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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Dormancy within *Staphylococcus epidermidis* biofilms: a transcriptomic analysis by RNA-seq

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Abstract The proportion of dormant bacteria within *Staphylococcus epidermidis* biofilms may determine its inflammatory profile. Previously, we have shown that *S. epidermidis* biofilms with higher proportions of dormant bacteria have reduced activation of murine macrophages. RNA-sequencing was used to identify the major transcriptomic differences between *S. epidermidis* biofilms with different proportions of dormant bacteria. To accomplish this goal, we used an in vitro model where magnesium allowed modulation of the proportion of dormant bacteria within *S. epidermidis* biofilms. Significant differences were found in the expression of 147 genes. A detailed analysis of the results was performed based on direct and functional gene interactions. Biological processes among the differentially expressed genes were mainly related to oxidation-reduction processes and acetyl-CoA metabolic processes. Gene set enrichment revealed that the translation process is related to the proportion of dormant bacteria. Transcription of mRNAs involved in oxidation-reduction processes was associated with higher proportions of dormant bacteria within *S. epidermidis* biofilm. Moreover, the pH of the culture medium did not change after the addition of magnesium, and genes related to magnesium transport did not seem to impact entrance of bacterial cells into dormancy.

Keywords Cytoscape · Dormancy · Gene ontology · Magnesium · RNA-seq · *S. epidermidis* biofilm · Transcriptome

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Introduction

Staphylococcus epidermidis is one of the major biofilm-producing bacteria which often colonize indwelling medical devices (Otto 2012). Establishment of biofilms is frequently associated with antimicrobial tolerance that can be explained, in part, by the presence of a subpopulation of cells in a reversible nonreplicative state which consequently can maintain a recalcitrant infection (Fauvart et al. 2011; Lewis 2007). These cells can be defined as being in a dormant state, generally presenting a low metabolism, allowing them to survive and resist under stressful conditions such as reduced availability of nutrients, oxygen starvation, temperature variation, salinity, and pH variation (Colwell 2000; Trevors 2011). Dormant cells may lead to misleading interpretations about the infection status of indwelling devices as they often do not grow on standard laboratory culture media (Zandri et al. 2012). Nevertheless, dormancy is a reversible state (Kaprelyants et al. 1993) and virulence properties of dormant bacteria may manifest as they transit into a growing state (Sun et al. 2008). Dormancy improves long-term bacterial survival and facilitates pathogenesis (Dworkin and Shah 2010) by increasing cellular tolerance to antibiotics (Kim et al. 2009; Shapiro et al. 2011; Williamson et al. 2012) and evasion of the host immune system (Cerca et al. 2011a; Yao et al. 2005).

Persisters have been defined as "a small subpopulation of cells that spontaneously go into a dormant, non-dividing state" (Lewis 2012) and tolerate high concentrations of bactericidal agents (Keren et al. 2004a). Despite being dormant, further characteristics of persisters are still under debate (Allison et al. 2011a; Balaban et al. 2013). Moreover, not all dormant bacteria are persisters (Jayaraman 2008; Orman and Brynildsen 2013). In order to characterize dormant persister bacteria, some approaches have been proposed and used to isolate these cells from *Escherichia coli* cultures (reviewed in Lewis 2010), namely by killing growing bacterial cells with antibiotics (Keren et al. 2004b) or by separation of cells based on the lack of expression of degradable green fluorescent protein (Shah et al. 2006). In addition, we recently described how SYBR® green, a fluorochrome used to evaluate microbial viability via binding to nucleic acids, can be used to assess the metabolic state of *S. epidermidis* bacteria (Cerca et al. 2011b). An in vitro model, using specific culture conditions, was used to modulate the proportions of dormant bacteria within *S. epidermidis* biofilms (Cerca et al. 2011c). According to this model, the culture medium acidification, due to glucose metabolism, was responsible for inducing bacteria into a dormant state (Cerca et al. 2011a). In contrast, this effect was found to be prevented by the addition of magnesium (Mg^{2+}) to the culture medium (Cerca et al. 2011c). Magnesium action on *S. epidermidis* adhesion was previously shown by Dunne and Burd (1992); however, the Mg^{2+} effect in biofilm maturation and its role on dormancy are still poorly understood (Piddington et al. 2000; Song and Leff 2006).

The purpose of our study was to describe the major transcriptomic features of *S. epidermidis* biofilms, with induced or prevented dormancy, using high-throughput RNA-sequencing (RNA-seq). RNA-seq has been used for transcriptome analysis as an alternative to other transcriptomic technologies such as microarrays, due to advantages such as a large dynamic range, high technical reproducibility, and the fact that a reference transcriptome is not a requirement (Raz et al. 2011; van Vliet 2010; Wang et al. 2009). RNA-seq has been applied to prokaryotic transcriptome studies (Croucher and Thomson 2010; Sorek and Cossart 2010), and some efforts have been made to use next-generation sequencing in clinical microbiology to test the properties of growing bacterial pathogens (Didelot et al. 2012). Global transcriptomic profile studies in *E. coli* and *Mycobacterium tuberculosis* dormant persister subpopulations have been previously carried out, being reported that the dormant state was associated with changes in transcription of genes involved in metabolism, biosynthetic pathways, or oxidative stress (Keren et al. 2011; Shah et al. 2006).

To achieve our goal, we performed RNA-seq, of three biological triplicates, from the two distinct conditions previously described, and we used a data analysis approach, based on direct and functional gene interactions, namely gene set enrichment and cluster analysis.

Materials and methods

Biofilm growth conditions

S. epidermidis strain 9142 (isolated from blood culture by Mack et al. 1992) (collection number 18857 at DSM, Braunschweig, Germany) was used to establish biofilms with higher and lower ratios of dormant cells, as previously described (Cerca et al. 2011a). Briefly, one colony of strain *S. epidermidis* 9142 was inoculated in tryptic soy broth (TSB) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubated at 37 °C in a shaker at 120 rpm for 18 h. The overnight culture was adjusted to an optical density at 640 nm of 0.250 (±0.05) with TSB, and 10 μL of this suspension was transferred into a 24-well plate (COSTAR, Corning Incorporated, Tewksbury, MA, USA) containing 1 mL of TSB supplemented with 0.4 % glucose (*v*/*v*) (TSB 0.4 % G) (American Bioanalytical, Natick, MA, USA) or TSB 0.4 % G enriched with 20 mM magnesium chloride ($MgCl_2$) (American Bioanalytical). The culture plates were then incubated at 37 °C in a shaker at 120 rpm for 24 h. After this period, the culture medium covering the biofilm was removed and replaced by fresh TSB supplemented with 1 % glucose (*v*/*v*) (TSB 1 % G) or TSB 1 % G containing 20 mM $MgCl_2$ (TSB 1 % G+Mg^{2+}). Biofilms were then allowed to grow in these same conditions for 24 additional hours. Next, the biofilm culture medium was removed and the biofilms were washed twice with 1× phosphate buffered saline (PBS) (Boston BioProducts, Boston, MA, USA). Then, the bacteria within the biofilms were recovered in 1 mL of PBS. All tubes were placed on ice to inhibit cell growth. Prior to preparation of the RNA-seq library, biofilm dormancy was assessed, as previously described (Cerca et al. 2011a). Briefly, biofilms from both conditions were grown with the same initial amount of cells. Number of colony forming units (CFU per milliliter) in each condition was determined using the spread plate method in Trypticase™ Soy Agar with 5 % Sheep Blood (TSA II™) (Becton, Dickinson and Company).

RNA extraction

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). To remove genomic DNA, Ambion® TURBO DNA-free™ kit (Invitrogen, Grand Island, NY, USA) was used followed by acid-phenol/chloroform precipitation (Ambion, Austin, TX, USA), both following the manufacturer's instructions. RNA integrity was determined using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), and samples with RNA Integrity Number (RIN) above 8 were selected for complementary DNA (cDNA) library preparation. Three independent biological experiments, each one from a pool of four biofilms, were performed.

cDNA library preparation and sequencing

Before library preparation, ribosomal RNA (rRNA) was depleted from total RNA samples using the Ribo-Zero™ Magnetic kit for Gram-positive bacteria (Epicentre, Madison, WI, USA), following the manufacturer's specifications. An Illumina® TruSeq™ RNA Sample Preparation kit (Illumina, San Diego, CA, USA) was used with the already purified mRNA, following the manufacturer's protocol. The construction of the libraries was rigorously validated by quantitative PCR and Hi-Sensitivity D1K TapeStation (Agilent 2200 TapeStation). Sequencing data were generated from paired end reads (2×150 bp) on a MiSeq® system (Illumina) using a RNA-seq library of 10 nM.

RNA-sequencing data analyses

The removal of sequence adapters, mapping to reference genome, and normalization of gene expression were performed using CLC Genomics Workbench software (V.5.5.1, CLC Bio.). RNA-seq reads were aligned to the reference genome of *S. epidermidis* strain RP62A (GenBank accession number NC_002976). Gene expression was normalized by calculating reads per kilobase per million mapped reads (RPKM) using the methods described by Mortazavi et al. (2008) in which normalization is adjusted by the counts of reads per kilobase per million mapped and gene length. Since consistency between experiments is a natural concern in RNA-seq, Pearson correlation was used to compare RPKM values between triplicates. To test for significant gene expression changes, Baggerley's test (beta-binominal) (Baggerly et al. 2003) with false discovery rate (FDR) correction was applied (Pawitan et al. 2005). A p value$\leq$0.05 was considered statistically significant. Venn diagram was generated using the Venny tool from BioinfoGP (Oliveros 2007) to identify the transcripts uniquely expressed in each condition.

Biological interactions

Gene ontology (GO) (Ashburner et al. 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (Kanehisa et al. 2004) analysis were performed to determine the function of differentially expressed genes, using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (version 9.05) (Franceschini et al. 2013). Classes with a p value$\leq$0.05 were considered statistically significant. Further analysis was carried out using Cytoscape software (version 2.8.3) (Shannon et al. 2003), namely a gene set enrichment analysis for over- and down-representation in the set of genes with a 1.50-fold expression change and their neighbors (Merico et al. 2010) and a cluster analysis with MCODE plugin (Bader and Hogue 2003). Biological interpretation was carried out using STRING bioinformatic tool.

Quantitative PCR

In order to validate the RNA-seq data, quantitative PCR (qPCR) was performed to quantify the mRNA transcripts of *pgi*, *gapA-2*, *acnA*, *ureA*, *ctsR*, and *rplE*. The primers were designed with Primer3 software (Rozen and Skaletsky 2000) and are shown in Supplementary material (Table S2). One microgram of total RNA was reverse-transcribed using RevertAid First strand cDNA synthesis (ThermoScientific, Waltham, MA, USA) using random primers (NZYTech, Lisbon, Portugal). The experiment was performed using iQ SYBR 2× green supermix (Bio-Rad, Hercules, CA, USA) and CFX96™ thermocycler (Bio-Rad) with the following cycling parameters: 10 min at 95 °C followed by 40 repeats of 5 s at 95 °C, 10 s at 60 °C, and finally 20 s at 72 °C. The cycle threshold values (C_T) were determined and the relative fold differences were calculated by the $2^{(C_T \text{ reference gene} - C_T \text{ target gene})}$ method (Livak and Schmittgen 2001) using 16S rRNA as the reference gene. Three independent experiments were run in triplicate. Statistical significance was analyzed using unpaired t test (with GraphPad Prism version 6, GraphPad Software Inc., La Jolla, CA, USA).

pH and Mg^{2+} measurements

To evaluate the culture pH and Mg^{2+} concentration in the culture medium over time, supernatant aliquots of *S. epidermidis* biofilms were collected every 3 h during the last 24 h of biofilm growth. Collected supernatants were centrifuged at 20,800g for 5 min at 4 °C and stored at −20 °C before use. Culture pH was determined with a pH meter (WTW pH 330, Sigma-Aldrich Inc., St. Louis, MO, USA). Mg^{2+} concentration was determined using atomic absorption spectrometry (Perkin-Elmer 5000, PerkinElmer, Inc., Waltham, MA, USA), according to the manufacturer's instructions. At least two independent experiments were performed.

Results

Transcriptome analysis

We used RNA-seq technology to characterize the transcriptome of *S. epidermidis* biofilms with different proportions of dormant cells. Total RNA was isolated from three biologic replicates of cells within *S. epidermidis* biofilms grown in excess glucose with (prevented dormancy) or without Mg^{2+} (induced dormancy). As expected (Cerca et al. 2011a), the number of culturable bacteria in biofilms grown in TSB 1 % G was lower than the number of CFU per milliliter of biofilms grown in TSB 1 % G+Mg^{2+} (above 0.8 $\log_{10}$ difference), as shown in the Supplementary material (Fig. S1).

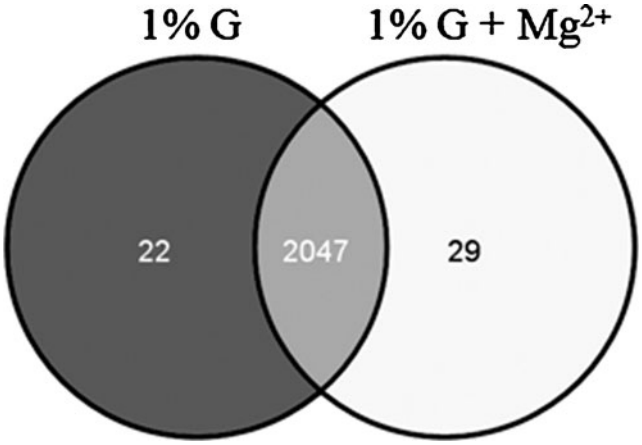

Fig. 1 Venn diagram summarizing the overlap between genes with RPKM value above 1.00 in *S. epidermidis* biofilms grown in glucose excess with (1 % G+Mg^{2+}) or without (1 % G) magnesium

After depletion of rRNA, cDNA libraries were constructed using Illumina® TruSeq™ RNA Sample Preparation kit and MiSeq® sequencing system. An average of 3,029,921 (TSB 1 % G) and 2,650,647 (TSB 1 % G+Mg^{2+}) sequencing reads were obtained for each cDNA library. A global comparison of genes between biological triplicates showed a high correlation (r>0.98, Pearson correlation coefficient for biofilms grown in TSB 1 % G and r≥0.95 for biofilms grown in TSB 1 % G+Mg^{2+}). Before trimming the raw data, a Venn diagram was constructed (Fig. 1) to summarize the overlap between genes expressed in each condition. The expressed genes uniquely found in each condition with a RPKM above 1.00 encoded many uncharacterized proteins with unknown functions as shown in the Supplementary material (Table S1). Nevertheless, among the identified genes with higher RPKM values, only found in *S. epidermidis* biofilms with induced dormancy was *mazE*, a gene which encodes an antitoxin component of a toxin-antitoxin module (*mazEF* module) and is related with the bacterial stress responses through mRNA degradation (Mittenhuber 1999).

For downstream applications, all genes with RPKM values under 1 were discarded. Thus, among the 2,047 genes identified as being transcribed in both conditions, 147 genes (7 %) were differentially expressed between *S. epidermidis* biofilms with different proportions of dormant bacteria, wherein 28 (1 %) were upregulated and 119 (6 %) were downregulated. Additionally, the distribution of the $\log_{10}$ RPKM among the differentially expressed genes of each biological triplicate also showed high agreement between the independent experiments, as in the Supplementary material (Fig. S2). To further validate our data, qPCR was performed using a selection of up- or downregulated genes, namely *pgi*, *acnA*, *gapA-2*, *ctsR*, *rplE*, and *ureA* (Fig. 2). GO annotation was used to classify the functions of the 147 differentially expressed genes, and a biological pathway-based analysis was performed using the KEGG pathway. All statistically significant categories are represented in Fig. 3. Oxidation-reduction and acetyl-CoA metabolic processes were the significant biological processes found in the differently expressed genes (Fig. 3a). Oxidoreductase functions and ribosome activity were the significant molecular functions (Fig. 3b). Cytoplasm and intracellular components were the main cellular components represented in the differently expressed genes (Fig. 3c). The enriched KEGG pathways were for the ribosome synthesis pathway and pyruvate metabolism (Fig. 3d). In order to provide a more detailed analysis through the pyruvate pathway, the KEGG metabolic network was consulted and the pyruvate cycle scheme was drawn to identify which enzymes involved in this pathway are encoded by the differentially expressed genes (Fig. 4). Relevant biological processes and KEGG pathways within differentially expressed genes on replicates are shown in a heat map, constructed to depict fold change values (Fig. 5). Gene expression analysis showed downregulation of oxidation-reduction, pyruvate metabolism, and acetyl-CoA metabolic processes in *S. epidermidis* biofilms with prevented dormancy, as compared to biofilms with induced dormancy. Conversely, ribosome synthesis pathway was upregulated in biofilms with dormancy prevented by Mg^{2+}. Twenty-eight of the differentially expressed genes encoded uncharacterized proteins. In an attempt to find homology with known proteins, we performed

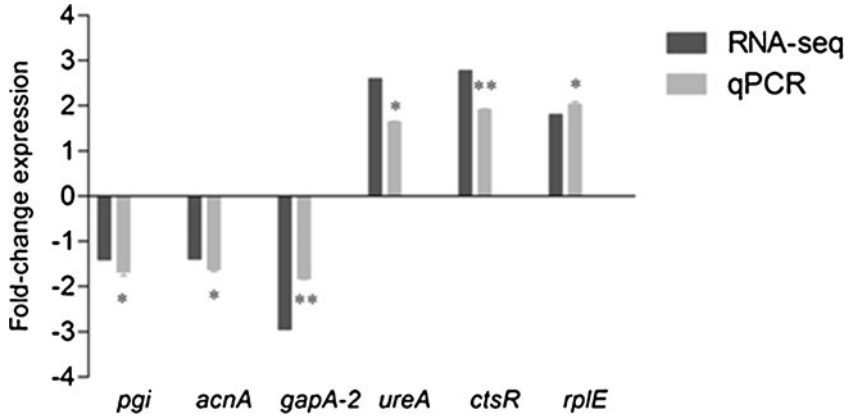

Fig. 2 qPCR validation of some differentially expressed genes. Fold change expression is related to *S. epidermidis* biofilms grown in 1 % G plus 20 mM of Mg^{2+}. The *bars* represent the mean and the *error bars* the standard deviation. Statistical significance was analyzed using unpaired *t* test (GraphPad Prism version 6). *p<0.05, **p<0.01

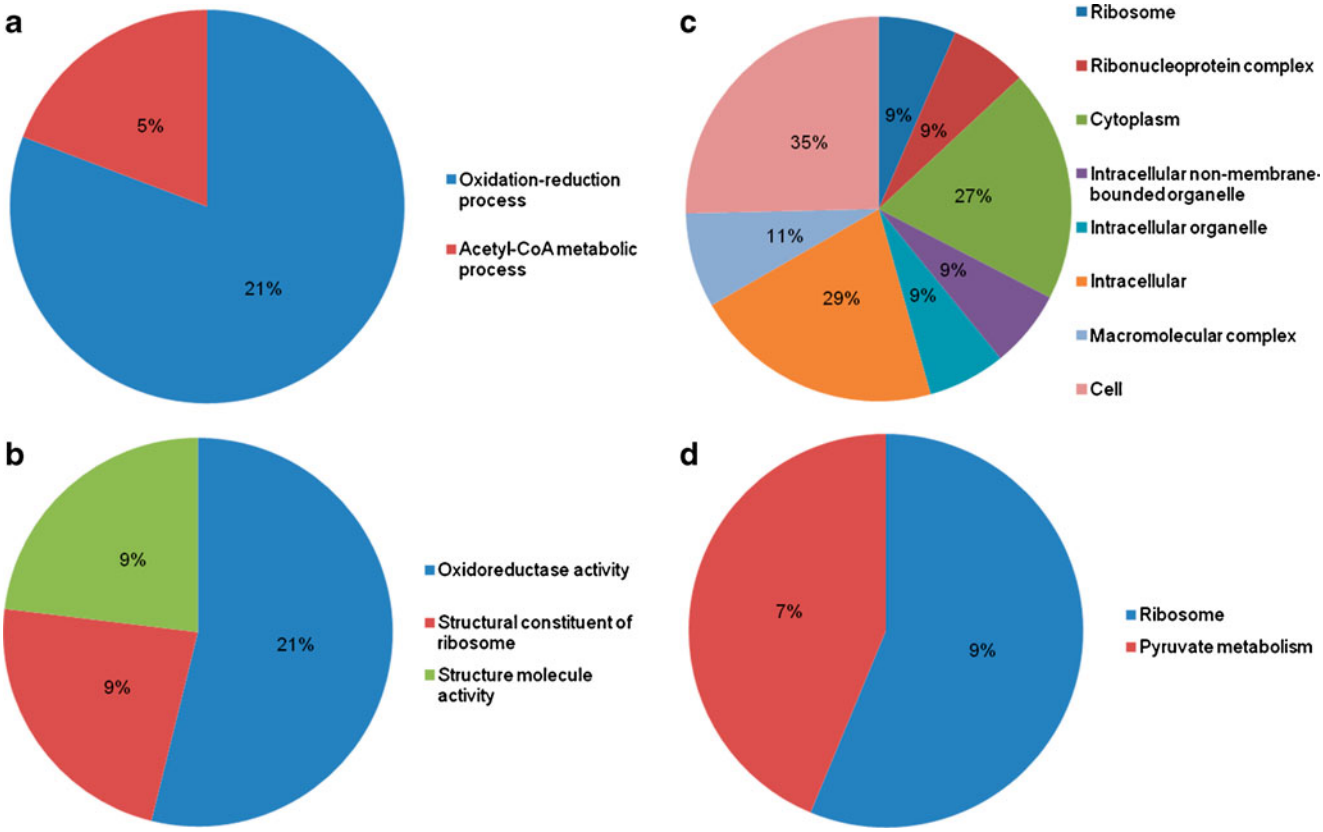

Fig. 3 Significant GO annotation and KEGG pathways of differentially expressed genes ($p<0.05$, FDR-corrected). Biological processes (**a**), molecular functions (**b**), cellular components (**c**), and KEGG pathways (**d**)

a BLAST analysis, a search in the Pfam database (version 27.0) for Pfam domains (Punta et al. 2012) and PSORTb program (v. 3.0) (Yu et al. 2010) to predict their subcellular localization (Table 1). Although BLAST analysis indicated some homology with genes encoding uncharacterized proteins from other species (all these genes showed higher proximity to *Staphylococcus aureus Mu50*, except the *serp2066* gene which showed higher homology to *Macrococcus*

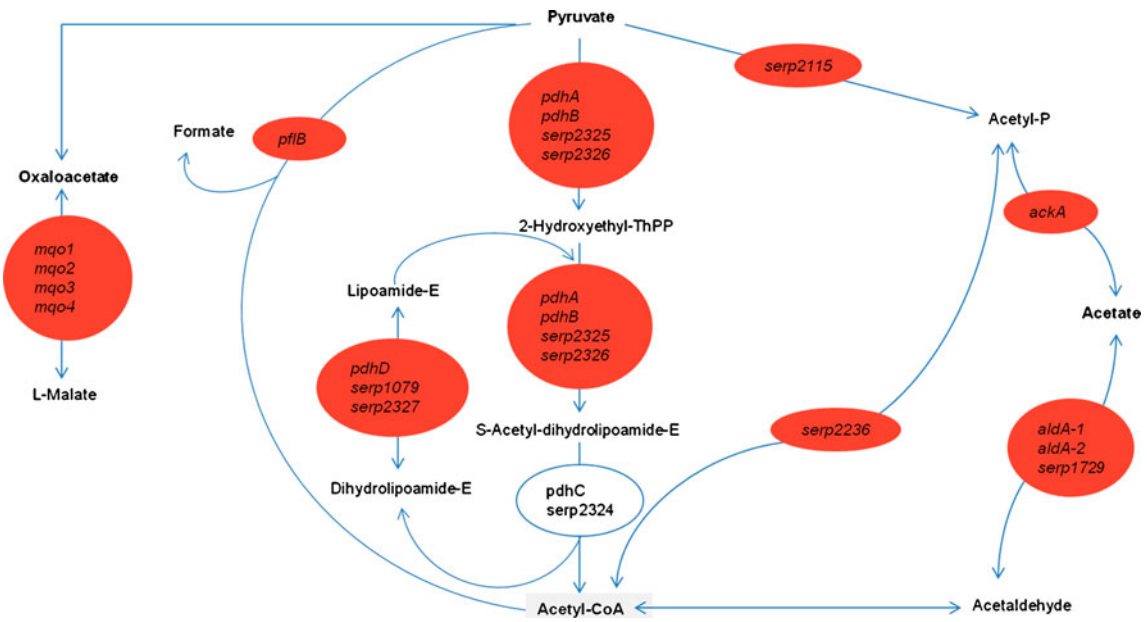

Fig. 4 Scheme of pyruvate metabolism and related products including genes which encode enzymes in each specific reaction. *Red circles* represent enzymes encoded by differentially expressed downregulated genes in *S. epidermidis* biofilms grown in 1 % G and Mg^{2+}

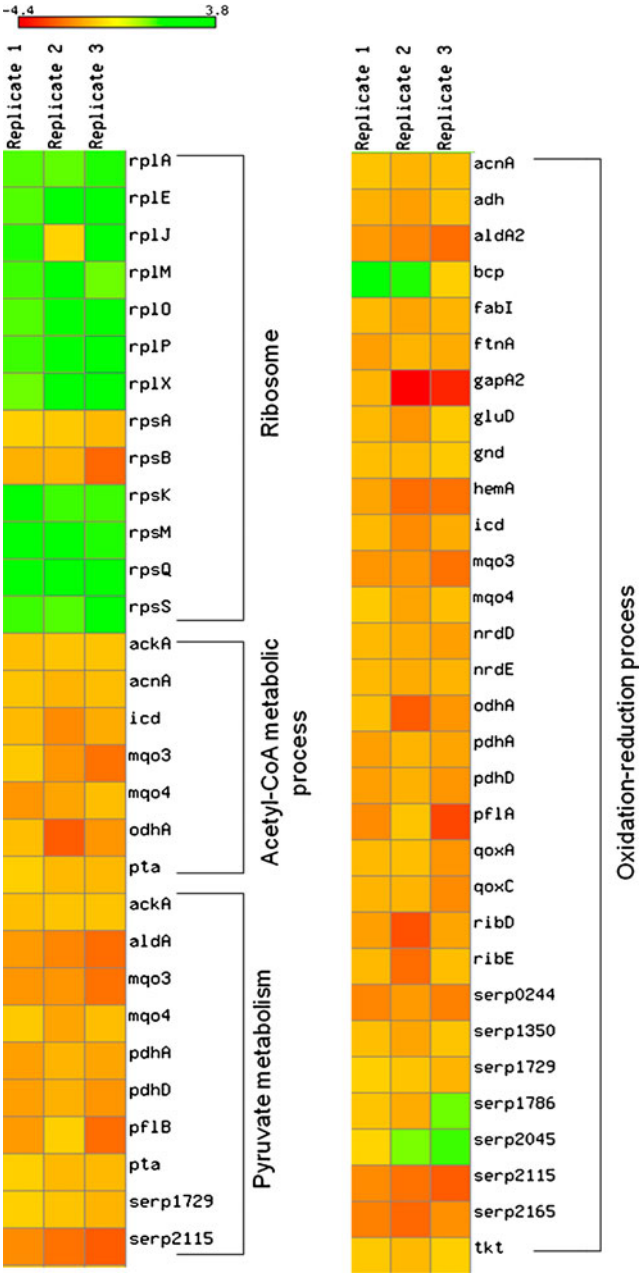

Fig. 5 Heat map of the main biological processes and KEGG pathways: statistically significant in differentially expressed genes between induced and prevented dormancy of *S. epidermidis* biofilms. Values represent fold change expression among biological triplicates. *Red* and *green colors* correspond to decrease and increase expression, respectively. Heat map was constructed using Matrix2png (Pavlidis and Noble 2003)

caseolyticus), most of the predicted translated proteins would have a cytoplasm or cytoplasmic membrane localization. However, there were 15 proteins that contained annotated domains in the Pfam database, indicating that these were primarily domains with unknown functions.

Table 2 lists the 10 most highly and the 10 less transcribed genes based on RPKM values from *S. epidermidis* biofilms grown in TSB 1 % G with Mg^{2+}. Among the downregulated and upregulated transcripts in these *S. epidermidis* biofilms were genes mostly associated with oxidation-reduction and metabolic processes, respectively. Interestingly, most of the differentially expressed genes had a relatively small fold change (−5.60 to 2.92), of which 13 of the upregulated genes had a fold change above 1.50 and 80 genes had a fold change under −1.50. Figure 6 shows RPKM values of a few *S. epidermidis* virulence genes and genes related to biofilm formation, accumulation, or modulation (Otto 2012; Resch et al. 2005) among the two distinct conditions. Although the differences were not statistically significant, virulence genes seemed to be transcribed to a greater level in biofilms with induced dormancy.

Enrichment map analysis and cluster analysis

Downstream analysis of transcripts was based in direct and functional gene interactions using Cytoscape (Cline et al. 2007). STRING was used to create a gene interaction network including all differentially expressed genes and neighbors, yielding a total of 1,442 nodes and 10,295 edges. GO analysis from the gene set enrichment is given in Table 3. Enrichment map analysis of the upregulated and neighbor genes associated with *S. epidermidis* biofilms grown with Mg^{2+} revealed distinct functions involved in translation and biosynthetic processes, such as macromolecular synthesis. Conversely, downregulated genes encoded catabolic processes and oxidoreductase activity. Gene clusters were obtained in Cytoscape with MCODE plugin. Among the differently expressed genes, we found two clusters (a and b in Fig. 7) with a score value above 2.0 and at least four nodes (complete gene network of differently expressed genes is shown in the Supplementary material Fig. S3). The main biological processes of each cluster were translation and biosynthetic processes (cluster a) and catabolic and oxidation-reduction processes (cluster b), which are in accordance with the results obtained in the gene set enrichment.

Evaluation of culture medium pH and Mg^{2+} levels

Since acidic pH conditions have been described as an inhibitor of bacterial growth (Wijtzes et al. 1995), we assessed whether the culture medium pH was altered and might be related to bacterial dormancy and consequently to transcriptomic differences observed when Mg^{2+} was added. We found that *S. epidermidis* biofilms grown in TSB 1 % G enriched with $MgCl_2$ did not have an altered pH in the final culture medium as shown in Supplementary material Fig. S4a. Furthermore, assessment of Mg^{2+} consumption over time showed that Mg^{2+} levels were constant, revealing that Mg^{2+} was not depleted from the culture medium in established biofilms, as shown in Supplementary material Fig. S4b. These results indicate that, similar to the culture medium pH, the Mg^{2+} levels are maintained throughout the culture.

Table 1 Differentially expressed genes representing uncharacterized proteins with significant Pfam domain, including predicted localization by PSORTb

Expression	Name	Function	Predicted localization	Protein family (Pfam) domain match
↑	*serp0087*	Uncharacterized protein	Cytoplasm	Uncharacterized conserved protein
↓	*serp0121*	Uncharacterized protein	Unknown	Protein of unknown function
↓	*serp0581*	UPF0413 protein	Cytoplasm	Thioredoxin
↓	*serp0701*	UPF0358 protein	Unknown	Protein of unknown function
↓	*serp0707*	Uncharacterized protein	Cytoplasmic membrane	Protein of unknown function
↑	*serp0741*	Uncharacterized *N*-acetyltransferase	Cytoplasm	Acetyltransferase (GNAT) domain
↓	*serp1053*	Uncharacterized protein	Cytoplasmic membrane	Protein of unknown function
↑	*serp1180*	Putative Holliday junction resolvase[a]	Unknown	Uncharacterized protein family
↓	*serp1210*	Uncharacterized protein	Cytoplasm	Protein of unknown function
↓	*serp1402*	Uncharacterized protein	Cytoplasmic membrane	Bacterial protein of unknown function
↓	*serp1720*	UPF0340 protein	Cytoplasm	Protein of unknown function
↓	*serp1754*	Uncharacterized protein	Cytoplasm	NADH(P) binding
↓	*serp1771*	Uncharacterized protein	Cytoplasmic membrane	Domain of unknown function: predicted membrane-bound metal-dependent hydrolase
↓	*serp1783*	Uncharacterized protein	Cytoplasmic membrane	Domain of unknown function: small integral membrane protein
↑	*serp2066*	Uncharacterized protein	Cytoplasm	Domain of unknown function: uncharacterized protein conserved in bacteria

↑ means upregulation and ↓ downregulation in *S. epidermidis* biofilms grown in TSB 1 % G enriched with Mg^{2+}

[a] Could be a nuclease that resolves Holliday junction intermediates in genetic recombination

Discussion

It has been reported that cells in the deeper layers of the biofilms are less active than the ones in the upper layers, although they are still viable but with a slower growth and a lower metabolic rate (Williamson et al. 2012). Bacterial phenotypes can change according to environmental conditions and chemical concentration gradients, generating distinct populations and contributing to heterogeneity within biofilms (Stewart and Franklin 2008). Dormancy can be considered an adaptive response to adverse environmental conditions. Since the survival capacity of dormant bacteria in biofilm-related infections is increased (Kim et al. 2009), it is of utmost importance to characterize *S. epidermidis* biofilm dormant subpopulation. In order to improve mRNA quantitation by RNA-seq technology, many transcriptomic studies have tried to increase the information content by depleting rRNA (Westermann et al. 2012). Previous bacterial RNA-seq studies suggest that a minimum of two to five million reads from an rRNA-depleted library are required for accurate coverage (reviewed in Westermann et al. 2012). Moreover, paired end sequencing is a more efficient strategy to characterize and quantify the transcriptome of bacteria without annotated genomes (Fang and Cui 2011). Besides biological variability, technical issues like coverage (Croucher and Thomson 2010), sequence depth (Tarazona et al. 2011), variation from one flow cell to another (Fang and Cui 2011), variation between the individual lanes within a flow cell (Fang and Cui 2011), and library preparation effect (Bullard et al. 2010) may also affect sequencing and cause variability within replicates. In order to overcome these limitations and to obtain more accurate results, we ran three biological replicates consisting of pools of four biofilms for each replicate. This approach contributed to the observed consistency in our data.

GO analysis has been used in genome-wide expression studies to reduce complexity and highlight biological processes (Young et al. 2010). GO analysis of the 147 differentially expressed genes between the two studied conditions identified ribosome activity, oxidation-reduction, and acetyl-CoA metabolic processes as the major classes with differences in mRNA transcripts. The main biological processes and KEGG pathways showed consistency within gene expression levels among biological triplicates and provided evidence that genes involved in oxidation-reduction, acetyl-CoA metabolic processes, and pyruvate metabolism were less expressed in biofilms with prevented dormancy. In the opposite, the ribosome pathway was more expressed in *S. epidermidis* biofilms grown with Mg^{2+}. Differentially expressed genes encoded transcripts mostly localized in the intracellular compartments, suggesting that the major changes related to the development of dormancy are occurring within the cell cytoplasm. These results were expected since *S. epidermidis* biofilms grown in excess glucose and Mg^{2+} have a lower number of dormant bacteria and, consequently, more metabolically active cells. In

Table 2 List of the 10 genes with higher RPKM values among the differentially expressed genes, in *S. epidermidis* biofilms grown in TSB 1 % G enriched with Mg^{2+}. Only genes with a fold change expression ≤−1.50 or ≥1.50 were included

	Gene	Definition	RPKM 1%G	RPKM 1%G+Mg^{2+}	Fold change	*p* value (FDR-corrected)
Rank downregulated						
1	*serp1782*	Alkaline shock protein 23	18,172.55	7,034.27	−2.58	0.003
2	*serp0244*	Aldo/ketoreductase	5,885.19	2,759.36	−2.13	<0.001
3	*pdhD*	Dihydrolipoamide dehydrogenase	4,352.90	2,433.65	−1.79	0.022
4	*pdhA*	Pyruvate dehydrogenase complex E1 component, alpha subunit	3,908.68	2,361.75	−1.65	0.026
5	*recA*[a]	Recombinase A	4,788.63	1,654.38	−2.89	<0.001
6	*serp1784*	Uncharacterized protein	4,967.70	1,624.00	−3.06	0.001
7	*mqo-4*	Malate:quinone oxidoreductase	3,457.27	1,590.09	−2.17	<0.001
8	*lexA*[a]	LexA repressor	3,307.97	1,571.21	−2.11	0.012
9	*aldA-2*	Aldehyde dehydrogenase	3,645.71	1,569.85	−2.32	0.011
10	*ptsI*	Phosphoenolpyruvate-protein phosphotransferase	2,156.90	1,350.52	−1.60	0.003
Rank upregulated						
1	*serp0419*	Ribosomal subunit interface protein	4,191.31	1,2241.98	2.92	0.018
2	*ctsR*	CtsR family transcriptional regulator	2,279.57	6,328.79	2.78	0.006
3	*serp0163*	UvrB/UvrC domain-containing protein	1,885.05	4,870.32	2.58	<0.001
4	*serp0164*	ATP:guanido phosphotransferase	1,775.17	4,289.27	2.42	0.031
5	*ppaC*	Manganese-dependent inorganic pyrophosphatase	443.67	861.75	1.94	<0.001
6	*rpsM*	30S ribosomal protein S13	425.78	698.09	1.64	<0.001
7	*serp1180*	Putative Holliday junction resolvase	368.63	583.88	1.58	<0.001
8	*rplE*	50S ribosomal protein L5	279.81	505.85	1.81	0.030
9	*ureA*	Urease subunit gamma	182.26	473.11	2.60	0.006
10	*rpsQ*	30S ribosomal protein S17	225.01	456.69	2.03	0.017

[a] lexA and recA "changes the occurrence of persister cells in bacterial populations" (Butala et al. 2011)

addition, half of the most downregulated gene transcripts in *S. epidermidis* biofilms grown with Mg^{2+} are involved in oxidoreductase activity, such as *serp0244*, *pdhA*, *pdhD*, *aldA*, and *mqo3* (Table 2). Conversely, the most upregulated genes are mainly related to metabolic processes. Previously, our group showed the impact on virulence of dormant *S. epidermidis* biofilm (Cerca et al. 2011a). In the present work, biofilms with lower levels of dormant cells had an increased, but not significant expression of some of the main virulence genes.

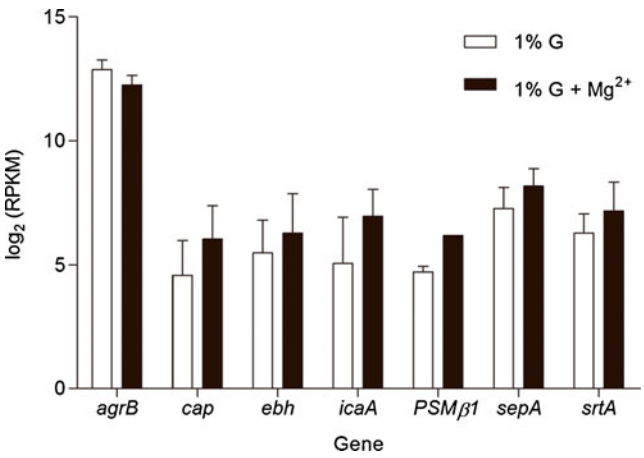

Fig. 6 Variation of $\log_2$(RPKM) among some known virulence genes in *S. epidermidis* biofilms grown with (1 % G+Mg^{2+}) or without (1 % G) magnesium. The *bars* represent the mean and the *error bars* the standard error of the mean

Glucose has a key role in biofilm physiology (Mack et al. 1992; Yao et al. 2005). The major pathways for glucose catabolism in Staphylococci are glycolysis, the pentose-phosphate pathways, and the tricarboxylic acid cycle (Blumenthal et al. 1974). Allison et al. (2011b) showed that metabolites from the upper glycolysis pathway and pyruvate, in combination with aminoglycosides, may be used to treat biofilms with higher levels of dormant, persister cells, proposing a metabolic-based strategy to eradicate bacterial persisters. Not surprisingly, glucose has influence in biofilm physiology since glucose metabolism leads to accumulation of acidic products decreasing the pH of the culture medium (Shu et al. 2000). The model that we previously described showed that dormancy was a consequence of excess of glucose catabolism that leads to the acidification of the culture medium and consequently to the accumulation of dormant bacteria within *S. epidermidis* biofilms (Cerca et al. 2011a). This phenomenon was prevented by the presence of high extracellular levels

Table 3 Statistically significant biological processes from gene set enrichment analysis

Gene set enrichment	Biological process	*p* value (Bonferroni correction)
Upregulation		
GO:0006412	Translation	4.56E−15
GO:0044267	Cellular protein metabolic process	2.52E−10
GO:0010467	Gene expression	3.75E−09
GO:0034645	Cellular macromolecule biosynthetic process	4.68E−09
GO:0019538	Protein metabolic process	8.31E−09
GO:0009059	Macromolecule biosynthetic process	8.58E−09
GO:0044249	Cellular biosynthetic process	2.89E−05
GO:0015986	ATP synthesis coupled proton transport	7.83E−05
GO:0009058	Biosynthetic process	1.84E−04
GO:0006754	ATP biosynthetic process	5.53E−04
GO:0044260	Cellular macromolecule metabolic process	1.07E−03
GO:0043170	Macromolecule metabolic process	4.97E−03
GO:0009142	Nucleoside triphosphate biosynthetic process	1.14E−02
GO:0034220	Ion transmembrane transport	1.14E−02
GO:0015992	Proton transport	2.65E−02
Downregulation		
GO:0008152	Metabolic process	9.76E−04
GO:0055114	Oxidation-reduction process	1.44E−03
GO:0006006	Glucose metabolic process	2.16E−02

of magnesium which did not change the pH of the culture medium, indicating that the Mg^{2+} effect on preventing cell dormancy was not related to the pH of the culture, since low pH was still maintained in the presence of Mg^{2+}. Low pH was previously associated with a loss of activity of glycolytic enzymes and damage to cell membrane and macromolecules such as proteins and DNA and was related to changes in biogenesis and maintenance of cell membrane integrity (revised in Cotter and Hill 2003).

Piddington and colleagues demonstrated that Mg^{2+} is essential to *M. tuberculosis* growth in acidic conditions, and it could not be replaced by other divalent cations (Piddington et al. 2000). A possible influence of Mg^{2+} in protein structure and physical properties was also described in *M. tuberculosis* (Bhatt et al. 2005). Since the Mg^{2+} levels in biofilm cultures were maintained over time, similar to the pH in the culture medium, we performed a search of *S. epidermidis* enzymes whose co-factor was magnesium (in http://enzyme.expasy.org). Interestingly, among 20 enzyme-encoding genes, only three of these were encoded by genes found to be differentially expressed between the two distinct biofilm conditions, with a lower expression in biofilms grown with $MgCl_2$, suggesting that Mg^{2+} is not increasing the expression of genes encoding proteins using Mg^{2+} as a co-factor. In addition, genes related to the Mg^{2+} transport system, such as *corA*, *mgtE*, and *serp1967* (Gill et al. 2005), were not differentially expressed, indicating that high Mg^{2+} level cultures are also not stimulating the expression of magnesium transport genes.

Previous work with dormant bacterial cultures demonstrated decreased levels of macromolecule synthesis, nutrient transport, and respiration rates (reviewed by Oliver 2005). Moreover, earlier transcriptomic studies of dormant persister cells of several microorganisms showed a downregulation of energy and metabolism pathways (Keren et al. 2011), increased reactive oxygen species levels (Bink et al. 2011), and downregulation of genes involved in energy production (Shah et al. 2006). Toxin overexpression from toxin-antitoxin modules has been linked to the persister fraction in *E. coli* and *M. tuberculosis* cultures (reviewed in Kint et al. 2012). The

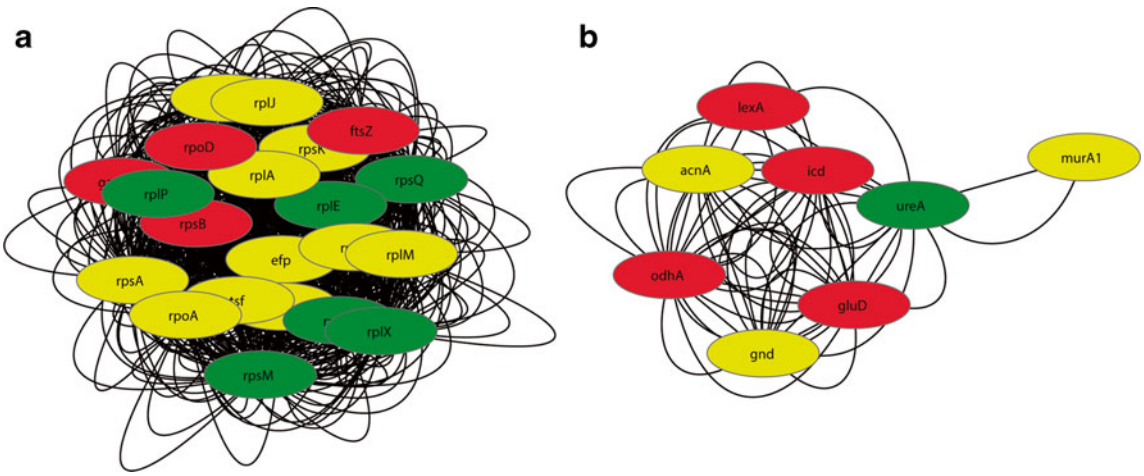

Fig. 7 Clusters generated by MCODE plugin in Cytoscape, including fold change expression. *Red*, *green*, and *yellow circles* represent fold change values under −1.50, above 1.50, and between −1.50 and 1.50, respectively. Biological processes are translation and biosynthetic processes (**a**); and tricarboxylic acid cycle, acetyl-CoA catabolic process, coenzyme catabolic process, co-factor catabolic process, acetyl-CoA metabolic process, aerobic respiration, cellular respiration, energy derivation by oxidation of organic compounds, coenzyme metabolic processes (**b**)

toxin-antitoxin module *mazEF* is highly conserved in *Staphylococcus* species and was described in detail in *S. aureus*, where antitoxin MazE binds toxin MazF to neutralize ribonuclease activity (Fu et al. 2007) and, more recently, in *Staphylococcus equorum* (Schuster et al. 2013). Despite no alterations in *mazF* expression between S. *epidermidis* biofilms grown in the absence or presence of Mg^{2+}, *mazE* gene RPKM was superior to 1.00 in biofilms grown without Mg^{2+}.

Taken together, these results reveal that the transcription of genes involved in oxidation-reduction processes as well as glucose metabolism was more active in dormant *S. epidermidis* biofilms, despite no differences in glucose consumption, probably due to lower metabolism of dormant bacteria. On the other hand, genes related to ribosome activity were upregulated in *S. epidermidis* biofilms when Mg^{2+} levels were higher in the growth medium. Additionally, these results provide evidence that the genes directly involved in Mg^{2+} transport do not seem to have a direct role in the shift to dormancy. However, the global changes found in this work do not provide information only confined to the dormant bacterial cells. Most probably, differences among readily culturable and dormant bacterial cells are greater than those referenced here. Single-cell analysis methods are being developed and may allow a more accurate comparison between these physiological states (Li and Xie 2011). Yet, dormant bacterial cell isolation is still a meaningful challenge.

Overall, these results contribute to a better understanding of the mechanisms underlying the dormancy phenomena. Nevertheless, these findings also raise questions regarding the role of dormant *S. epidermidis* biofilms in virulence, since it is still debatable if there is a lower virulence potential due to lower metabolism, or if expression of a specific gene or a set of genes could lead to the suppression of the host immune response.

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