Small Molecules Produced by Commensal *Staphylococcus epidermidis* Disrupt Formation of Biofilms by *Staphylococcus aureus*

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ABSTRACT The microbiota influences host health through several mechanisms, including protecting it from pathogen colonization. *Staphylococcus epidermidis* is one of the most frequently found species in the skin microbiota, and its presence can limit the development of pathogens such as *Staphylococcus aureus*. *S. aureus* causes diverse types of infections ranging from skin abscesses to bloodstream infections. Given the increasing prevalence of *S. aureus* drug-resistant strains, it is imperative to search for new strategies for treatment and prevention. Thus, we investigated the activity of molecules produced by a commensal *S. epidermidis* isolate against *S. aureus* biofilms. We showed that molecules present in *S. epidermidis* cell-free conditioned media (CFCM) caused a significant reduction in biofilm formation in most *S. aureus* clinical isolates, including all 4 *agr* types and *agr*-defective strains, without any impact on growth. *S. epidermidis* molecules also disrupted established *S. aureus* biofilms and reduced the antibiotic concentration required to eliminate them. Preliminary characterization of the active compound showed that its activity is resistant to heat, protease inhibitors, trypsin, proteinase K, and sodium periodate treatments, suggesting that it is not proteinaceous. RNA sequencing revealed that *S. epidermidis*-secreted molecules modulate the expression of hundreds of *S. aureus* genes, some of which are associated with biofilm production. Biofilm formation is one of the main virulence factors of *S. aureus* and has been associated with chronic infections and antimicrobial resistance. Therefore, molecules that can counteract this virulence factor may be promising alternatives as novel therapeutic agents to control *S. aureus* infections.

IMPORTANCE *S. aureus* is a leading agent of infections worldwide, and its main virulence characteristic is the ability to produce biofilms on surfaces such as medical devices. Biofilms are known to confer increased resistance to antimicrobials and to the host immune responses, requiring aggressive antibiotic treatment and removal of the infected surface. Here, we investigated a new source of antibiofilm compounds, the skin microbiome. Specifically, we found that a commensal strain of *S. epidermidis* produces molecules with antibiofilm activity, leading to a significant decrease of *S. aureus* biofilm formation and to a reduction of previously established biofilms. The molecules potentiated the activity of antibiotics and affected the expression of hundreds of *S. aureus* genes, including those associated with biofilm for-

mation. Our research highlights the search for compounds that can aid us in the fight against *S. aureus* infections.

The human microbiota is considered a crucial factor that influences the state of health and disease of the host. In recent years, studies have correlated the micro-biota composition with several human diseases, and its role in protection against infections has been addressed (1). Most of these studies have focused on the gut microbiota, and relatively little is known about the complexity of the skin microbiota and the interactions among its components. The skin is the most extensive and the most exposed organ of the human body and is considered a first line of defense against environmental conditions, harmful chemicals, and exogenous pathogens (2). It is currently known that the composition of the skin microbiota can vary according to the skin site and host characteristics, such as age, gender, and lifestyle (3). In general, *Staphylococcus*, *Propionibacterium*, *Micrococcus*, and *Corynebacterium* are the most abundant bacterial genera on the superficial layers of the human skin (4). *Staphylococcus epidermidis* is one of the most frequently isolated species from human epithelial samples, being considered ubiquitous in the human skin (5). Studies have shown that this species has an arsenal of self-defense mechanisms aiming to limit the establish-ment and growth of possible skin pathogens by the production of bacteriocins and proteases (5, 6). Moreover, it has been described that molecules produced by *S. epidermidis*, known as phenol-soluble modulins, have antimicrobial activity against pathogens such as *Streptococcus pyogenes* and *Staphylococcus aureus* (7, 8). Further-more, during the treatment of atopic dermatitis patients, which includes a regimen of dilute bleach baths, topical steroids, bland emollients, and, in some cases, systemic antibiotics, a significant decrease in the numbers of *S. aureus*, the major pathogen related to the aggravation of skin lesions, with a concomitant increase of *S. epidermidis* colonization, has been observed (9). In addition, the introduction of *S. epidermidis* strains producing the protease Esp into the nasal cavities of *S. aureus* asymptomatic carriers resulted in clearance of *S. aureus* colonization (6). Therefore, a possible antag-onistic relationship has been suggested to exist between these species, supporting the hypothesis that pathogens are kept under control by skin microbiota members.

S. aureus is associated with high morbidity and mortality rates, causing ailments that range from skin and soft tissue infections to invasive diseases such as pneumonia, bacteremia, and meningitis (10). Resistance to several antibiotics is a common trait of *S. aureus* clinical strains, and methicillin-resistant *S. aureus* (MRSA) isolates are a huge concern for public health due to the limited treatment options available (11). One of the virulence factors that contributes to the success of *S. aureus* infections is the ability to form biofilms on biotic (bones and heart valves) and abiotic (catheters, prostheses, and other medical devices) surfaces (12). The process of biofilm formation induces many phenotypic alterations, including reduced growth rate and higher tolerance to antibi-otics and host immune responses (13, 14). Some antibiotics act efficiently on planktonic cells but not on cells grown in biofilms, leading to recurrent infections (15). Thus, biofilm formation is associated with chronic and recurrent infections caused by *S. aureus* and is currently considered a worldwide challenge for the treatment of infec-tions caused by this pathogen (15).

Due to the ability of *S. aureus* to produce biofilms and the high antimicrobial resistance rates found in this pathogen, therapeutic alternatives are limited. Therefore, the search for new compounds to combat *S. aureus* infections is urgent. Recent studies showed that the human microbiota is a source of antivirulence molecules, indicating a potential novel source of substances against multidrug-resistant pathogens (16–18). The major advantage of employing an antivirulence approach is to impose lower selective pressure when compared to conventional antibiotics, delaying the develop-ment of resistant variants. In addition, antivirulence molecules will likely only cause a

TABLE 1 Characteristics of *S. aureus* clinical strains used in this study[a]

Strain	Isolation site	Methicillin susceptibility	Agr type	Biofilm production	Matrix type	Reduction of biofilm formation by CFCM (%)[b]
1348	**Blood**	**MSSA**	**3**	**Strong**	**Mix**	**96.8**
1452	**Blood**	**MSSA**	**1**	**Strong**	**Ptn**	**86.3**
74	**Skin lesion**	**MSSA**	**3**	**Moderate**	**Ptn**	**77.0**
1453	**Blood**	**MSSA**	**4**	**Strong**	**Ptn**	**66.0**
1602	**Blood**	**MRSA**	**3**	**Moderate**	**Mix**	**85.0**
1173	**Blood**	**MRSA**	**3**	**Moderate**	**Ptn**	**80.6**
14	**Skin lesion**	**MRSA**	**2**	**Strong**	**Ptn**	**78.1**
1580	**Blood**	**MRSA**	**3**	**Moderate**	**Mix**	**75.4**
833	**Urine**	**MRSA**	**3**	**Strong**	**Ptn**	**73.9**
1125	**Prosthesis**	**MRSA**	**3**	**Strong**	**Pol**	**63.2**
ATCC 33591	**—**	**MRSA**	**1**	**Strong**	**Ptn**	**62.1**
527	**BAL**	**MRSA**	**3**	**Strong**	**Ptn**	**60.3**
920	**BAL**	**MRSA**	**3**	**Strong**	**Ptn**	**52.2**
1035	Skin lesion	MSSA	3	Strong	ND	48.2
26	Skin lesion	MSSA	2	Moderate	ND	45.9
297	Skin lesion	MSSA	2	Moderate	ND	45.3
244	Skin lesion	MSSA	2	Moderate	Mix	40.4
1445	Blood	MSSA	1	Moderate	Ptn	32.7
326	Skin lesion	MSSA	1	Moderate	Mix	37.4
1166	Blood	MSSA	1	Moderate	ND	35.5
41	Skin lesion	MSSA	1	Moderate	ND	28.0
100	Skin lesion	MSSA	1	Strong	Mix	20.0
10	Skin lesion	MSSA	1	Moderate	Ptn	18.0
57	Skin lesion	MSSA	1	Moderate	Mix	−7.5
112	Skin lesion	MSSA	2	Moderate	Ptn	−4.0
1349	Blood	MSSA	3	Strong	Mix	−7.9
1380	Blood	MRSA	3	Moderate	Ptn	23.0
1581	Blood	MRSA	3	Moderate	ND	14.0
255	Skin lesion	MRSA	2	Moderate	ND	9.6

[a]MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; BAL, bronchoalveolar lavage; Mix, mixed matrix composition (similar reduction with proteinase K and sodium metaperiodate); Ptn, mostly protein-based matrix composition (major reduction with proteinase K); Pol, mostly polysaccharide matrix composition (major reduction with sodium periodate); bold, strains with over 50% reduction in biofilm formation; ND, not determined; —, information not provided in source references.
[b]Reduction compared to control CFCM.

minor impact on host commensals, acting efficiently and specifically on the targeted virulence factors (19). Considering that the microbiota competes with exogenous pathogens for host colonization, we hypothesized that molecules produced by members of the skin microbiota would impact the virulence of skin pathogens. Here, we show that molecules produced by the commensal *S. epidermidis* have a drastic impact on *S. aureus* global gene expression and on its ability to form biofilms.

RESULTS

Cell-free conditioned media (CFCM) from different *S. epidermidis* isolates grown for 24 h in Trypticase soy broth (TSB) media were collected and concentrated, and their activity on *S. aureus* biofilm formation was tested. CFCM from all *S. epidermidis* strains tested showed significantly inhibited *S. aureus* biofilm formation, with CFCM from one commensal isolate (RF1) showing the strongest reduction (Fig. S1A). This isolate was chosen for further analysis. The activity of RF1 CFCM was tested against biofilm production using 29 previously characterized *S. aureus* clinical isolates, both methicillin-susceptible *S. aureus* (MSSA) and MRSA, belonging to the 4 *agr* types, and isolated from diverse types of infections (Table 1). When *S. aureus* was grown in the presence of CFCM, biofilm production was reduced over 30% in 20 (69%) strains, compared to biofilm formation with the addition of control CFCM. Biofilm formation without the addition of control CFCM or CFCM was also used for comparison, and the vast majority of strains tested were not affected by control CFCM. Nevertheless, CFCM significantly reduced biofilm formation for all strains tested, regardless of whether control CFCM or no-addition controls were used for comparison (Fig. S1B). A more pronounced reduction (over 50%) in biofilm formation was observed for 13 (65%) of these strains, which
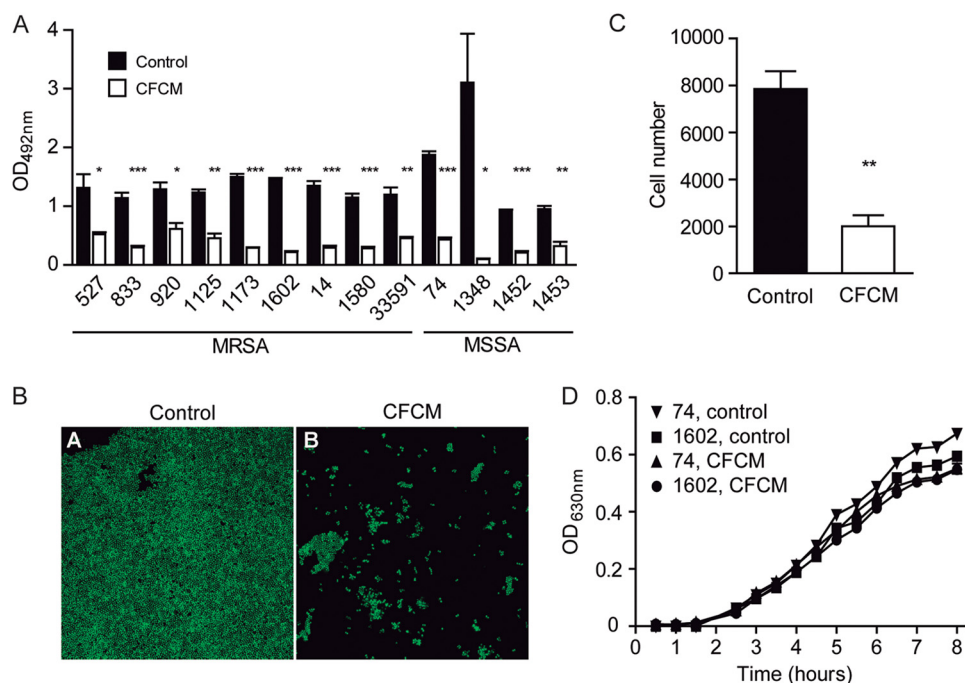
FIG 1 Cell-free conditioned media (CFCM) from commensal *S. epidermidis* represses biofilm production of *S. aureus* clinical isolates. (A) Biofilm formation of selected *S. aureus* clinical isolates grown in the presence or absence of *S. epidermidis* CFCM. (B) Confocal microscopy of biofilms produced by *S. aureus* clinical isolate (MRSA isolate 1602) grown in the presence or absence of *S. epidermidis* CFCM. (C) Cell count on *S. aureus* (MRSA isolate 1602) biofilm cells grown in the presence or absence of *S. epidermidis* CFCM. (D) Growth curves of selected *S. aureus* clinical isolates with or without *S. epidermidis* CFCM. OD, optical density; upside-down triangles, MSSA isolate 74 control; squares, MRSA isolate 1602 control; triangles, MSSA isolate 74 with CFCM; circles, MRSA isolate 1602 with CFCM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

were selected for further analyses of CFCM activity (Fig. 1A). Curiously, among the strains showing an increased susceptibility to the effect of CFCM on biofilm formation (over 50%), 69.2% were MRSA. In contrast, 66.6% of the strains whose biofilm formation was not impacted (less than 30% reduction) by CFCM were MSSA (Table 1). We also analyzed the composition of biofilms formed by all 13 isolates that showed greater reduction (over 50%) with CFCM, as well as 3 isolates with reduction between 30 and 50% and 6 isolates with less than 30% reduction or no reduction. Among the 13 strains with greater biofilm reduction, 9 had biofilms composed mainly of protein, and 1 strain had a polysaccharide-based biofilm. Three other strains had mixed biofilms, with similar biofilm reduction caused by proteinase K or sodium metaperiodate. These data show that RF1 CFCM has activity against biofilms with different matrix compositions (Table 1). Among the isolates that showed intermediate reduction (30 to 50%) and little or no reduction (<30%), the composition of the biofilm matrix was also heterogeneous (5 mixed and 4 proteinaceous). We then selected the strain that showed the greatest reduction in biofilm formation by CFCM (strain 1602) and performed confocal microscopy analyses, the results of which confirmed the initial observation of the impact of CFCM on biofilm production and cell number (Fig. 1B and C). The impact of CFCM on *S. aureus* planktonic growth was verified, and no significant effects were observed (Fig. 1D).

We then investigated if the effect of CFCM during the formation of biofilms could also be observed on established *S. aureus* biofilm. The molecules secreted by *S. epidermidis* showed activity on established *S. aureus* biofilms, a desirable feature for the treatment of biofilm-associated infections (Fig. 2A). The effect was variable, depending on the *S. aureus* strain tested, with biofilm reduction ranging from 7.2% to 58.8%. Most tested strains (12/13; 92.3%) had a reduction greater than 18% on the biofilm after addition of *S. epidermidis* CFCM (Fig. 2A).
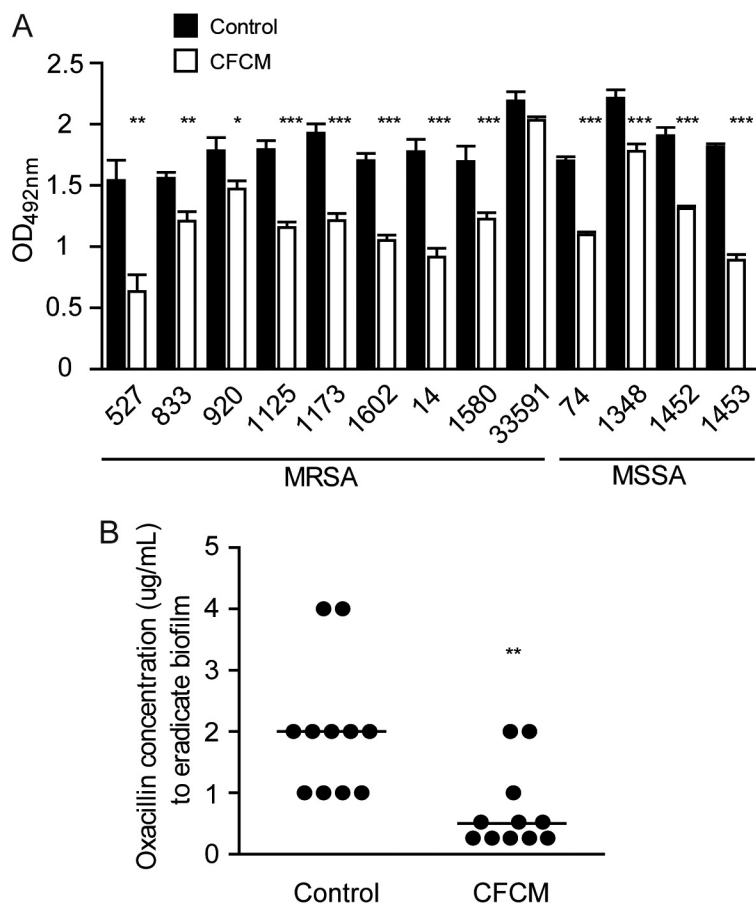
FIG 2 Activity of *S. epidermidis* cell-free conditioned media (CFCM) on preestablished *S. aureus* biofilm. (A) Optical density of biofilm matrix staining after treatment with either the control medium or *S. epidermidis* CFCM of 24-h preestablished biofilms. (B) Oxacillin concentration used to obtain nonviable preestablished biofilms (MSSA isolate 74) cells with or without *S. epidermidis* CFCM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

Next, we tested the ability of CFCM to reduce the minimal inhibitory concentration (MIC) of oxacillin required to eradicate previously established *S. aureus* biofilms. The addition of CFCM alone did not affect the viability of biofilm cells. However, when CFCM was used in combination with oxacillin, there was a reduction of the concentration of the antibiotic necessary to eliminate viable biofilm cells. When oxacillin alone was used to treat biofilms, an average concentration of 2 μg/ml of the antibiotic was required to disrupt biofilms. On the other hand, when CFCM and oxacillin were used in combination, an average concentration of 0.25 μg/ml of the antibiotic was enough to achieve the same level of biofilm disruption (Fig. 2B), suggesting an additive effect between CFCM and oxacillin.

Transcriptomic analysis was then performed by transcriptome sequencing (RNA-seq) to determine the effect of CFCM on *S. aureus* gene expression. In particular, we sought to determine if the expression of genes involved in biofilm formation by *S. aureus* was modulated by *S. epidermidis*-secreted molecules. By doing so, we found 90.6% (2,601 out of 2,872) of the genes were expressed in at least one of the conditions (control or CFCM). Of these, 30.5% of all detected genes (876) had their expression levels modulated by CFCM. Of these, 407 genes were upregulated and 469 were downregulated when *S. aureus* was grown in the presence of *S. epidermidis* CFCM (Table S1; Fig. 3). Additionally, we found eight genes that had no identifiable orthologs in the reference strain as regulated by CFCM (2 activated and 6 repressed; Table S2). Within the group of genes that were differentially expressed in the presence of CFCM, nine have been
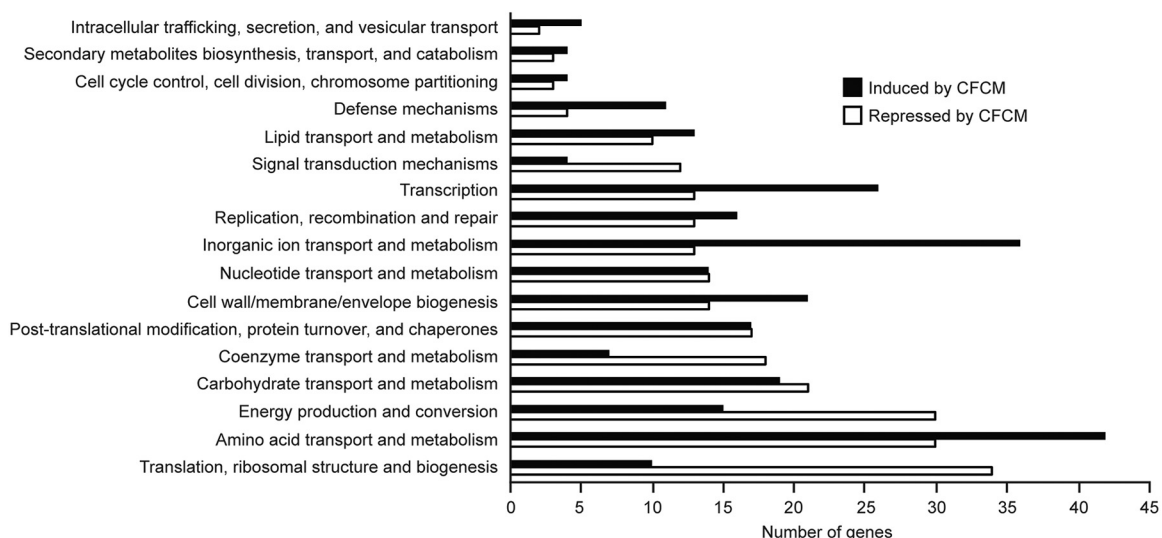
FIG 3 Metabolic pathways affected by *S. epidermidis* cell-free conditioned media (CFCM) on *S. aureus* biofilms, as determined by RNA-seq.

previously associated with *S. aureus* biofilm formation (Table 2). Among these, expression of *icaR*, an important negative regulator of the *icaADBC* operon, which encodes the major *S. aureus* biofilm polysaccharide matrix PIA/PNAG [polysaccharide intercellular adhesin/poly-β(1-6)-*N*-acetylglucosamine], was upregulated (3.7-fold). Transcription of *rsp*, an AraC-type transcriptional regulator that inhibits attachment and biofilm formation in *S. aureus*, was also induced by CFCM (2.7-fold) (20, 21). Some of the genes associated with biofilm formation showed reduced expression, including *sasG* (4.1-fold), which encodes *S. aureus* surface protein G, *hla* (5.1-fold), which encodes an alpha-hemolysin, *spa* (4.4-fold), which encodes protein A, and the genes of the *agr* system (*agrA* [2.1-fold], *agrB* [2.4-fold], and *hld* [9.5-fold]). Transcription of *clpC*, encoding an ATPase required for stress tolerance and biofilm formation in *S. aureus*, was also repressed (10.5-fold) (22). In order to validate the RNA-seq results showing differential expression of genes involved in biofilm formation, we evaluated the effect of CFCM on the expression of *icaR* and *icaA* by real-time quantitative PCR (RT-qPCR). Our results confirmed the upregulation of *icaR* and also showed a significant reduction of *icaA* expression in response to CFCM, in agreement with the biofilm phenotype observed (Fig. 4).

Next, we initiated the characterization of the active molecules present in the CFCM of *S. epidermidis*. Boiling the CFCM for 40 min did not reduce its effect on *S. aureus* biofilm formation, and neither did proteinase K, trypsin, and sodium periodate treatments followed by heat inactivation (Fig. 5A). We also treated the CFCM with a protease inhibitor cocktail since some *S. epidermidis* isolates are known to produce the serine protease Esp, which has antibiofilm activity (6). Treatment with this cocktail, which

TABLE 2 Transcriptional analysis of genes associated with *S. aureus* biofilm formation in response to *S. epidermidis* cell-free conditioned media

| Gene	Predicted function	Fold change	*P* value
icaR	*ica* operon transcriptional regulator	3.74	0.00005
rsp	AraC-type transcriptional regulator	2.69	0.00005
clpC	ATP-dependent Clp protease ATP-binding subunit ClpC	−10.52	0.00005
hld	Delta-hemolysin	−9.50	0.00005
hla	Alpha-hemolysin	−5.10	0.00005
spa	Protein A	−4.45	0.00005
agrB	AgrB transmembrane protein	−2.40	0.00015
agrA	AgrA response regulator	−2.15	0.00005
sasG	*S. aureus* surface protein G	−4.15	0.00005
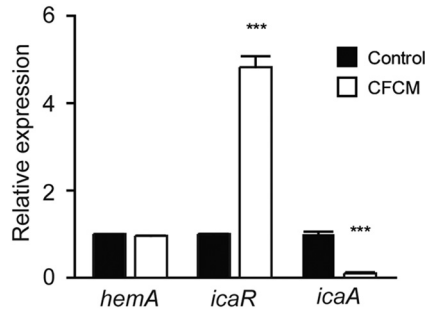
FIG 4 Expression of *icaR* and *icaA* genes in response to *S. epidermidis* cell-free conditioned media (CFCM) on *S. aureus* (MRSA isolate 1602) biofilms, as determined by RT-qPCR. ***, $P < 0.0001$.

includes inhibitors of serine proteases, did not abolish CFCM antibiofilm activity. Additionally, extraction of the CFCM with ethyl acetate resulted in the recovery of a biologically active fraction, indicating that the active molecules are soluble in this solvent and therefore may be either nonpolar or only mildly polar (Fig. 5B). Fractionation of the ethyl acetate-soluble fraction of CFCM by use of centrifugal filters indicated that the active molecules are between 3 and 10 kDa (Fig. 5B). Altogether, these results indicate that the antibiofilm molecules present in the CFCM of commensal *S. epidermidis* are heat, proteinase K, trypsin, sodium periodate, and protease inhibitor resistant, between 3 and 10 kDa, and hydrophobic in nature.

In order to determine if the *agr* quorum-sensing system is involved in the inhibition of biofilm formation by CFCM, we tested CFCM activity on two *agr*-deficient *S. aureus* strains, RN4220 and Mu50 (23, 24). CFCM significantly reduced biofilm formation on both strains; biofilm formation by Mu50 was reduced by 52.4% in the presence of CFCM, and a slightly more pronounced effect was observed in RN4220, where biofilm formation was reduced by 58.8%. Altogether, these data show that a functional *agr* system is not required for CFCM antibiofilm activity (Fig. 5C).

DISCUSSION

The skin is the largest organ in the human body and is directly exposed to the environment, where it is constantly challenged by potential exogenous pathogens. In a similar way to the intestinal microbiota, the skin microbiota is associated with
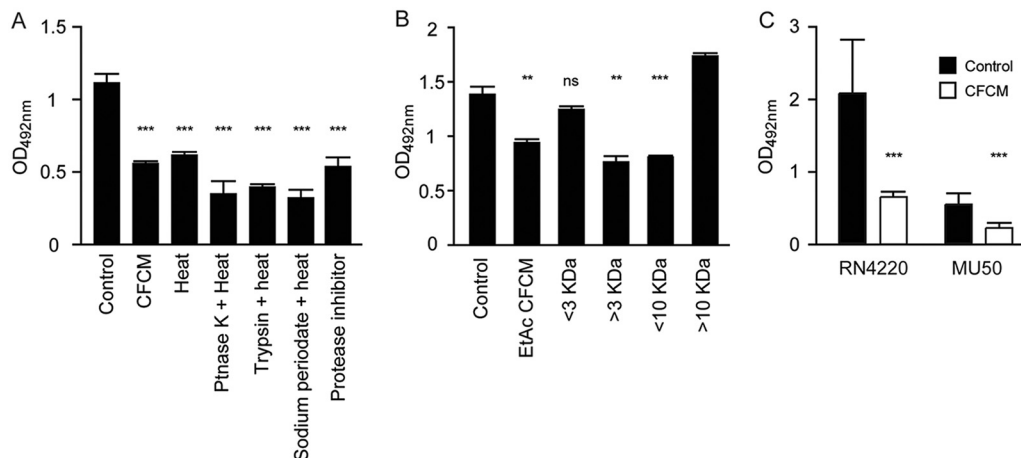

FIG 5 Characterization of the antibiofilm molecules present in *S. epidermidis* cell-free conditioned media (CFCM). (A) Biofilm formation of the *S. aureus* clinical isolate 1602 grown in the presence of *S. epidermidis* CFCM treated with heat, proteinase K, trypsin, sodium periodate, or protease inhibitors. (B) Biofilm formation of the *S. aureus* clinical isolate 1602 grown in the presence of *S. epidermidis* CFCM fractionated by molecular weight (kDa) after ethyl acetate extraction. (C) Biofilm formation of *S. aureus* RN4220 and MU50 *agr*-defective strains grown in the presence of *S. epidermidis* CFCM. **, $P < 0.01$; ***, $P < 0.0001$.

protection against pathogens (5). In recent studies, our group showed that the intestinal microbiota produces compounds with antivirulence activity, which may lead to new alternative therapeutic strategies (17, 25). Moreover, in addition to the bacteriocins produced by *S. epidermidis* (5), *Cutibacterium acnes*, another commensal bacterium from skin, was shown to suppress the growth of MRSA isolates (26). These and other studies have produced a solid body of evidence that the skin microbiota can also be a potential source of new compounds that may aid the treatment of drug-resistant infections (27–29).

The ability of *S. aureus* to acquire antimicrobial resistance genes and to produce biofilms makes infections increasingly hard to treat (30). Some molecules that act on *S. aureus* virulence have been described. For instance, savirin, a small molecule that acts on the important quorum-sensing regulator AgrA (31), is currently in the preclinical phase, and other molecules that act on *S. aureus* toxins are currently part of ongoing phase II studies (19). Because of the importance of biofilms in the persistence and recurrence of *S. aureus* infections, some studies have looked for alternative therapies that act on this virulence trait with molecules from various sources (32–35). Antibiofilm compounds derived from marine microorganisms were described, and recently, butenolide, a nontoxic compound derived from a marine *Streptomyces* sp., was shown to be effective in inhibiting MRSA biofilm formation and eradicating established biofilms (35). Also, a cell-free supernatant from commensal *Staphylococcus chromogenes* was shown to have antibiofilm properties against coagulase-negative *Staphylococcus* clinical isolates and a limited impact on *S. aureus* clinical isolates (36). Unlike the bioactivity described herein, the authors found that the activity was lost when the supernatant was treated with heat or proteinase K (36).

Here, we describe that CFCM of a commensal *S. epidermidis* strain isolated from the skin of a healthy donor contains molecules with a significant impact on biofilm formation of *S. aureus* clinical isolates without affecting bacterial growth. The effect is not universal, since some *S. aureus* clinical isolates were not affected. However, our data suggest a certain degree of correlation between CFCM activity and resistance to methicillin, which could be due to the proteinaceous composition of the biofilm matrix of MRSA isolates. In the literature, MRSA strains are known to produce protein-based biofilms, whereas MSSA strains usually produce polysaccharide-based structures (37). We analyzed the matrix composition of *S. aureus* biofilms, focusing on the 13 strains that showed the greatest reduction by CFCM, alongside a few representatives of strains that showed little or no reduction. These analyses showed that biofilms of most MRSA strains showed greater reduction when treated with proteinase K. However, one MRSA isolate showed greater reduction with sodium periodate, and two isolates showed similar reduction with both treatments, suggesting that they produce mixed biofilms. Among MSSA strains, 6 showed protein-based biofilm matrices, whereas the other 6 produced mixed biofilm matrices. Therefore, our results did not show a correlation between methicillin susceptibility, biofilm matrix composition, and susceptibility to the antibiofilm effect of CFCM. Even though proteinase K and sodium periodate treatments have been used in many studies to define the major components of *S. aureus* biofilms, biofilm matrices contain a variety of macromolecules. For example, in a study with *Staphylococcus lugdunensis*, known to produce protein-based biofilms, researchers showed, using specific fluorescent dyes and confocal laser scanning microscopy, that its biofilm contains both PIA and proteins (38). Furthermore, another study showcased the presence of PIA/PNAG in MRSA biofilm matrices using staining with a fluorochrome associated with an anti-PNAG antibody (39).

In addition to the effect shown by CFCM during biofilm formation, the active molecules produced by *S. epidermidis* were also able to reduce preestablished *S. aureus* biofilms and, when tested in combination with oxacillin, decreased the antibiotic concentration necessary to eliminate biofilm cells, suggesting that the active molecules could be used in combination with antibiotics to improve their activity against *S. aureus* biofilms.

A transcriptomic analysis of biofilms grown in the presence of *S. epidermidis* CFCM

showed an increase of *icaR* expression. IcaR is an important negative regulator of the *icaADBC* operon, binding to its promoter region and repressing PIA/PNAG, a major biofilm component of *S. aureus* (40). Even though *icaABDC* was not shown as significantly repressed in our RNA-seq data, RT-qPCR experiments showed a reduction of *icaA* expression by biofilm cells grown in the presence of *S. epidermidis* CFCM, supporting the notion that activation of *icaR* expression by CFCM results in *icaADBC* operon repression, leading to decreased biofilm formation. Together, these data suggest that molecules secreted by *S. epidermidis* can promote inhibition of *S. aureus* biofilm through the repression of PIA/PNAG in an *icaR*-dependent manner. Another transcriptional regulator induced by *S. epidermidis* CFCM was Rsp, which was shown to repress genes that affect biofilm formation, including *icaABC* (21). Recently, this gene was shown to be repressed in *S. aureus* biofilms (73). Furthermore, Rsp was shown to have proteolytic properties, which correlates with the biofilm reduction when expression of this gene is induced.

Another important molecule for biofilm formation in *S. aureus* is the SasG adhesin, relevant for the attachment and early accumulation steps of *ica*-independent biofilm formation (15). Our transcriptomic analysis showed significant repression of *sasG* by *S. epidermidis* CFCM during biofilm formation, suggesting that repression of *icaADBC* is not the only pathway involved in the biofilm disruption phenotype. Transcriptome analysis also showed repression of multifunctional proteins that have been related not only to the accumulation step in *S. aureus* biofilm formation but also to its virulence, such as protein A (*spa*) (15) and alpha-hemolysin (*hla*) (41). The role of protein A in *S. aureus* evasion is well-known due to its ability to bind the Fc chain of immunoglobulins (42). In addition, the proinflammatory properties of protein A contribute to inflammation in patients with atopic dermatitis, who are commonly colonized by *S. aureus* (43). Alpha-hemolysin can bind to erythrocytes, monocytes, platelets, endothelial cells, and lymphocytes, as well as epithelial cells (44), and increased expression of *hla* is an important marker of the shift from commensalism to pathogenic behavior in *S. aureus* (27, 45). Another relevant gene whose expression was modulated by CFCM is *clpC*; CFCM strongly repressed its expression. This gene encodes an ATPase that was previously shown to be required for stress tolerance and biofilm formation in *S. aureus*. In the absence of this gene, biofilm formation was shown to be significantly reduced (22).

In addition to the effects on *icaR* and *sasG*, our transcriptomic analysis showed that molecules secreted by *S. epidermidis* affected the *S. aureus agr* system; addition of CFCM to *S. aureus* cultures inhibited the expression of *agrA*, *agrB*, and *hld*. The *agr* system is a major *S. aureus* virulence regulator; it can induce the expression of several toxins, such as α-, β-, and γ-hemolysin; leukotoxins; and toxic shock syndrome toxins (TSSTs), as well as proteases (46). Some studies have proposed compounds with the ability to inhibit *agr* as a new therapeutic approach against *S. aureus* infections (47). However, besides causing a decrease in toxin expression, inactivation of *agr* has been associated with increased biofilm formation in some *S. aureus* strains (46, 48, 49). Compounds that not only decrease *agr* activity but also impair biofilm formation, like the ones present in *S. epidermidis* CFCM described here, may have important therapeutic potential.

Altogether, our data show that the antibiofilm activity displayed by *S. epidermidis* CFCM is not due to an autoinducing peptide (AIP) effect on the *S. aureus agr* system. Canovas et al. showed that AIPs from some *Staphylococcus* spp., including one out of the four *S. epidermidis* isolates tested, downregulate the *S. aureus agr* system (50). However, as mentioned earlier, data from several papers show that inhibition of the *S. aureus agr* system does not lead to biofilm reduction; rather, *agr* inhibition is associated with a significant increase in biofilm formation (46, 48, 49). Vuong et al. showed that AIP from *S. epidermidis* significantly improved biofilm formation by *S. aureus* (51). When the study was published, the occurrence of different *agr* types in *S. epidermidis* had not yet been described, and therefore, we do not know what type of AIP was tested. However, other studies have since shown that only AIP I of *S. epidermidis* can inhibit the *agr* system of *S. aureus* in all but one (type IV) *agr* types (52, 53). Our data show that an *S. epidermidis agr* type II strain (RF1) can inhibit biofilm formation by several *S. aureus*

strains from all *agr* types, including previously characterized *agr*-defective strains (MU50 and RN4220) (23, 24). Therefore, CFCM antibiofilm activity does not appear to be due to AIP regulation. Nevertheless, the activity of CFCM on *S. aureus* gene expression appears to act through multiple pathways, including *agr* signaling, as, according to transcriptomic data, CFCM inhibits *agr* expression, along with downstream virulence genes regulated by quorum sensing.

Recently, phenol-soluble modulins (PSMs) present in the supernatants of *S. epidermidis* and *S. aureus* cultures were identified as having a negative impact on *S. aureus* persister cell frequency after treatment with antibiotics (54). It has been suggested that biofilms may be formed by a significant proportion of persister cells, and therefore, the effect of PSMs on persister cell frequency could be extrapolated to an effect on biofilm formation as the one described herein. However, it is unlikely that the active molecule present in RF1 CFCM is a PSM due to its molecular size and the protease-resistant nature of its activity on biofilm formation. Other studies investigating the effect of bioactive compounds on *S. aureus* biofilm formation have been published. While studying molecules produced by *C. acnes* (formerly *Propionibacterium acnes*), Wollenberg et al. have been able to identify coproporphyrin III, a molecule present in a *C. acnes*-conditioned medium that increases *S. aureus* biofilm formation (55). Altogether, studies in this area will aid in our understanding of the intricate chemical interplay between different species inhabiting the human body.

The goal of the present study was to shed light on the interactions between a major human skin commensal and one of the most important human skin pathogens, which is currently a global health issue. To do this, we focused on a specific trait of *S. aureus* that is directly linked to its virulence and ability to resist antimicrobial therapy, namely, the development of biofilms. Additionally, the analysis of the impact of *S. epidermidis*-secreted molecules on the *S. aureus* transcriptome enabled us to look at how this commensal can affect this important pathogen at the whole-genome level. However, it is safe to assume that the gene expression changes observed are not caused solely by the molecule responsible for the antibiofilm activity of CFCM. Thousands of molecules are likely present in CFCM, and many of these molecules may have specific effects on *S. aureus* physiology. Therefore, despite our interest in seeking antibiofilm molecules as new therapeutic strategies, the activity of CFCM on the *S. aureus* transcriptome may be a result of multifactorial events that occur between these species. Although the use of CFCM allowed us to probe the global effects that may occur during these interactions, it also represents a limitation of our study with regard to the identification of potential therapeutic agents. Nevertheless, we have generated some significant data that will aid in the identification of the active molecule, and our work opens an avenue for further bioprospection of the human skin microbiome for bioactive molecules. Future work will focus on determining the exact chemical nature of the bioactive compound and on describing its mode of action. In the future, this and other bioactive molecules from the human microbiome could be used as alternatives to the currently available antibiotics, with the goal of circumventing the phenomenon of antimicrobial drug resistance. Additionally, and perhaps in a more short-term approach, such molecules may be used in combination with antibiotics in order to disturb bacterial biofilms and decrease the antimicrobial concentrations required to treat biofilm-related infections.

MATERIALS AND METHODS

Ethics statement. This study was carried out in accordance with the recommendations and approval of the Research Ethics Committee of the National School of Public Health, Oswaldo Cruz Foundation, Brazil (protocol number, 2.919.895). All subjects provided written informed consent.

Bacterial strains. For two volunteer subjects who were included in the study, swabs previously moistened with sterile saline (0.85%) were rubbed against the heel area and cultured on mannitol salt agar and blood agar. Three commensal *S. epidermidis* isolates were obtained and named RF1 (one subject), RF3, and RF5 (another subject). Initial experiments were performed with these three strains as well as other clinical *S. epidermidis* isolates, and strain RF1 was selected for further studies. All *S. epidermidis* strains were identified by conventional biochemical tests (56) and matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF microflex LT; Bruker Daltonics, Leipzig, Germany). The RF1 strain was characterized as *agr* type II by PCR (57), and it is a nonbiofilm

producer. *S. aureus* clinical isolates categorized as strong or moderate biofilm producers (29 isolates) (58), including 13 MRSA and 16 MSSA, were selected from a previously characterized collection of strains of the Laboratório de Infecção Hospitalar at Universidade Federal do Rio de Janeiro (Table 1). The *agr* type of each of the strains was determined by PCR (59), and all 4 *agr* types were represented (type 1, 9 strains; type 2, 6 strains; type 3, 13 strains; and type 4, 1 strain). Two *agr*-defective strains, MU50 (23) and RN4220 (24), were also used in this study.

Biofilm matrix composition assay. To determine if biofilms were composed mainly of proteins or polysaccharides, established biofilms were treated with either proteinase K (100 μg/ml) or sodium periodate (40 mM) for 2 h at 37°C. After incubation, biofilms were washed with phosphate-buffered saline (PBS) and stained with 0.1% safranin as described for biofilm assays (21). The relative biofilm reduction elicited by each treatment compared to PBS (control) indicated the role of each component (proteins or polysaccharides) on biofilm matrix composition.

Cell-free conditioned medium preparations. After growth on Trypticase soy agar (TSA), the RF1 strain was inoculated in 50 ml of TSB (BD Diagnostics, Oxford, UK) for 24 h at 37°C with shaking (250 rpm). To obtain the CFCM, cultures were centrifuged at 3,100 $\times$ *g* for 15 min and filtered using 0.22-μm filters. CFCM was then evaporated to dryness using a Speed Vac Concentrator plus (Eppendorf, Hamburg, Germany), and the dried residue was resuspended in sterile 0.85% saline at a relative concentration of 20$\times$. This concentrated CFCM was used at 1:10 in the experiments described herein. Therefore, the final concentration of the CFCM in the experiments, relative to the original bacterial cultures, was 2$\times$. The same procedures were performed with culture media without bacterial inoculum, and the extract produced was used as a control for all experiments. For preliminary characterization of the active molecules, the stability of active molecules in CFCM was assayed after boiling for 40 min. CFCM was also treated with proteinase K (20 mg/ml) (Invitrogen, CA, USA), trypsin (20 μg/ml) (Promega, WI, USA), and sodium periodate (40 mM) (Sigma-Aldrich, MO, USA) for 1 h at 37°C, and all the treatments were inactivated by boiling the mixture for 40 min. In order to exclude effects of proteases that can be secreted by *S. epidermidis*, we also added 0.2% of a protease inhibitor cocktail P8340 (Sigma-Aldrich, MO, USA) to the CFCM. Ethyl acetate extraction was performed by adding ethyl acetate (1:1) to RF1 filtered supernatants and manually agitating and incubating the solution at room temperature for up to 20 min for phase separation. The soluble phase was collected, and ethyl acetate was evaporated using a Speed Vac. Dried residues were resuspended in saline, and the solution was fractionated by centrifugation using Amicon filters (Merck Millipore, MA, USA) with 3- and 10-kDa cutoff membranes for 30 min at 5,000 $\times$ *g*. Each of the fractions was also concentrated 20$\times$ using a Speed Vac.

Bacterial planktonic growth. Growth curves of *S. aureus* clinical isolates in the presence of CFCM or the control medium were performed on 96-well plates in triplicate. Overnight growth on TSB was diluted to an optical density at 630 nm (OD_{630}) of 0.05 in TSB with 10% CFCM or a control medium. The microplate was incubated with agitation at 37°C, and the OD_{630} was recorded every 30 min with an enzyme-linked immunosorbent assay (ELISA) auto reader (Thermoplate TP Reader, Thermoplate, MN, USA).

Biofilm formation. *S. aureus* biofilm formation was assessed by the microtiter plate test, as described elsewhere with modifications (58). Bacterial colonies were used to prepare cell suspensions in sterile distilled water with densities adjusted to the 0.5 McFarland standard. For analysis of the impact of the CFCM on biofilm formation, 120 μl of TSB 1% glucose was supplemented with 15 μl of CFCM, or control CFCM and 15 μl of the bacterial suspension were added. Additionally, *S. aureus* biofilm formation was also assayed without any additions. After incubation for 24 h at 37°C, the content of each well was removed, and the wells were carefully washed 3 times with 200 μl of PBS (pH 7.2) (Laborclin, Paraná, Brazil). The plates were then incubated at 60°C for 1 h and stained with 150 μl per well of 0.1% safranin for 15 min at room temperature. Excess stain was removed by rinsing the wells twice with PBS. The dye was then solubilized using 150 μl of a 95% ethanol solution, and the OD of each well was measured at 492 nm with an ELISA plate reader after 30 min. Results were obtained by subtracting the average ODs of the negative controls (uncultured media) from the average ODs of the experimental wells. Experiments were performed using three biological replicates and repeated at least three times.

Activity of the CFCM on established biofilms. The wells of a sterile 96-well flat-bottomed tissue culture plate (manufacturer no. TPP 92096; Trasadingen, Switzerland) were filled with 135 μl of TSB 1% glucose, and 15 μl of bacterial suspension, prepared as described earlier, was added into each well, and the plate was incubated for 24 h at 37°C. After two washing steps with 200 μl of sterile PBS (pH 7.2), 10% CFCM or control (in PBS) was added, and the plate was incubated for an additional 24 h at 37°C (60). Safranin staining and measurement were then performed as described above. Experiments were performed in triplicate at least three times.

Combined activity of the CFCM and oxacillin on the viability of established biofilm cells. Established biofilms of a selected MSSA strain (isolate 74) were inoculated as described above. After 24 h of incubation at 37°C and two washing steps with 200 μl of sterile PBS (pH 7.2), 10% CFCM or control with or without increasing concentrations of oxacillin (0.25 to 256 μg/ml) were added, and plates were incubated for 24 h at 37°C. After a single washing step with PBS, the viability of biofilm cells was revealed using resazurin 0.02% for 3 h. A blue color indicated dead cells, whereas pink indicated live cells (60).

Confocal microscopy and image processing. Biofilms of a selected MRSA strain (isolate 1602) were formed on Nunc Lab-Tek Chambered Coverglass (Thermo Scientific, MA, USA) with either the CFCM or control under the same conditions used for biofilm formation on microplate assays. After incubation, chambers were rinsed three times with 200 μl of 0.1 M HEPES buffer (pH 7), fixed with 100 μl of 2.5% glutaraldehyde, and incubated overnight at 4°C. Following three washes with HEPES buffer, 150 μl of DAPI (4',6-diamidino-2-phenylindole dihydrochloride) in HEPES (1:200) was added, and biofilms were incubated at room temperature for 10 min. Two washes with HEPES buffer were performed, and 300 μl

TABLE 3 Primers used in gene expression analyses by RT-qPCR

Target gene	Primer	Sequence (5′–3′)
icaR	icaRfw	CAGAGAAGGGGTATGACGGTAC
	icaRrev	GGTAATCAAAACAACATTTAACACTTTGTTCG
icaA	icaAF	GTGCAGTTGTCGACGTTGGC
	icaAR	CACATGGCAAGCGGTTCATAC
hemA	hemAfw	GAGATGATGCCTTACGAATTGCC
	hemArev	GTAACGACCTGTGTGAATTTGATCAAC

of a solution of 20% acrylamide/bis-acrylamide, 0.5 μl of TEMED (*N,N,N′,N′*-tetramethylethylenediamine), and 0.5 μl of 10% ammonium persulfate was added. Biofilms were then observed using a Leica TCS-SPE microscope (Leica Microsystems, Germany) on fluorescence mode using a 63× objective lens and a 1.5× digital zoom. For excitation, a 405-nm laser line was used, and emission was collected using a 580-nm bandpass filter. All images were collected and processed using the Leica Application Suite X (LAS X) software. For cell quantification, the first 19 sections of each confocal series were selected, representing a total volume of 8.8×10^4 μm³. The bacterial cells present in each biofilm volume were automatically segmented and quantified using the daime software (61).

RNA extraction. Biofilms of a selected MRSA strain (isolate 1602) were formed in 24-well flat-bottomed tissue culture plates (TPP; Trasadingen, Switzerland) with either the CFCM or control medium (the CFCM was prepared without bacterial inoculum) under the same conditions used for biofilm formation on microplate assays. After incubation, wells were rinsed three times with 500 μl of PBS, 1 ml of RNAprotect bacteria reagent (Qiagen, Hilden, Germany) was added in each well, and biofilms were scraped. After 5 min at room temperature, the sessile cell suspension was centrifuged, and pellets were treated with a lysis solution (20 mg/ml lysozyme, 40 μg/ml lysostaphin, and 4 mg/ml proteinase K). Purification was performed with High Pure RNA isolation kit (Roche, Basel, Switzerland), and RNA was treated with the RNase-Free DNase set (Qiagen, Hilden, Germany).

RNA sequencing. rRNA depletion was performed with the Ribo-Zero rRNA removal kit (Illumina) according to the manufacturer's recommendations (74). Removal of rRNA is achieved with magnetic spheres that bind to removal probes that hybridize to rRNA, producing an RNA sample ready for library construction. Library construction was performed with the TruSeq Stranded mRNA sample preparation kit according to the manufacturer´s recommendations (catalog no. RS-122-9004; Illumina). This protocol enables the acquisition of a suitable library for subsequent cluster generation for nucleotide sequencing. First, poly(A) RNA molecules were purified using magnetic spheres bound to oligonucleotide poly(T). After purification, mRNA was fragmented in small pieces using divalent cations under high temperature. Cleaved RNA fragments were reverse transcribed to cDNA (first chain) using reverse transcriptase and arbitrary primers. Chain specificity was achieved by substitution of dTTP for dUTP on the second strand marking mix (SMM), following the synthesis of the second cDNA chain using DNA polymerase I and RNase H. This results in removal of the RNA template with concomitant synthesis of the second strand. The presence of actinomycin D on the first strand synthesis act D mix (FSA) prevents spurious DNA-dependent synthesis, allowing only RNA-dependent synthesis, improving chain specificity. The 3´ ends of cDNA fragments were then adenylated with a single A base, and indexing adapters were ligated. Products were then purified using complementary adapters and beads and were enriched by PCR to create the final cDNA library.

The RNA obtained was used for transcriptome analyses using an Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) at the Plataforma de Sequenciamento de Ácidos Nucleicos de Nova Geração–RPT01J of the Fundação Oswaldo Cruz (Rio de Janeiro, Brazil). Data processing and analyses were performed at the Plataforma de Bioinformática–RPT04A of the Fundação Oswaldo Cruz. The bcl files containing the raw sequencing reads were converted to fastq files using the bcl2fastq software version 2.17 (Illumina). Technical and low-quality sequences were removed using Trimmomatic with the following parameters: ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN:40 (62). Read quality was evaluated with FastQC (Babraham Bioinformatics). Filtered reads were mapped with Bowtie2 (–no-mixed –no-discordant) to the *S. aureus* NCTC8325 genome (GenBank accession no. NC_007795.1) as a reference (63). The SAMtools package was used to extract and sort only the mapped reads (-G 77, -G 141) (64). Finally, the Cufflinks suite was used to compare global gene expression between both conditions (65–67). Genes that showed a differential expression of 2-fold with $P < 0.05$ between conditions (CFCM and control) were considered significantly regulated by CFCM. Unmapped reads were extracted from the original files into two files, one containing forward reads and another containing reverse reads, and sorted using SAMtools. Forward and reverse reads from all samples were concatenated into two files and then used for *de novo* transcriptome assembly using Trinity 2.8.5 (68, 69). Using the downstream scripts available within Trinity, the reads used to assemble the transcriptome (originally unmapped to the reference genome) were then mapped to the newly assembled transcriptome using Salmon (70), and differential expression was calculated using DESeq2 (71). Genes that showed a differential expression of 4-fold with $P < 0.001$ between conditions (CFCM and control) were selected. These genes were extracted and annotated against the NCBI RefSeq genome database for bacteria (taxid, 2) using online BLASTn with the default parameters.

For a final annotation, the top 10 genomes for each entry were downloaded and used for a local BLASTn using the individual gene sequences as queries and the most differentially expressed transcripts

as subjects. Metabolic pathways for each of the affected genes were determined using the EggNOG pathway database v. 4.5.1 (http://eggnogdb.embl.de) (72). To do this, gene IDs were searched manually in the database, and genes were categorized based on the resulting classification.

Real-time quantitative PCR. RT-qPCR was performed to confirm the effect of the CFCM on the expression of genes related to biofilm formation obtained by RNA-seq. To obtain cDNA, RNA extracted as described above, with either the CFCM or control medium, was subjected to a reverse transcriptase reaction using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Primers were designed for this study and are listed in Table 3. The relative expression levels of tested genes were normalized according to the expression levels obtained with the housekeeping gene *hemA*, which encodes the glutamyl tRNA reductase.

Statistical analysis. All comparisons were performed using unpaired Student's *t* tests (Prism 5, GraphPad Software, CA, USA). Differences were considered statistically significant when values of $P < 0.05$ were obtained.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.6 MB.

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