Revista da Sociedade Brasileira de Medicina Tropical
Journal of the Brazilian Society of Tropical Medicine
Vol.:52:e20180001: 2019
doi: 10.1590/0037-8682-0001-2018

Short Communication

Stress conditions in the host induce persister cells and influence biofilm formation by *Staphylococcus epidermidis* RP62A

***Rafael Ovando Fraiha*[1], *Ana Paula Ramos Pereira*[1], *Eliana da Costa Alvarenga Brito*[1], *Clayton Luiz Borges*[2], *Ana Flávia Alves Parente*[3], *Renata Trentin Perdomo*[1], *Maria Ligia Rodrigues Macedo*[4] and *Simone Schneider Weber*[1],[5]**

[1]. Laboratório de Biociência, Faculdade de Ciências Farmacêuticas, Alimentos e Nutrição, Universidade Federal do Mato Grosso do Sul, Campo Grande, MS, Brasil.
[2]. Laboratório de Biologia Molecular, Universidade Federal de Goiás, Goiânia, GO, Brasil.
[3]. Universidade de Brasília, Brasília, DF, Brasil.
[4]. Laboratório de Purificação de Proteínas e suas Funções Biológicas, Faculdade de Ciências Farmacêuticas, Alimentos e Nutrição, Universidade Federal do Mato Grosso do Sul, Campo Grande, MS, Brasil.
[5]. Instituto de Ciências Exatas e Tecnologia, Universidade Federal do Amazonas, Itacoatiara, AM, Brasil.

Abstract

Introduction: Studies have demonstrated that pathogens react to the harsh conditions in human tissues by inducing mechanisms that promote survival. **Methods:** Persistence and biofilm-forming ability were evaluated during stress conditions that mimic those in the host. **Results:** Carbon-source availability had a positive effect on *Staphylococcus epidermidis* RP62A adhesion during hypoxia, accompanied by a decrease in pH. In contrast, iron limitation led to decreased surface-adherent biomass, accompanied by an increase medium acidification and lactate levels. Interestingly, iron starvation and hypoxia induced persister cells in planktonic culture. **Conclusions:** These findings highlight the role of host stress in the virulence of *S. epidermidis*.

Keywords: Persistence. Starvation. Virulence.

The pathogenesis of *Staphylococcus epidermidis* in medical device-associated infections is mainly attributed to its ability to form adherent biofilms[1], and more recently to its persistence[2]; thus, both are important virulence factors. During the invasion of human tissues, microorganisms are exposed to different hostile conditions.

Biofilm development in staphylococci is a two-step process consisting of the adherence and accumulation and maturation phases. Staphylococcal biofilms can form in a polysaccharide intercellular adhesin-dependent manner. Polysaccharide intercellular adhesin (PIA/PNAG) is a beta-1,6-N-acetylglucosamine polymer that is essential for bacterial cell-to-cell adhesion. The synthesis of PIA/PNAG is mediated by the intracellular adhesion (*ica*) operon, which encodes three enzymatically active membrane proteins (IcaA, IcaC, and IcaD) and one extracellular protein (IcaB) that are involved in polysaccharide synthesis[3]. Host environmental factors, including osmotic stress, oxygen limitation, temperature, and carbon-source availability [glucose, N-acetylglucosamine (GlcNAc), or glucosamine], may also influence PIA expression[4]. Studies have demonstrated that pathogens react to the harsh conditions encountered in the host by regulating their metabolism in response to the stress to survive. Many studies have focused on the effects of the host environment on the virulence of *S. aureus*. However, relatively few such studies on *S. epidermidis* have been reported. The absence of free iron in host tissues and the mechanisms of host iron restriction demand

Corresponding author: Simone Schneider Weber.
e-mail: weberblood@gmail.com
Orcid: 0000-0002-4181-142X
Received 2 January 2018
Accepted 24 July 2018

that pathogens develop strategies to combat the nutritional limitation. While some varying results have been reported for the genus *Staphylococcus*, many studies have demonstrated that iron positively regulates biofilm formation. In staphylococci, biofilm formation is a complex process that is strongly influenced by the external conditions encountered during its interactions with host tissues, including nutrient availability, osmolarity, temperature, and oxygen availability[5].

One way that bacterial cells overcome stress conditions is by producing persisters. Persister cells are antibiotic-tolerant cells that increase dramatically in number from mid-exponential phase to stationary phase[6], probably due to decreasing nutrient availability[7]. It is not clear how the stress conditions in the host induce persistence in *S. epidermidis*, but understanding the mechanisms involved in survival during infection is very important. The main goal of this research was to provide comprehensive insight into the sophisticated machineries of *S. epidermidis* virulence in response to the stress encountered in the host by studying the host-bacteria interaction *in vitro*.

The *S. epidermidis* strains used in the study included a known slime-producing INCQ 00650 strain, ATCC 35984 (RP62A), and a non-slime-producing strain, ATCC 12228, both were provided by CMRVS, FIOCRUZ-INCQS (Rio de Janeiro, RJ, Brazil). The bacterial strains were grown on Mueller-Hinton agar overnight at 36.5°C, and bacterial suspensions in 0.9% NaCl corresponding to a 0.5 McFarland standard (1.5×10^8 CFU mL^{-1}) were used in the experiments. The bacterial cultures were subjected to different stress conditions, which can be encountered during the host infection, such as hypoxia and glucose and iron starvation. Iron deprivation was analyzed using brain heart infusion (BHI) medium supplemented with an iron chelator (50 μM bathophenanthroline disulfonate, BPS; Sigma-Aldrich). The control cells were incubated in pre-chelated medium supplemented with 3.5 μM $Fe(NH_4)_2(SO_4)_2$. Carbon-source availability was evaluated by culturing the cells on BHI, which contains residual amounts of glucose (carbon starvation), or BHI supplemented with 0.5% glucose (carbon supplemented). The cultures were also subjected to either hypoxic conditions by incubation in an environment with low levels of available oxygen (1% O_2) and 5% CO_2 or normoxia (20% O_2). *In vitro* biofilm production was assessed by a crystal violet (CV) assay in 96-well microtiter plates, as previously described for staphylococci[8]. Biofilm formation was determined over time and was correlated with the presence of each stressor. All assays were performed in triplicate, and comparisons between groups were performed using Student's t-test or one-way ANOVA. Differences among groups were considered significant when the *p*-values were less than 0.05. Lactate levels were assessed by *VITROS® dry technologies* (Ortho-Clinical Diagnostics, Johnson & Johnson), and the pH was measured using a microelectrode coupled to a pH meter (Thermo Scientific Orion 2-Star Plus pH meter).

We observed biofilm accumulation with the addition of 0.5% glucose (**Figure 1A**), which was associated with the acidification of the culture medium and increased lactate levels in the supernatant (**Figure 1B-C**). It has been established that staphylococci growing in a biofilm shift their physiology toward microaerobic or anaerobic metabolism. Thus, the effect of carbon-source availability on *S. epidermidis* adhesion was assessed under low oxygen availability, and an increase in biofilm accumulation, along with medium acidification, was observed under hypoxia with low glucose (**Figure 1)**.

Sequence similarity searches were performed to identify the main homologous proteins in the *S. epidermidis* RP62A strain that have been reported to play a role in the regulation of biofilm formation in *S. aureus* under low glucose, oxygen, or iron availability. The *S. aureus* protein sequences of interest were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/) in FASTA format. To infer homology, we used BLASTp, and sequences with expectation values (*E-values*) $\geq 10^{-5}$ were considered to be similar.

In the host environment, human pathogens are exposed to fluctuating oxygen levels, and adaptive responses to these sudden changes often occur at the metabolic level[10]. Hence, we deemed it important to examine the effect of 0.5% glucose on *S. epidermidis* adhesion during low oxygen availability. The results showed biofilm accumulation as well as acidification during hypoxia. This acidosis may have been the result of extracellular lactic acid accumulation, which is produced by fermentative metabolism. In addition, our results showed that the presence of a carbon source had a positive effect on *S. epidermidis* adherence. Rbf and SarX are part of a regulatory cascade that promotes PIA-dependent biofilm formation in response to glucose in *S. aureus*. Interestingly, homologs for both proteins were identified in our *in silico* analysis (**Table 1)**, suggesting that this regulatory system might also exist in *S. epidermidis* RP62A and that there is substantial overlap between the biofilm responses to glucose availability n *S. aureus* and *S. epidermidis*.

The sugar nucleotide UDP-GlcNAc, which is synthesized from glucose, is an important precursor for PIA production[11]. Thus, we speculated that the availability of glucose and oxygen might regulate biofilm formation in *S. epidermidis* via an *ica*-dependent pathway, which involves metabolism adaptation as a strategy to improve virulence. We would like highlight this result, since the induction of biofilm formation in response to higher glucose is particularly important in diabetic individuals, who are more susceptible to persistent infections and/or infections associated with catheters during hospitalization, which, given the limited number of effective antibiotics to treat such infections, makes treatment difficult.

The effect of iron limitation on biofilm formation by *S. epidermidis* was examined by using a static-culture biofilm assay with the iron-chelating compound BPS (50 μM). The addition of BPS led to a decrease in the surface-adherent biomass (**Figure 2A**), which was accompanied by a decreased pH and increased lactate in the supernatant (**Figure 2B-C**). Iron starvation has been reported as a requirement for metabolic shifts to anaerobic growth by pathogens in host environments because iron is an important cofactor for the 24 enzymes of the tricarboxylic acid (TCA) cycle. As shown in **Figure 2A**, *S. epidermidis* biofilm formation was restricted under iron-limiting conditions, followed by an increase in lactate release

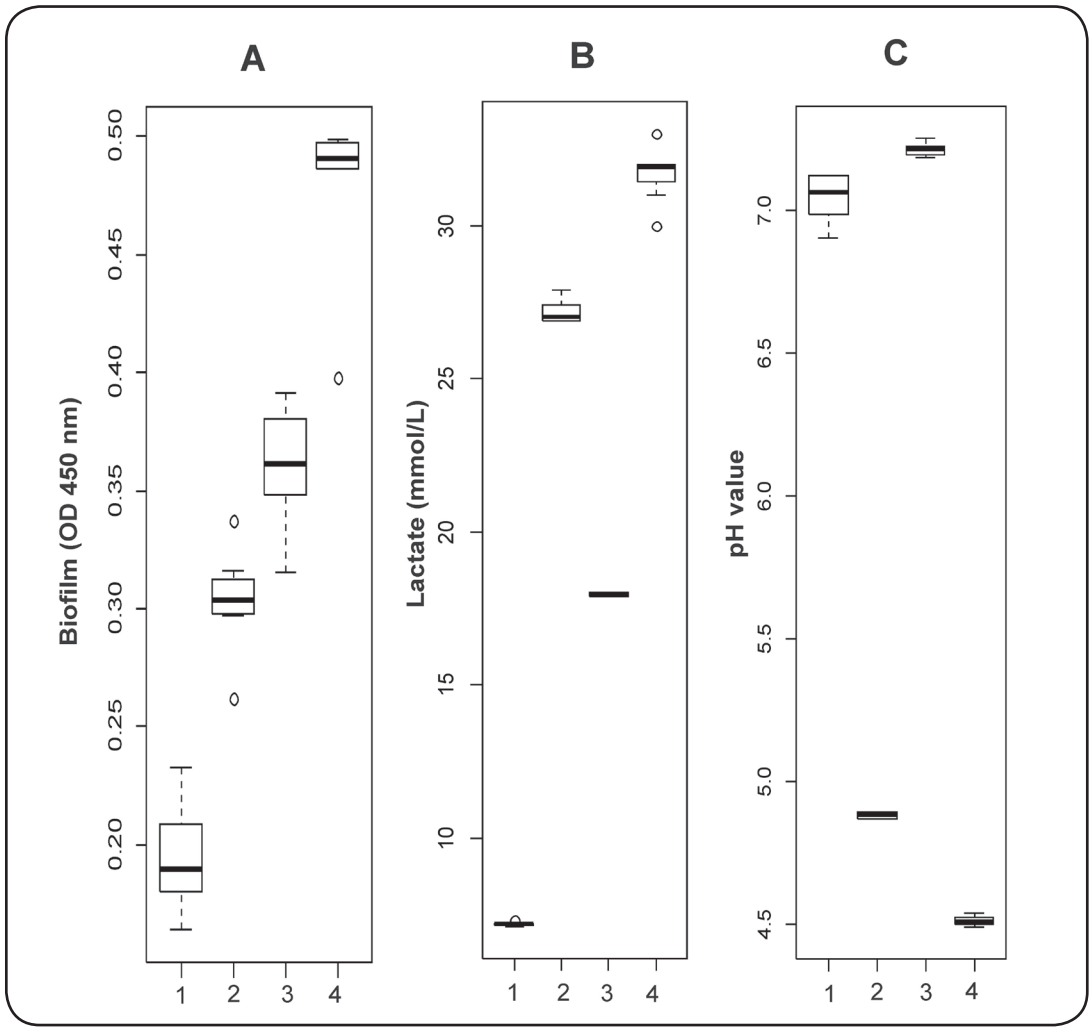

FIGURE 1: **Quantification of *S. epidermidis* biofilm formation, lactate production, and medium pH changes in response to hypoxia and the addition of 0.5% glucose**. (A) A biofilm assay was performed in the absence (1 and 3) or presence of 0.5% glucose (2 and 4). (B) The extracellular lactate concentration in the cell-free supernatant of the culture for the biofilm assay (A) was evaluated by the VITROS350 enzymatic test (Ortho-Clinical Diagnostics). (C) The pH was monitored by a microelectrode coupled to a pH meter. The data showed that both glucose and hypoxia significantly increased biofilm formation, extracellular lactate production, and medium acidification. The differences among groups were considered significant when the p-values were less than 0.05. Legend: 1- BHI with basal glucose in normoxia; 2: BHI with 0.5% glucose in normoxia; 3- BHI with basal glucose in hypoxia; 4- BHI with 0.5% glucose in hypoxia.

(**Figure 2B**). In *S. aureus*, the genes regulated by the ferric uptake regulator (Fur) are involved in a synchronized mechanism in response to iron deprivation that inhibits Fur repression and is reflected by increased iron acquisition and glycolysis[12]. Since the enzymes of the TCA cycle are downregulated during iron deprivation, the pyruvate produced by glycolysis is directed toward lactate production by lactate dehydrogenase, which leads to a decrease in the pH, favoring the shutdown of iron binding by transferrin and leading to increased levels of free iron in the host. Other *S. aureus* virulence factors are positively regulated by Fur under low-iron conditions, including Eap and Emp, which are extracellular proteins that promote adhesion to fibronectin, fibrinogen, vitronectin, and collagen. Although our *in silico* analysis did not reveal homologous genes in the *S. epidermidis* RP62A genome (**Table 1)**, we found an ortholog of *fur*, which suggests that *S. epidermidis* may possess other Fur-regulated adhesins that promote the virulence of this strain. In contrast, Johnson et al.[13] demonstrated that the expression of the *ica*-operon was required for biofilm regulation under iron starvation. Nevertheless, the factors involved in iron-mediated adhesion, whether regulated by Fur and/or by the *ica* operon, have yet to be identified. Here, we performed a biofilm assay with two *S. epidermidis* strains, one known slime-producing strain, RP62A, and one strain with a deletion of the entire *ica* operon, ATCC 12228, and we showed that only the RP62A strain was able to adhere to the plate under variable glucose, oxygen, and iron availability. These environmental conditions are relevant to those encountered by *S. epidermidis in vivo*,

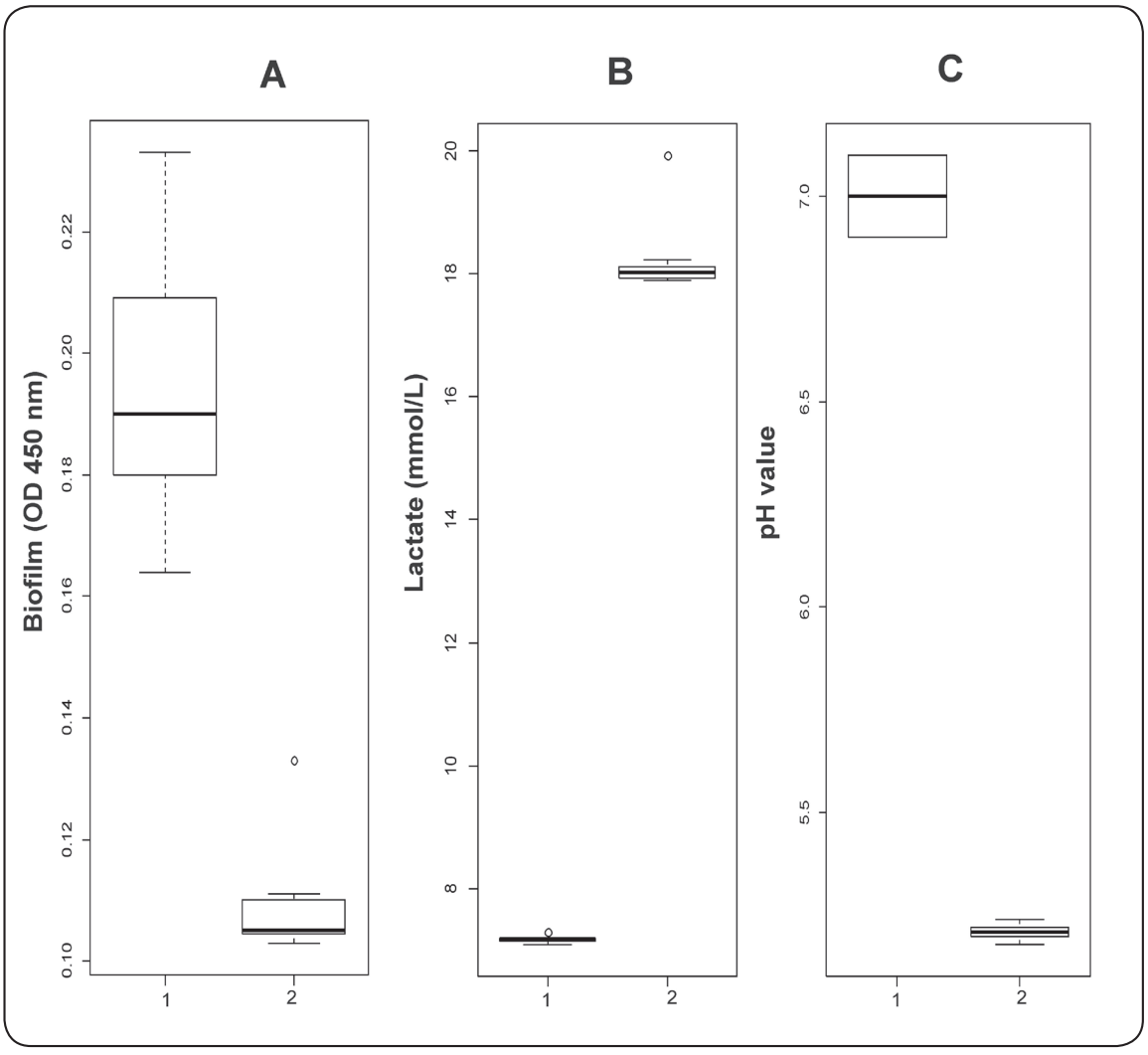

FIGURE 2: **Quantification of *S. epidermidis* biofilm formation, lactate production, and medium pH in response to iron starvation.** (A) A biofilm assay was conducted in BHI medium supplemented with an iron chelator (BPS), and control cells were incubated in previously chelated medium supplemented with Fe $(NH_4)_2(SO_4)_2$. (B) The extracellular lactate concentration in the cell-free culture supernatant during the biofilm assay was evaluated by an enzymatic test (VITROS350; Ortho-Clinical Diagnostics). (C) The pH was monitored with a microelectrode coupled to a pH meter. The data showed that iron restriction significantly decreased biofilm formation, increased extracellular lactate, and reduced the pH of the medium. The differences among groups were considered significant when the p-values were less than 0.05. Legend: 1- BHI and 2- BHI with BPS.

suggesting that our experimental results correlate with what may be occurring during host-bacteria interactions.

S. epidermidis RP62A persister cells were isolated as described by Shapiro et al[9]. using vancomycin (Sigma). The cells were isolated over time and were correlated with the presence of each stressor. Briefly, a standard culture was used to prepare a 100 mL culture of *S. epidermidis* RP62A in BHI as described above. At different time points during the growth cycle (0–18 h), one sample was removed for the plating assay, and another aliquot was treated with the optimal concentration of vancomycin (50 μg mL^{-1}). To enumerate the persisters, samples collected at each indicated time point were washed twice in 1 mL of cold BHI, centrifuged for 2 min at 10,000 × *g*, pelleted, and serially diluted in saline. Then, 10 μL aliquots of the dilutions were plated on BHI agar. The number of "persisters" is reported as CFU mL^{-1}. "Persisters" were defined as the cells that survived at 25 times the MIC of vancomycin (2 μg mL^{-1}) for 12 h (stationary phase). *Here, we used* persister-derived isolates obtained after vancomycin exposure (50 μg mL^{-1}) within 12 h to evaluate the influence of hypoxia and iron starvation on persistence. Persisters were not detected after planktonic growth under both low oxygen and iron availability conditions. However, a substantial number of persister cells remained viable after incubation of persister-derived isolates with vancomycin under hypoxia or iron deprivation compared with the number remaining after incubation with vancomycin alone (data not shown). Persisters are important as they have the potential to improve bacterial adaptation to environmental change, and may lead to recalcitrance and relapse of persistent bacterial infections[14]. In this study, we presented evidence that host stress

TABLE 1: Identification of *S. epidermidis* RP62A proteins with similarity to *S. aureus* proteins involved in biofilm formation by BLASTp.

Protein	Query sequence[1]	Protein name	Function	Accession number in RP62A[2]	E-value[3]
SarX	AIU86729.1	TH-type transcriptional regulator	Positive transcriptional regulation of *icaADBC* and PIA/PNAG expression by inhibiting the expression of *icaR*	AAW53683.1	7e-51
Rbf	YP501450.1	HTH-type negative transcriptional regulator IcaR (*helix-turn-helix*)	Positive transcriptional regulation of *icaADBC* and PIA/PNAG expression via upregulating SarX and inhibiting *icaR* expression	WP_002497698.1	7e-89
Eap	ALY17655.1	Extracellular adherence protein Eap	Mediates the adherence of *S. aureus* to host extracellular matrix components	-	-
Emp	P0C6P1.1	Extracellular matrix protein-binding protein	Vitronectin-binding cell surface protein (host protein-adhesin)	-	-
Fur	AAF21313.1	Iron uptake regulatory protein	Transcriptional repressor	WP_001831103.1	8e-104

[1] NCBI accession number for the *S. aureus* query sequence (http://www.ncbi.nlm.nih.gov/).

[2] NCBI accession number for *S. epidermidis* RP62A protein sequence (http://www.ncbi.nlm.nih.gov/).

[3] Statistical estimate score or expectation value (E-value), which was used to infer homology between the protein sequences.

conditions induce the appearance of persister *S. epidermidis* RP62A cells, presumably via stringent response. This result corroborates the study by Mordukhova et al.[15], who reported that the bacterial response to unfavorable environmental conditions, such as methionine starvation, enhances the persistence phenotype in *Escherichia coli*, while re-establishment of methionine reduced persistence. Here, we described, for the first time, that both oxygen and iron starvation upregulated persister cells, while Fe supplementation and normoxia decreased cell tolerance. Therefore, these results suggest that the stress response induced in host tissues could be a mechanism that leads to high antibiotic tolerance and an increase in resistant strains, contributing to the increased number of chronic infections, which is an emerging problem. Our findings advance our understanding of host-bacteria interactions and present evidence regarding the role of host stress conditions in the regulation of relevant virulence factors of *S. epidermidis*, namely, biofilm formation and persistence. However, more comprehensive studies are necessary to explain the sophisticated machineries used by *S. epidermidis* to establish an infection in the host.

Acknowledgments: We thank to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for scholarship for APRP and ECAB, respectively.

Financial support: This work was supported by grant from Universal/CNPq/2016 (432867/2016-3).

Conflict of Interest Statement: The authors declare that there is no conflict of interest.

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