expression at 24 and 48 h, respectively. Controls with isotype-matched antibodies against *P. aeruginosa* and with goat sera alone confirmed that binding was specific to Embp (data not shown). Confocal images also indicated a distinct difference in biofilm morphology due to the induced osmotic stress. As seen in Fig. 4b, confocal micrographs show biofilms to be thicker and less dense in TSB supplemented with 1.5 M NaCl compared to TSB only controls. Live/dead staining revealed many more live cells grown in 1.5 M NaCl + TSB culture after 24 h growth compared to the TSB only cultures. In addition, the size of cells grown under hyperosmotic stress was increased compared to the TSB alone. Table 2 displays the biofilm thickness, roughness, and surface area-to-volume ratios of *S. epidermidis* biofilms accumulated under the two osmotic pressure conditions. Compared to the control without NaCl, all three variables increased significantly under the osmotic stress induced by 1.5 M NaCl yielding a thicker, more heterogeneous biofilm.

We also noticed marked macroscale differences in biofilms formed under high osmotic pressure conditions compared to the controls. Biofilms cultured in TSB supplemented with 1.5 M NaCl appeared much more filamentous and thicker than biofilm formed in TSB alone (Fig. 4c). Osmotically stressed biofilms appeared to adhere less to the polystyrene itself and were easily dislodged from the plate surface. Rather, these *S. epidermidis* cells were bound to each other by thick filaments. Individual cells grown under higher osmotic stress were larger than their control counterparts. This phenomena was also reported by Vijaranakul et al. [21] for *S. aureus* cells grown in high NaCl and KCl concentrations though not in those grown in the presence of glycerol or sucrose. These authors concluded that the high ionic strength of the salt solution and not the osmotic effect alone was responsible for the larger size of the cells. Kuroda et al. [10] postulated that the Ebh protein of *S. aureus* might be involved in tolerance to hyperosmotic pressures as seen by commensal organisms on human skin. Extreme fluctuations in water activity and salt concentrations are common due to interactions between the skin's sebaceous secretions, and the external environment [10, 22]. Our results suggest that there is a role for Embp in cell survival under such osmotic stresses.

Conclusion

In this work, we have characterized the expression patterns of the *embp* gene in planktonic and biofilm cultures, and in response to changes in NaCl-induced osmotic stress. The *embp* gene and protein were found to be expressed by various clinical and laboratory *S. epidermidis* strains at low levels in both planktonic and biofilm cultures cultivated under normal growth conditions. *Emb p* gene and protein regulation was not correlated to either *icaA* or *agr* expression, genes related to biofilm and stationary phase growth, respectively.

However, under biofilm culture conditions of sustained hyperosmotic stress, *embp* gene and protein expression were highly upregulated, and both cell and biofilm morphology were substantially altered at the micro- and macroscales. Results suggest that *embp* is upregulated by increases in osmotic pressure and may be involved in protein mediated resistance to plasmolysis due to osmotic stresses, such as those found on the skin. This upregulation results in increased *Embp* production, cell size, and survival, and correlates with altered biofilm morphology indicating a possible role for commensal biofilm maintenance through cell:cell adhesion properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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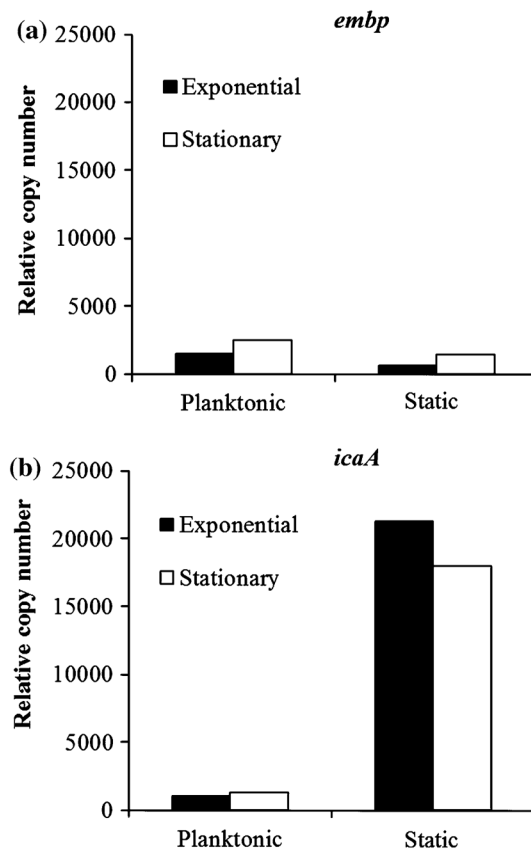


Fig. 1. Relative copy number for *embp* (a) and *icaA* (b) during exponential (4 h) and stationary (24 h) growth in planktonic and adherent cultures. Gene expression determined by qRT-PCR and normalized to guanylate kinase (*gmk*) housekeeping gene

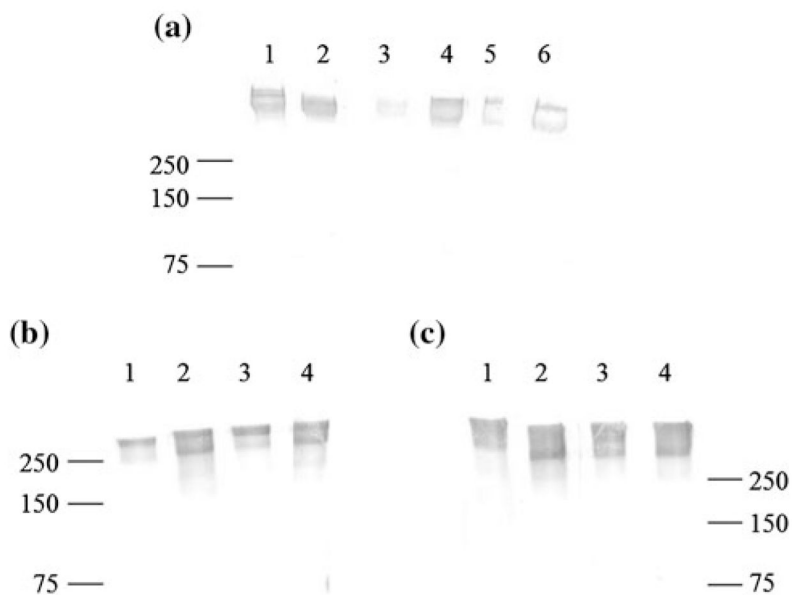


Fig. 2.

Western Blot of rabbit-antiEmb2588 binding to Embp expressed in various *S. epidermidis* strains and conditions. **a** Embp expression by *S. epidermidis* laboratory strains 1457 (*lane 1*) and 12228 (*lane 2*) plus four separate clinical isolates of *S. epidermidis* (*lanes 3–6*). **b** 4 h and **c** 16 h Embp expression by *S. epidermidis* strain 1457 (*lanes 1 and 2*) and *agr*- strain CV2 (*lanes 3, 4*) in planktonic cultures (*lanes 1 and 3*) versus adherent cultures (*lanes 2 and 4*). Molecular weight standards (in kDa) labeled for reference

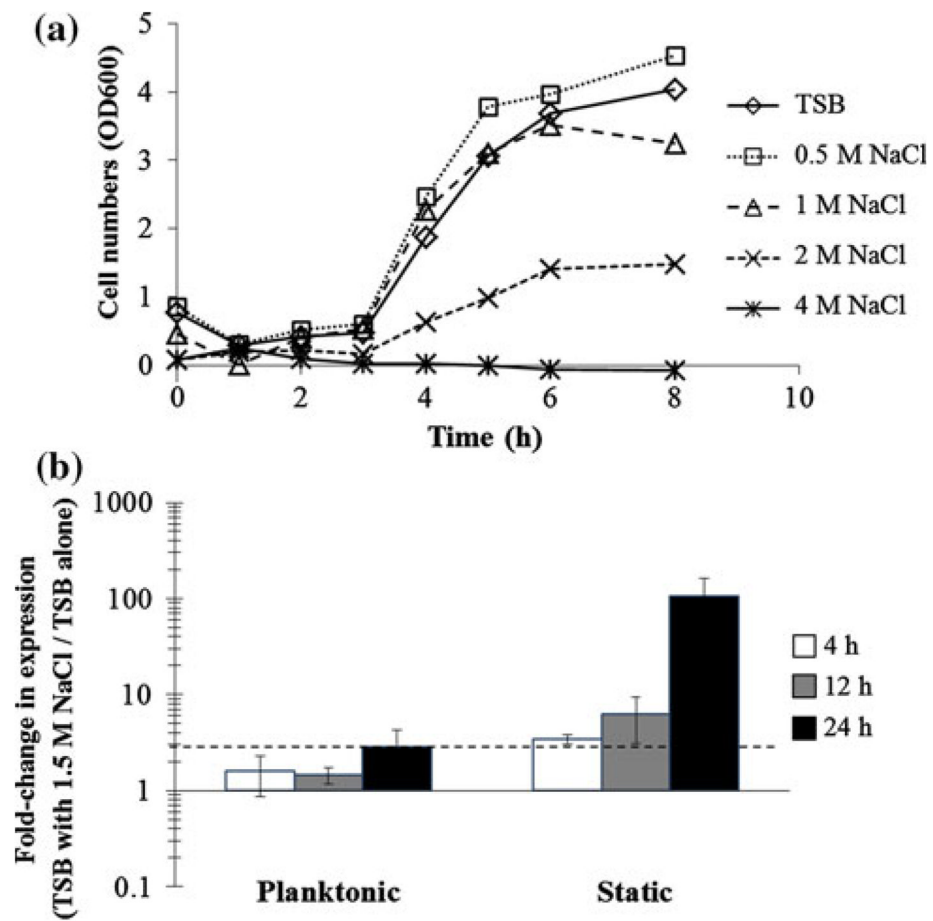


Fig. 3. *S. epidermidis* 1457 cell growth in TSB with increasing NaCl concentrations and relative *embp* gene expression by cells cultured with and without 1.5 M NaCl. **a** *S. epidermidis* 1457 planktonic growth in TSB alone or TSB with increasing NaCl. **b** Fold-change in *embp* expression from *S. epidermidis* cells grown in TSB with 1.5 M NaCl versus TSB only. Fold-change represents *embp* transcription in the presence of NaCl stress normalized expression levels in TSB alone. A twofold change was considered significant (*dashed line*). *Error bars* are standard error of the mean of 3 separate cultures and RNA preparations

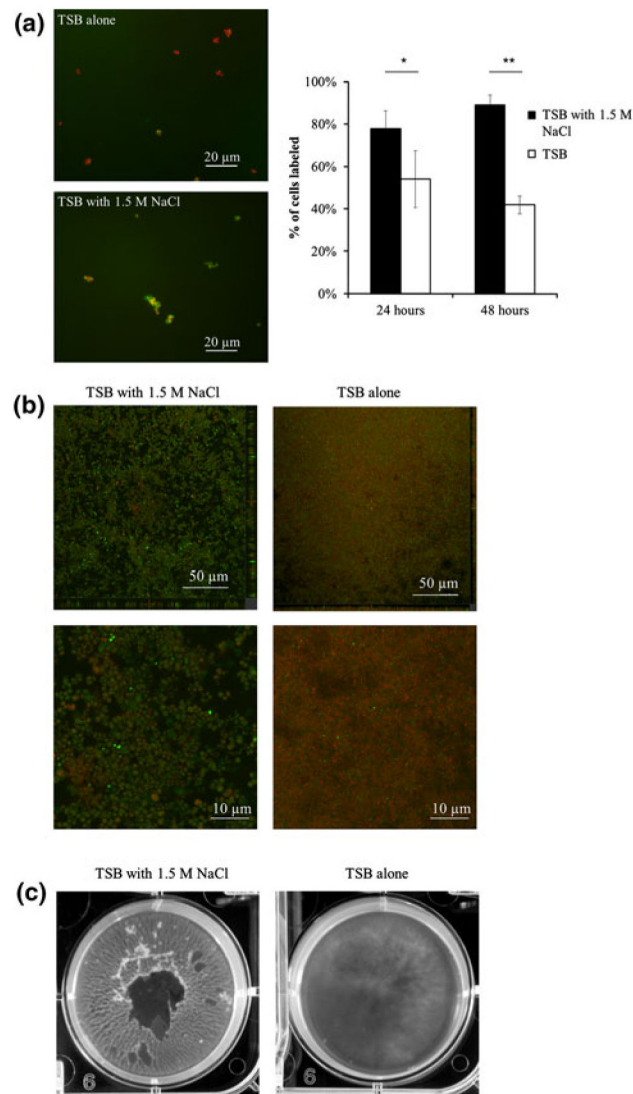


Fig. 4. Effects of high osmotic pressure conditions on biofilm formation. **a** Immunofluorescence microscopy of *S. epidermidis* cell Embp expression at 24 h (anti-rEmb25882 antibodies, green) and counterstained with SYTO9 (red) in TSB alone (Top) or TSB plus 1.5 M NaCl (bottom). Chart quantifies anti-Emb p25882 staining in TSB with 1.5 M NaCl (black bars) or TSB alone (white bars) at 24- and 48-h biofilm growth. Error bars standard deviations of the mean. * $P < 0.01$, ** $P < 0.00001$. **b** Confocal micrograph of *S. epidermidis* biofilm formation in TSB with 1.5 M NaCl (left) versus TSB alone (right) with close ups of depicting cell morphology (below). Staining performed with BacLightLive/Dead stain. **c** Photograph of *S. epidermidis* biofilm formation at 48 h in TSB supplemented with 1.5 M NaCl (left) versus TSB alone (right)

Table 1

RT-PCR and qRT-PCR primers used in RNA expression studies

Gene	RT-PCR type	Forward primer	Reverse primer	PCR product size (bp)
<i>gmk</i> (SE0776)	Standard and quantitative	tcaggtgttgaaaggaac	cgctcaaattctccttg	153
<i>embp</i> (SE1011)	Quantitative	tgacggtccggaagtattg	Ttagctccctgccatcttc	–
<i>icaA</i> (SERP2293)	Quantitative	ttatcaatgccgcagttgctc	Accgttgatattgcctctg	–

Table 2

Effect of osmotic pressure on *S. epidermidis* biofilm structure analysis by means of COMSTAT (mean \pm standard deviation)

Osmotic stress	Thickness (μm)	Roughness coefficient	Surface area to volume ratio ($\mu\text{m}^2 \mu\text{m}^{-3}$)
None	6.16 \pm 0.75	0.32 \pm 0.06	0.75 \pm 0.08
1.5 M NaCl	13.16 \pm 2.28	0.85 \pm 0.12	3.01 \pm 0.40
<i>P</i> value	<i>P</i> < 0.005	<i>P</i> < 0.0005	<i>P</i> < 0.0001