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ABSTRACT

Interest has been rekindled in the old antibiotic fosfomycin, partly because of its ability to penetrate biofilm. Using a transcriptomic approach, we investigated the modifications induced by fosfomycin in sessile cells of a clinical *S. aureus* isolated from a device associated infection. Cells still able to form biofilm after 4h of incubation in the presence of sub-inhibitory concentrations of fosfomycin and cells from 24h-old biofilm later submitted to fosfomycin had 6.77% and 9.41%, respectively, of differentially expressed genes compared to their antibiotic-free control. Fosfomycin induced mostly down-regulation of genes assigned to nucleotide, amino acid and carbohydrate transport and metabolism. Adhesins and capsular biosynthesis proteins encoding genes were down-regulated in fosfomycin-grown biofilm, whereas the murein hydrolase regulator *lgrA* and a D-lactate dehydrogenase-encoding gene were up-regulated. In fosfomycin-treated biofilm, the expression of genes encoding adhesins, the cell wall biosynthesis protein ScdA and to a lesser extent the fosfomycin target MurA was also decreased. Unattached cells surrounding fosfomycin-grown biofilm showed greater ability to form aggregates than their counterparts obtained without fosfomycin. Reducing their global metabolism and lowering cell-wall turnover would allow some *S. aureus* cells to grow in biofilm despite fosfomycin stress while promoting hyperadherent phenotype in the vicinity of the fosfomycin-treated biofilm.

Introduction

Chronic human infectious diseases are commonly caused by biofilm-forming bacteria such as *Staphylococcus aureus* (1) in prosthetic joint infections (PJIs), owing to the pathogen's ability to colonize the implants.

Bacteria inside biofilms have increased resistance not only to the action of the innate and adaptive immune defense systems, but also to the action of antimicrobial agents (2). Several factors are responsible for biofilm-associated resistance, including the density and the physiological state of the cells and the physical structure of the biofilm. The biofilm matrix can also act as a barrier to diffusion and thus reduces penetration of antibiotics into biofilms. The effectiveness of this barrier varies between antibiotics; gentamicin, tigecycline and daptomycin penetrate better than clindamycin, linezolid and vancomycin (3, 4). The effects of antibiotics can also be affected by the particular microenvironment of biofilm, such as acidic pH and low levels of oxygen encountered in the deep layers of the aggregates (5). Moreover, the presence of antibiotics can per se enhance biofilm formation, particularly at sub-minimum inhibitory concentrations (sub-MIC) (6–9) and thus contributes to treatment failure.

MICs are determined *in vitro* with planktonic cells growing exponentially under conditions that are optimal for the action of the drug but do not provide a true estimation of the concentration of antibiotics required to treat a bacterial biofilm. *In vitro* and *in vivo* experiments demonstrated that minimum bactericidal concentrations for biofilm bacterial cells are usually much higher (about 10-10,000 times) than their counterpart planktonic cells (4, 10) and therefore impossible to reach by conventional antibiotic administration. A combination of antibiotics with different and synergic

killing mechanisms is the best current solution for the treatment of biofilm infections. Rifampicin and mainly fosfomycin-based combinations have shown enhanced activity *in vitro* against biofilm-embedded *Staphylococcus aureus* isolates (11, 12). Several studies have evaluated the effect of rifampicin on the biofilm but little is known about the action of fosfomycin.

Fosfomycin is an old antimicrobial agent with powerful bactericidal activity against several Gram-negative and Gram-positive bacteria (13, 14). It has recently rekindled interest as a treatment option for infections caused by multiple drug resistant (MDR) bacteria (15–17). Fosfomycin inhibits the initial step in peptidoglycan synthesis by inactivating the cytosolic uridine diphosphate (UDP)-N-acetylglucosamine enolpyruvyl transferase (MurA), thereby preventing the formation of UDP-N-acetylglucosamine-3-O-enolpyruvate from UDP-N-acetylglucosamine and phosphoenolpyruvate (PEP), which ultimately leads to bacterial cell lysis (18).

Fosfomycin is able to penetrate biofilm and several studies have reported a positive effect against biofilm (16) but the underlying mechanisms involved remain to be elucidated. Our previous work indicated that fosfomycin at sub-MICs inhibited *in vitro* biofilm formation by a *S. aureus* strain isolated from a bone infection while viable but unattached cells were still detectable in the suspensions covering the biofilm, even at the highest antibiotic concentrations tested (4).

The aim of the present study was to investigate the mechanisms by which fosfomycin influences biofilm formation and affects a 24h-old biofilm of *S. aureus*. RNA sequencing (RNA-Seq) technology was used to compare the transcriptomes of *S. aureus* biofilms formed or treated with sublethal concentrations of fosfomycin. We also investigated the ability of non-adhering biofilm surrounding bacteria to form biofilm after fosfomycin treatment, since this portion of the population could be

involved in the development of chronic and recurrent infections.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Methicillin-susceptible *S. aureus* (MSSA) strains isolated from patients suffering from PJI were used (LYO-S2, LYO-S22 and LYO-S31) (4). The whole-genome of strain LYO-S2 has been deposited at DDBJ/EMBL/GenBank under the accession number FCOV01000001 to FCOV01000024 (19). The isolates were stored at −80°C in Trypticase Soy (TS) broth containing 15% glycerol (v/v) and were subcultured onto Columbia agar (bioMérieux, Marcy l'Etoile, France). Further cultures were performed with TS agar (TSA) (Conda, Madrid, Spain) or modified Mueller-Hinton (mMH) (bioMérieux, Saint Louis, USA) broth.

Fosfomycin disodium (Ercros, New York, USA) was used supplemented with glucose-6-phosphate (G6P) (Sigma-Aldrich, St Louis, USA). Vancomycin chlorhydrate was provided by Sigma-Aldrich (St Louis, USA). The Minimal Inhibitory Concentration (MIC) of fosfomycin for strain LYO-S2 was 4 mg/L and its Minimal Biofilm Eradication Concentration superior to 25,600 mg/L (4).

Biofilm formation

Biofilms were formed on polystyrene pegs of an MBEC^{TM} device (Innovotech, Edmonton, Canada) or on polystyrene disc coupons in a CDC Biofilm Reactor device (BioSurface Technologies Corp, Bozeman, USA).

When the MBEC^{TM} device was used, biofilms were formed on polystyrene pegs of the cover with a bacterial inoculum of $8x10^5$ CFU/mL in mMH broth supplemented with 25 µg/mL of G6P without or with 0.25µg/mL of fosfomycin. After 4h of incubation at 37°C with slow shaking (6 rpm per min), the capacity of non-adhering bacteria

surrounding the biofilm to form new aggregates was determined by harvesting and rinsing the cells with saline, transferring them to new microtiter plates at a bacterial inoculum of 10^6 CFU/mL in mMH broth. After 1 to 4h incubation at 37°C with shaking, aggregates formed on the pegs were rinsed with saline, transferred to new microtiter plates, and the number of sessile viable, culturable cells was counted by plating serial dilutions on TS agar after sonication.

For the assays with the CDC Biofilm Reactor device, biofilms were formed for 4h on polystyrene disc coupons with a bacterial inoculum of 10^7 CFU/mL in mMH supplemented with 25 µg/mL of G6P and supplemented when necessary with 0.25 µg/mL of fosfomycin or in mMH supplemented with 0.5 µg/mL of vancomycin.

To assess the effect of fosfomycin on mature biofilm, 24h-old biofilms formed without antibiotics on polystyrene disc coupons of CDC Biofilm Reactor device were further incubated for 24h in the presence of 16 µg/mL of fosfomycin and 25 µg/mL of glucose-6-phosphate or in the presence of 8 µg/mL of vancomycin.

RNA extraction

After incubation at 37°C with shaking, biofilms formed on the polystyrene disc coupons of the CDC Biofilm Reactor device were rinsed with saline, transferred to saline and sonicated. To have enough biomass for RNA extraction, 24h-old biofilm treated with or without fosfomycin from both reactors were pooled for each sample. The resulting cell suspensions were filtered with the Swinnex Filter Holder system (0.2 µm – Millipore, Molsheim, France). The filters were then transferred into RNA extraction buffer: glucose 50 mM, Ethylenediaminetetraacetic acid (EDTA) 10 mM, Tris(hydroxymethyl)aminomethane (Tris) 25 mM pH = 8 into RNAse-free water (ThermoFisher Scientific, Waltham, Massachusetts, USA). After mechanical shaking

to disperse the cells, the filters were removed and EDTA 0.5M pH = 8 was added. After vigorous mixing, lysozyme (50 mg/mL – Euromedex, Souffelweyersheim, France) was added and incubated for 15 min at 37°C with gentle shaking. Sodium dodecyl sulfate (SDS) 20% (ThermoFisher Scientific) was then added and the mixture incubated for 5 min at room temperature without shaking. Cells were transferred into Precellys tubes containing beads with one volume of phenolic acid/ chloroform (ThermoFisher Scientific) for mechanical lysis with the PreCellys 24 system (Bertin Technologies, Montigny le Bretonneux, France) at a speed of 6,000 rocks per minute for two consecutive cycles of 30 s. After centrifugation for 5 min at 20,000 g at 4°C, the aqueous phase was recovered and transfered to a tube containing 1 volume of chloroform (ThermoFisher Scientific). After centrifugation for 5 min at 20,000 g at 4°C, the aqueous phase was recovered and transfered to a tube containing a 1/10 volume of ammonium acetate 5M and 2 volumes of ethanol 100% (ThermoFisher Scientific). The samples were incubated overnight at -20°C. After centrifugation for 20 min at 20,000 g at 4°C, the supernatant was removed and the pellet washed with ethanol 70%. After centrifugation for 10 min at 20,000 g at 4°C, the RNA pellets were dried with a SpeedVac Vacuum Concentrator device (Genevac Ltd, Ipswich, United Kingdom). The RNA pellets were dissolved into RNAse-free water and treated with 10 units of TURBO DNAse (ThermoFisher Scientific). After a second phenol-chloroform extraction and ethanol precipitation, RNA pellets were suspended in RNAse-free water. RNA concentrations were quantified with the Qubit System ThermoFisher Scientific) and RNA quality was assessed with Agilent RNA 6000 Pico Chip (Agilent Technologies, Santa Clara, USA). Ribosomal RNA was removed from each total RNA sample with the RiboZero kit (Bacteria – Illumina, San

Diego, California, USA) by GenoScreen (Lille, France). Three biological replicates were assessed for each condition.

RNA sequencing and idenfication of differentially expressed genes

RNA-sequencing (RNAseq) was performed by GenoScreen (Lille, France). Libraries were produced by the ScriptSeq Complete Kit (Bacteria - Illumina) and sequenced with the Hiseq2500 system (Illumina) with a paired-end protocol and read lengths of 100bp. Reads were mapped against the genome of the reference strain *S. aureus* NCTC 8325 with Bowtie2 v2.2.6 software. Only reads without mismatch were counted. Differentially expressed genes between biofilm treated with fosfomycin and its untreated control were determined by RSEM v1.2.23 software. Genes with an adjusted p-value < 0.01 and |fold-change| > 2 were considered as being differentially expressed. RNA seq was performed with three independently isolated RNA samples.

Transcriptome annotation

Transcripts were identified with BLASTn searches against the reference strain *S. aureus* NCTC 8325 (NC_007795.1). The EggNog 4.5 database was used to obtain COG annotation for transcripts.

RT-qPCR

Reverse transcription was performed with 5 ng of total RNA prepared as described above. cDNA were obtained with iScript cDNA Synthesis kit (Bio-Rad, Hercules, California, USA) under the following conditions: 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. qPCRs were carried out in the CFX96 Real Time System (Bio-Rad,

Hercules, California, USA) with the SsoAdvanced SYBR Green Supermix (Bio-Rad) under the following conditions: initial denaturation at 95 °C for 30 s, and 40 cycles of 5 s at 95 °C and 20 s at 59 °C in a total volume of 10 µL per well with 1X SYBR® Green, 625 nM of each gene-specific primer and 2 µL of 20X diluted cDNA. Primers were designed on the basis of *S. aureus* NCTC 8325 genome data and are listed in Table 1. Melting curve analysis was used to verify the specific single-product amplification. The gene expression levels were normalized relative to the expression levels of the *gyrB* housekeeping gene, and relative quantifications were determined with CFX Manager software v3.1 (Bio-Rad) by the E(−Delta Delta C(T)). The amplification efficiency (E) of each primer pair used for quantification was calculated from a standard amplification curve obtained with four dilution series of genomic DNA. All assays were performed in technical triplicates with three independently isolated RNA samples.

Statistical analysis

CFU measures were compared with Student's *t*-test. Correlation between RNAseq and RT-qPCR was analyzed by Pearson’s correlation test in GraphPad Prism 5. Fold-change (log_{10}) values were used as a matrix to perform a heatmap with Heatmap.2 (gplots) with R sofware (version i386 3.2.4 Revised). Statistical analyses of RT-qPCR data were performed using the U Mann-Whitney test in GraphPad Prism 5.

Availability of supporting data

The RNA-seq data sets supporting the results of this article have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series

accession number GSE83269.

RESULTS

Transcriptome assembly and annotation

Transcriptome studies were performed with two different *S. aureus* LYO-S2 biofilm materials: biofilm cells formed for 4h in the presence of fosfomycin at sub-MIC, and pre-formed 24h-old biofilm later submitted to sub-MBEC of fosfomycin for 24 hours, hereafter referred to as fosfomycin-grown biofilm and fosfomycin-treated biofilm, respectively. Controls (without fosfomycin treatment) were run in parallel.

About 11 and 13 million RNA-seq reads (minimum 100 bp each) were obtained for each sample: fosfomycin-treated biofilm and its control (not treated), fosfomycin-grown biofilm and its control (without fosfomycin), respectively. The genome sequence available for the *S. aureus* strain NCTC 8325 was used for mapping and to identify differentially expressed genes. Only reads aligned to the reference genome without any mismatch were counted. One triplicate of the fosfomycin-treated biofilm control generated less than 30% of reads that mapped with the genome reference and so was not taken into account for further analysis. More than 80% perfect matches were obtained for sequence reads of the fosfomycin-grown biofilm sample, fosfomycin-treated biofilm sample and their respective controls.

Transcriptome analysis

RNAseq analysis indicated that of the 2,538 annotated genes of *S. aureus* LYO-S2 deduced from the sequence of the reference strain NCTC 8325, 172 and 239 were differentially expressed in fosfomycin-grown biofilm and in fosfomycin-treated biofilm, respectively ($|\text{fold-change}| > 2$ and adjusted p-value < 0.01), with fold-changes ranging from −35.7 to 25.9 (Table S1). Only 38 genes were shared by the two sets of comparison. To be highly specific, we focused mainly on genes differentially expressed with a $|\text{fold-change}| > 5$ (p-value < 0.01).

Under these stringent conditions and after analysis of fosfomycin-grown biofilm, only 38 genes were differentially expressed (2 up- and 36 down-regulated) compared to the control (biofilm formed without fosmomycin). This clustering was further supported by the cluster structure as revealed by heatmap analysis (Figure 1). When categorized into different functional groups based on the COG database (Cluster of Orthologus Groups), 37 genes could be classified into 12 categories (Figure 2b). Among the latter, 43.6% were assigned to the COG category of metabolism, comprising essentially nucleotide and amino acid transport and metabolism, and 28.20% were assigned to the poorly characterized COG category. Twenty-three point one percent were assigned to the COG category of cellular processes and signaling, comprising essentially cell wall / membrane / envelope biogenesis. Only 5.1% were assigned to the COG category of information storage and processing, comprising essentially transcription and translation, ribosomal structure and biogenesis (Figure 2a).

Analysis of fosfomycin-treated biofilm data showed that 52 genes were differentially expressed (13 up- and 39 down-regulated) compared to the control (without fosfomycin). This clustering was further supported by heatmap analysis (Figure 1). Categorization of the selected genes into different functional groups

based on the COG database indicated that only 49 of them could be classified into 14 categories (Figure 2b). Among the latter, 65.3% were assigned to the COG category of metabolism comprising essentially nucleotide, amino acid and carbohydrate transport and metabolism. Fourteen point three percent were assigned to the COG poorly characterized category and 10.2% were assigned to the COG category of information storage and processing, comprising essentially translation, ribosomal structure and biogenesis. Finally, only 10.2% were assigned to the COG category of cellular processes and signaling (Figure 2b).

Under the most stringent condition (|fold-change| > 5), only 12 genes were detected in both conditions (fosfomycin-grown biofilm and fosfomycin-treated biofilm versus their respective controls) and were down-regulated (Figure 1 and Table 2). Four of them belong to an operon that encodes proteins involved in purine biosynthesis, and another gene was identified as encoding a protein involved in pyrimidine biosynthesis. Two other genes belong to an operon that encodes proteins involved in arginine biosynthesis and two other genes in the same operon encoding ABC transporter were identified. Finally, three of them encode hypothetical or unknown proteins (Table 2).

RNA-seq results verification by RT-qPCR

To validate the RNAseq efficiency, eight genes differentially expressed with a |fold-change| > 5 were randomly selected and their relative expression levels determined by RT-qPCR with total RNA extracted from *S. aureus* LYO-S2 fosfomycin-grown biofilm, fosfomycin-treated biofilm and their respective controls. Results indicated a likeness between fold-change obtained with RNAseq and RT-qPCR (Figure 3).

RNAseq and RT-qPCR data for the eight genes tested in RT-qPCR were analyzed by a Pearson's correlation test, which indicated a high correlation between RNAseq and RT-qPCR results with a $r = 0.99$ (p-value < 0.0001).

Expression of target genes with biofilm extracts from two other *S. aureus* strains and in the presence of vancomycin

RT-qPCRs were performed with a set of selected genes and extended to two other *S. aureus* strains (LYO-S31 and LYO-S22 in addition to LYO-S2); results are shown in Figure S1. The variation of expression of the target genes was quite similar between the three strains in the fosfomycin-grown biofilm condition (Figure S1b). More heterogeneous data were observed with fosfomycin-treated biofilm extracts (Figure S1a), although variations were comparable for some genes (*scdA*, *fib-like*), or involved only two strains out of the three tested in parallel (*atl*, *lacC*, *PTS*, *purS*, *vraS*).

To determine how specific was the fosfomycin action on biofilm cells, we assessed the effect of another membrane targeting antibiotic, *i.e.* vancomycin, on LYO-S2 biofilm cells. Globally vancomycin-grown biofilm and vancomycin-treated biofilm cells display different patterns of expression compared to their respective fosfomycin-treated extracts (Figures S2a and b), with only a few statistically comparable levels of mRNA detected for *PTS* (PTS system lactose-specific transporter subunit), *purS* (phosphorybosylformylglycinamide synthase PurS), and *fib-like* (fibrinogen-binding protein like protein) in antibiotic-treated condition and *atl* (autolysin) in antibiotic-grown biofilm condition.

Adhesion capacity of unattached cells surrounding fosfomycin-grown biofilm

Determination of the adherence ability of these unattached cells indicated that they were more effective than their control (unattached cells harvested in the vicinity of biofilm formed in absence of fosfomycin) to form aggregates in a fairly short time (as of 1h) (Figure 4).

DISCUSSION

S. aureus LYO-S2 is a clinical strain isolated from a patient suffering from a PJI after total knee arthroplasty. The patient underwent three antibiotic treatments over 147 days including fosfomycin combined with oxacillin for 55 days in the middle of the treatment period. The outcome was favorable after 3-year clinical follow-up. We previously demonstrated this strain's ability to form biofilm *in vitro*, and the *in vitro* anti-biofilm activity of fosfomycin at sub-MICs (4). We also showed that the effective concentrations of fosfomycin required for 24h-old biofilm eradication were unattainable by conventional antibiotic administration (4). We therefore used *S. aureus* LYO-S2 as the model strain to study the molecular effects of fosfomycin on sessile cells using RNA-seq technology with both biofilm cells formed for 4h in the presence of fosfomycin at sub-MIC and pre-formed 24h-old biofilm cells later treated with sub-MBEC of fosfomycin for 24 hours. About 12 million RNA-seq reads were obtained for each sample, which is consistent with other studies that frequently obtained some 10 million reads per sample. In addition, with the exception of one set of data that was not included in the analysis, 80% of perfect matches were obtained when compared with the reference genome from *S. aureus* NCTC 8325, which illustrates the suitability of these RNA-seq results for comparative transcriptome analysis.

Previous transcriptional profiling studies performed with *S. aureus* strains and cell wall active antibiotics showed the induction of a "cell wall stress stimulon" (CWSS), i.e. a set of genes responding to cell wall active antibiotics but not to other external stresses such as temperature, osmotic and pH extremes (20). Fosfomycin acts on the first step of peptidoglycan biosynthesis pathway by irreversibly inhibiting MurA, an enolpyruvyl transferase. Petek *et al.* showed that this antibiotic at sub-MICs was able to up-regulate cell wall stress stimulon genes, including the genes encoding VraSR (Petek 2010). VraSR is a two-component sensory regulatory system that plays an essential role in the CWSS response, which leads to increased resistance against cell wall-active antibiotics (21). Dengler *et al.* showed that among several antibiotics tested fosfomycin triggered the highest induction of the promoters of CWSS genes in planktonic cells (22). In our study, the expression of *vraSR* was not modified whatever the tested condition (fosfomycin-grown biofilm and fosfomycin-treated biofilm). This suggests that sessile cells are less affected by fosfomycin stress than their planktonic counterpart. Shen *et al.* already observed such discrepancies in response to another external stress (sub-inhibitory concentrations of Licochalcone A [LLA] a natural plant product) with an increased expression of *vraSR* in planktonic cells compared to their sessile counterparts (23). However, in our study, genes under the control of VraSR system were down-regulated, although to a small extent (with a $|\text{fold-change}| > 2$, Table S1), including *murA* in the fosfomycin-treated condition and *mgt* (encoding the monofunctional glycosyltransferase MGT) in the fosfomycin-grown condition. Since both enzymes are involved in peptidoglycan synthesis, this would reduce peptidoglycan synthesis within sessile cells. The quantity of peptidoglycan in *S. aureus* is also controlled by the activity of autolysins, mainly the murein hydrolase Atl, which is involved in cell wall turnover, cell division,

366 cell separation and biofilm formation. In our study, the expression of *atl* was down-
367 regulated with a |fold-change| > 2 in presence of the antibiotic under the two tested
368 conditions (fosfomycin-grown biofilm and fosfomycin-treated biofilm). The presence
369 of fosfomycin, in addition to impairing the synthesis of peptidoglycan, could prevent
370 its degradation in sessile cells. Similar observations were made with planktonic *S.*
371 *aureus* cells treated with different cell-wall-active antibiotics (20, 21). In our study, the
372 gene encoding a regulator of murein hydrolase LrgA was up-regulated under the
373 fosfomycin-grown biofilm condition. This regulator negatively regulates murein
374 hydrolase activity (24). Thus, by preventing further degradation of peptidoglycan
375 under fosfomycin stress, some sessile cells would keep their peptidoglycan integrity
376 despite the decreased expression of the peptidoglycan biosynthesis pathway enzyme
377 gene, and would contribute to biofilm growth.

378 Treatment with fosfomycin not only modified the expression of peptidoglycan
379 synthesis-related genes but also that of genes involved in surface structure
380 synthesis. Lower levels in the expression of the protein A encoding gene *spa* and in
381 the expression of two genes encoding proteins involved in capsular polysaccharide
382 biosynthesis (including Cap8C protein) were observed when biofilm was formed in
383 the presence of fosfomycin and with fosfomycin-grown biofilm sessile cells,
384 respectively. Similar results were obtained by Petek *et al.* with sub-MICs of
385 fosfomycin and *S. aureus* planktonic cells (25). Qin *et al.* also showed that biofilm
386 cells challenged with resveratrol respond with a significant under-expression of
387 genes encoding capsular polysaccharide biosynthesis proteins (26). Although opinion
388 differs on the role of the capsule (27), these two surface structures are important
389 virulence factors that play multiple roles in bacterial adherence and biofilm formation
390 by *S. aureus*. Hence, reduction in the expression of their encoding genes is likely to

391 result in a decrease in bacterial adhesion and biofilm formation, as observed in our
392 study.

393 Extension of our study to two other *S. aureus* strains showed that most target
394 genes underwent similar changes in their expression (Figure S1), meaning that the
395 data from the transcriptome analysis were not strain specific but correspond to a
396 more general behavior. Additional assays performed with vancomycin instead of
397 fosfomycin also revealed that the antibiotic stress responses induced by the two
398 peptidoglycan-targeting molecules were different (Figure S2), probably related to the
399 unique molecular response induced by each stress as previously demonstrated in a
400 metabolomic approach (28).

401 Numerous studies including ours have determined the impact of antibiotics on
402 biofilm formation and sessile cell viability (6, 8). However, the concomitant outcome
403 of non-adhering bacteria has been poorly investigated, despite the possibility that this
404 portion of the population could actively participate in the development of chronic
405 infections. For this reason, we also investigated in the present study the fate of
406 unattached cells surrounding 4h-old biofilm, not knowing whether these cells were
407 initially adhering to the biofilm or whether they remained unattached throughout the
408 incubation time. Surprisingly, such cells harvested in the assay performed in the
409 presence of fosfomycin had a greater ability to form aggregates than their
410 counterparts obtained without fosfomycin. It is therefore tempting to speculate that
411 sub-MICs of fosfomycin, while partially impairing bacterial incorporation within
412 biofilms, also affect the non-adhering surrounding bacteria, giving rise to hyper
413 adherent cells in the vicinity of the biofilm.

414 A recent study characterizing the *in vivo* transcriptome of *S. aureus* cells
415 isolated from the joint fluid of a PJI reported typical transcriptomic and metabolomic

patterns when compared to those of *in vitro* planktonic cells, with increased expression of virulence associated-genes (29). Bacteria harvested in the patient's joint fluid could correspond to unattached cells surrounding the biofilm formed on the prosthesis. However, any further comparison would be inappropriate since our study was conducted in aerobic conditions and PJI-associated biofilm formation and dissemination occur in anaerobic/hypoxic conditions, as illustrated by the elevated number of related catabolic pathways genes up-regulated in the study performed by Xu *et al.* (29). Nonetheless, therapeutic molecules used to prevent or cure *S. aureus* infections of prosthetic material should be effective against both sessile and free living cells to avoid emergence of biofilm-associated PJIs. In our study, the relative effectiveness of fosfomycin in the prevention of biofilm formation and against 24h-old biofilm was counterbalanced by a stimulating effect on the ability of unattached cells surrounding 4h-old biofilm to form new aggregates. Further investigation with a large panel of MRSA isolates is required to confirm these findings and determine whether fosfomycin has overall beneficial effects on the reduction of *S. aureus* biofilm-mediated infections.

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Legends of figures

Figure 1. Heatmap with 80 differentially expressed genes with a |fold change| > 5 in two different growth conditions of *S. aureus* biofilm (p-value adjusted < 0.01). The fold-change was presented as a $\log_{10}$ fold-change between -1.5 and 1.5. Blue and red match to genes under- or overexpressed, respectively, in presence and absence of fosfomycin.

Figure 2. Histogram of Clusters of Orthologous Group (COG) classification of the differentially expressed genes with a |fold change| > 5 (*p*-value adjusted < 0.01) in two different growth conditions of *S. aureus* biofilm. (a) COG affiliation of genes differentially expressed between biofilm formed with or without fosfomycin. (b) COG affiliation of genes differentially expressed between 24h-old biofilm treated or not with fosfomycin. COG function classes are indicated along the *x*-axis and the number of genes differentially expressed for each COG function class along the *y*-axis. COG function class: [C] Energy production and conversion, [D] Cell cycle control, cell division and chromosome partitioning, [E] Amino acid transport and metabolism, [F] Nucleotide transport and metabolism, [G] Carbohydrate transport and metabolism, [H] Coenzyme transport and metabolism, [J] Translation, ribosomal structure and biogenesis, [K] Transcription, [M] Cell wall/membrane/envelope biogenesis, [ND] COG classification not determined, [O] Posttranslational modification, protein turnover and chaperones, [P] Inorganic ion transport and metabolism, [S] Function unknown, [T] Signal transduction mechanisms, [U] Intracellular trafficking, secretion, and vesicular transport and [V] Defense mechanisms.

Figure 3. Expression ratio (log_{10}) obtained by RT-qPCR and RNAseq. Expression ratio obtained by RT-qPCR (black) and RNAseq (grey) of 8 genes differentially expressed between biofilm formed with or without fosfomycin and between 24h-old biofilm treated or not with fosfomycin. These genes encode [1] D-lactate dehydrogenase, [2] murein hydrolase regulator LgrA, [3] immunoglobulin G-binding protein Sbi, [4] amino acid ABC transporter ATP-binding protein, [5] dihydroorotate dehydrogenase 2, [6] L-lactate dehydrogenase, [7] ferritin and [8] glycolytic operon regulator. *gyrB* was used as a reference gene for normalization of RT-qPCR data. All gene expression ratios from RT-qPCR between treatment groups and controls were significantly different (*p*-value < 0.05). The *x*-axis indicates the genes, and the *y*-axis shows the gene expression ratios. Bars represent the error standard (n=3).

Figure 4. Determination of the ability of non-adhering *S. aureus* collected in the vicinity of biofilm to form aggregates by enumeration of viable bacteria. Non-adhering cells surrounding biofilm formed without fosfomycin (control) are represented in black and non-adhering cells surrounding fosfomycin-grown biofilm are indicated in gray. Length of incubation (h) is indicated along the *x*-axis and cell viability along the *y*-axis (CFU/mL). Bars represent the error standard (n=3, each assay including at least three replicates) and the threshold of detection was 10 CFU/ml. The significant differences between CFU/mL values were calculated by a Student test. *Significant differences (* $p< 0.05$ and *** $p<0.001$).