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Transcriptomic analysis of stress response to novel antimicrobial coatings in a clinical MRSA strain

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ABSTRACT

Multi-drug resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) cause nosocomial infections that can have deleterious effects on human health. Thus, it is imperative to find solutions to treat these detrimental infections as well as to control their spread. We tested the effect of two different antimicrobial materials, functionalised graphene oxide (GOX), and AGXX® coated on cellulose fibres, on the growth and transcriptome of the clinical MRSA strain *S. aureus* 04-02981. In addition, we investigated the effect of a third material as a combination of GOX and AGXX® fibres on *S. aureus* 04-02981. Standard plate count assay revealed that the combination of fibres, GOX-AGXX® inhibited the growth of *S. aureus* 04-02981 by 99.98%. To assess the effect of these antimicrobials on the transcriptome of our strain, cultures of *S. aureus* 04-02981 were incubated with GOX, AGXX®, or GOX-AGXX® fibres for different time periods and then subjected to RNA-sequencing. Uncoated cellulose fibres were used as a negative control. The antimicrobial fibres had a huge impact on the transcriptome of *S. aureus* 04-02981 affecting the expression of 2650 genes. Primarily genes related to biofilm formation and virulence (such as *agr*, *sarA*, and those of the two-component system SaeRS), and genes crucial for survival in biofilms (like arginine metabolism *arc* genes) were repressed. In contrast, the expression of siderophore biosynthesis genes (*sbn*) was induced, a probable response to stress imposed by the antimicrobials and the conditions of iron-deficiency. Genes associated with potassium transport, intracellular survival and pathogenesis (*kdp*) were also differentially expressed. Our data suggest that the combination of GOX and AGXX® acts as an efficient antimicrobial against *S. aureus* 04-02981. Thus, these materials are potential candidates for applications in antimicrobial surface coatings.

1. Introduction

Multidrug resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) cause severe nosocomial infections and can develop resistance to all classes of antibiotics [1]. In addition, *S. aureus* can tolerate starvation, desiccation, and acid stress conditions on a variety of surfaces, making it extremely difficult to combat [2,3]. MRSA was also listed as a high priority pathogen by the World Health Organization (WHO) in 2017 [4,5]. Thus, pathogens, specifically multidrug resistant pathogens like MRSA continue to pose a serious threat to human health [6]. This notion has prompted researchers to develop novel antimicrobial strategies to stop the spread of infectious diseases. Some of these strategies include using heavy metal or carbon-based nanomaterials such as gold or silver nanoparticles, carbon nanotubes,

and graphene-based antimicrobials [6].

MRSA is a leading cause of nosocomial infections. These infections cause around 50,000 deaths per year in Europe [7]. Implant devices account for significant nosocomial infections. *S. aureus* is the most prevalent pathogen in orthopaedic and spinal infections [8,9]. Bacteria can form firm biofilms on the surfaces of medical devices. Thus, medical devices serve as microbial reservoirs leading to serious infections. If caused by dangerous pathogens like MRSA they are resistant to antibiotics and host defences. Antimicrobial surface coatings inhibiting biofilm formation such as AGXX® can be considered as candidates to minimise this problem [10].

Bacterial adhesion to the implant surface is the crucial and primary step in implant infections [11]. Targeting this step using certain chemical or biological compounds is another approach that could aid in

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diminishing infections. Graphene oxide (GO) can be used to achieve this aim. GO is derived from graphene and has hydroxyl and epoxy groups on the basal surface and carboxyl groups on the edges. These functional groups impart hydrophilic and dispersing properties to GO making it suitable for medical applications [12]. GO can readily adsorb metal or inorganic compounds due to its variety of functional groups [13]. It has an antibacterial effect on Gram-negative pathogens such as *Pseudomonas aeruginosa* [14] as well as on Gram-positive bacteria like the dental pathogens *Streptococcus mutans*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum* [15]. This antibacterial effect is caused by graphene-like parts of GO wrapping the bacterial membranes or by producing reactive oxygen species (ROS) [12,14,16,17]. ROS create oxidative stress and cause damage to bacterial cell membranes, and macromolecules such as lipids, proteins and DNA, ultimately leading to cell death [18].

Apart from causing infections, bacterial pathogens also pose a great challenge by contaminating basic sources of water by for instance contaminating sanitary areas and thus causing water-borne infections. Globally, more than 700 million people lack access to clean drinking water and a total of 1.8 billion people have to obtain their drinking water from sources that are at least temporarily contaminated with faecal matter [7].

Hence, there is an urgent need to develop new strategies to deal with the problem of infection and contamination by multidrug resistant biofilm-forming bacteria such as MRSA. We aim to develop a two-component antimicrobial coating on cellulose where component one is functionalised graphene oxide (GOX) and component two is a carrier material coated with the antimicrobial AGXX®. GO has previously been used in combination with other antimicrobial metals such as silver and titanium dioxide [6,19,20]. In recent studies, we used confocal and atomic force microscopy to show that GOX immobilises bacterial cells by tightly binding to them. GOX wraps around the cells and inhibits bacterial proliferation [21]. Thus, it has a bacteriostatic effect. AGXX® consists of two transition metals, silver and ruthenium, and has been shown to work as an antibacterial by generating ROS. It may also play a role in inhibiting biofilm formation in bacteria [10,22–24]. The putative mechanism of this two-component antimicrobial coating consisting of GOX and AGXX® is as follows: First, component one, GOX (functionalised GO), binds free bacteria. Adsorption of bacteria (which have an overall negative charge) is ensured by modification of the GO layers by grafting polymers with cationic groups. These flexible and micrometre sized GOX sheets multivalently bind and capture bacterial cells via electrostatic attraction. Second, AGXX® as component two catalytically produces ROS, which will kill the captured bacteria.

In this study, several antimicrobial materials consisting of GOX and AGXX® coated on cellulose fibres were tested. To understand the molecular mechanism of GOX and AGXX®, *S. aureus* 04-02981 (MRSA) was exposed to GOX, AGXX®, or the combination of both and the bacterial response was investigated via RNA sequencing. Our data suggest that GOX, AGXX® and their combination act as antimicrobials as GOX individually arrests bacterial growth and AGXX® and GOX-AGXX® efficiently kill bacteria. They mainly affect the expression of genes involved in pathogenesis and virulence and those associated with biofilm formation and metabolism essential for survival in biofilms.

2. Materials and methods

2.1. Preparation of GOX and AGXX® fibres

Methylene bisphenyl di-isocyanate (0.1 mg) was dissolved in 1 mL dry dimethyl formamide (DMF). Cellulose fibres (100 mg) were dispersed in 1 mL di-isocyanate/DMF solution for 15 min. In a separate flask, 10 mg of GOX was dispersed in 9 mL dry DMF. GOX solution was added to the fibres and allowed to react at room temperature for 24 h under constant stirring. The functionalised fibres were washed 10 times by centrifugation at 4000 rpm (Heraeus™ Megafuge 8 benchtop

centrifuge) for 5 min to get rid of unattached GOX, di-isocyanate and DMF and lyophilised to obtain dry, functionalised GOX cellulose fibres.

AGXX® fibres were prepared by chemical reduction of silver and ruthenium as previously described [10,22] and then chemically coated on cellulose fibres (patent pending).

2.2. Inhibition assay

Different amounts of GOX and AGXX® coated on cellulose fibres of 20 × 700 µm (Vitacel) were tested against *S. aureus* 04-02981 (MRSA). 10, 20, 30, 50, and 100 mg of uncoated cellulose, GOX, AGXX®, or GOX-AGXX® were tested. To this end, MRSA was grown overnight in Tryptic Soy Broth (TSB; Carl Roth GmbH and Co. KG, Karlsruhe, Germany) without NaCl. The culture was diluted to ~10⁵ colony forming units (CFU) mL⁻¹, after which either uncoated cellulose fibres, or GOX, or AGXX®, or GOX-AGXX® was added to the *S. aureus* 04-02981 culture. The cultures were incubated for 5 h at 37 °C with shaking (150 rpm), serially diluted and plated on Tryptic Soy agar (1.5%) plates. After 16–24 h at 37 °C the CFU mL⁻¹ were determined.

2.3. RNA extraction

S. aureus 04-02981 was exposed to GOX, AGXX®, and the combination of the two materials and uncoated cellulose fibres as negative control for different time periods, after which the bacterial response was investigated via RNA sequencing. For this purpose, overnight cultures of *S. aureus* 04-02981 were diluted to an optical density at 600 nm (OD₆₀₀) of 0.05 and then incubated at 37 °C, 150 rpm until the mid-exponential phase (4 h). Subsequently, either cellulose (30 mg fibres/30 mL culture) or GOX (30 mg fibres/30 mL culture) or AGXX® (15 mg fibres/30 mL culture) or GOX-AGXX® fibres (30 mg GOX fibres plus 15 mg AGXX® fibres/30 mL culture) were added to the culture. The cultures were incubated at 37 °C, 150 rpm for 0, 30, 120, or 180 min. The cells were harvested by centrifugation at 10,000 rpm in a Heraeus Multifuge X3R Centrifuge (Thermo Electron LED GmbH, Osterode am Harz, Germany). Cell pellets were immediately frozen in liquid nitrogen and stored at –80 °C or directly used for RNA extraction using the ZR Fungal/Bacterial RNA MiniPrep™ Kit (ZymoResearch, Freiburg, Germany) following the manufacturer's instructions. To recover total RNA including small RNAs, 1.5 volumes of absolute ethanol were added in step 5 of the protocol. Total RNA was eluted with 50 µL DNase- and RNase-free water and stored at –80 °C. RNA quantity and quality were assessed with a NanoDrop 2000c UV–Vis Spectrophotometer (Thermo Scientific, Osterode am Harz, Germany) as well as on agarose gels. Residual contaminating DNA was eliminated with TURBO DNA-free™ Kit Ambion according to the protocol (Life Technologies, Darmstadt, Germany).

2.4. RNA sequencing

RNA sequencing was performed by Novogene Company LTD., Cambridge, United Kingdom. The protocol was performed using the following steps; quality control by Agilent 2100; rRNA removal by Ribo-Zero rRNA Removal Kit (Bacteria) (Illumina), followed by strand-specific cDNA library preparation, templating, enrichment and sequencing via Illumina sequencer.

2.5. RNA-sequencing data analysis

Raw sequencing reads were aligned to the reference genome of *S. aureus* 04-02981 using Bowtie2 [25] version 2.2.3 with optimal settings (D 20 -R 3 -N 1 -L 20 -i S,1,0.50 -local) for the IonProton™ Sequence. Post-processing of the SAM files into sorted BAM files was carried out with SAMtools ([26], version 1.2-207). The samples uncoated cellulose, GOX, AGXX®, and GOX-AGXX® were normalised against the untreated control of the respective time-points. *S. aureus* 04-02981 cultures

without addition of any fibres served as the untreated control. Length-normalised confidence interval RPKM (= Reads per Kilobase of transcript per Million mapped reads) values were obtained with Cufflinks [27]. For better prediction of the variation in gene expression, an extra sample (Control_0) was added to the input for T-Rex. Finally, statistical analysis was carried out using the T-Rex RNA-Seq analysis pipeline [28]. A gene was considered significantly differentially expressed when the fold change was $\geq |2.0|$ and the false discovery rate (FDR) adjusted p-value ≤ 0.05 . The data presented in this paper have been deposited at NCBI and are accessible through GSE149013.

2.6. Reverse transcription quantitative PCR (RT- qPCR)

To verify the results obtained from RNA-sequencing, RT-qPCR was performed on four genes representing a group of genes that was differentially expressed in RNA-seq. To this end, total RNA extracted from MRSA cultures exposed to uncoated cellulose, GOX, AGXX® or GOX-AGXX® for 0 or 120 min was used. Time-points 0 and 120 min were selected for validation of RNA-seq data since GOX-AGXX® affected the expression of most genes at these time-points. cDNA was synthesized with the RevertAid™ First Strand cDNA Synthesis kit (Thermo Fisher Scientific Inc., Walham, UK) as per the manufacturer's instructions using 200 ng of total RNA as template and random hexamer primers. cDNA was diluted with DNase- and RNase-free water and amplified in a LightCycler®480 II (Roche Diagnostics GmbH, Mannheim, Germany).

The genes encoding 6-phospho- β -glucosidase (*bglA*), staphylococcal accessory regulator A (*sarA*), carbamate kinase (*arcC*), and transcriptional regulator protein *kdpE* were selected to verify the data obtained through RNA-seq; *gyrB* was used as the house-keeping gene. These genes were amplified using TaqMan chemistry according to the instructions provided in LightCycler®480 Probes Master Kit (Roche Diagnostics). All RT-qPCR reactions were carried out in a total volume of 20 μ L. The amplification step was performed 45 times and with 'Quantification' analysis mode at 95 °C for 10 s, with a ramp rate of 4.4 °C/s, followed by annealing at the respective annealing temperature for 50 s, with a ramp rate of 2.2 °C/s and finally an extension at 72 °C for 1 s, with a ramp rate of 4.4 °C/s. All primers and probes used in the study are listed in Supplementary Table 1. All RT-qPCR experiments were done in triplicate and each experiment was repeated at least twice. Data were analysed by LightCycler® 480 Software release 1.5.0. Data represent fold changes, calculated by normalising to the *gyrB* gene and relative to the untreated culture of MRSA using the Livak method of relative quantification [29]. Means of three Cp values each were used to calculate the fold changes in gene expression.

Fig. 1. Effect of GOX-AGXX® on the growth of *S. aureus* 04-02981. Impact of GOX and AGXX® coated on cellulose fibres on *S. aureus* 04-02981 cultures. Mean CFU mL⁻¹ values were calculated from three independent CFU values each. Error bars indicate standard deviation. The percentage value above each sample column indicates the percent reduction in CFU mL⁻¹ in that sample relative to an untreated control (culture without addition of antimicrobials = Culture).

3. Results

3.1. AGXX® and the combination of GOX and AGXX® fibres effectively inhibit the growth of *S. aureus* 04-02981

To examine the effect of different amounts of GOX and AGXX® materials on *S. aureus* 04-02981, inhibition assays were performed. The cultures were exposed to either cellulose, GOX, AGXX® or GOX-AGXX® for 5 h after which CFU mL⁻¹ was determined using standard plate assays. Optimum results were obtained with 30 mg GOX fibres and 15 mg AGXX® fibres per 30 mL culture. The effect of the materials on *S. aureus* 04-02981 growth is presented in Fig. 1; CFU mL⁻¹ values are given in Supplementary Table 2.

GOX-AGXX® fibres had a pronounced effect on *S. aureus* 04-02981. The combination fibres killed 99.98% of *S. aureus* 04-02981 cells. Therefore, GOX (30 mg/30 mL) and AGXX® (15 mg/30 mL) coated on cellulose fibres were used in further experiments.

3.2. Cellulose-based fibres have a huge impact on the transcriptome of *S. aureus* 04-02981

The raw RNA-seq sequences were aligned to the genome of *S. aureus* 04-02981. All the sequenced samples had satisfactory read depth, ranging from approximately 10 million reads (Control_180) to approximately 20 million reads (GOX-AGXX®_120). An average sequence depth of approximately 14 million reads was achieved. The library sizes for all the sequenced samples are provided in Supplementary Table 3.

Fig. 2 shows that after normalisation of the samples to their respective controls (untreated culture) the gene counts were equally distributed. Outliers were mostly present only in the high- or low-fold range. The data from Figs. 1 and 2 clearly indicate the remarkable effect of the cellulose-based fibres on *S. aureus* 04-02981. Intriguingly, cellulose fibres without any antimicrobial coating also had a tremendous unexpected effect on gene expression. Upon exposure to uncoated cellulose, GOX, AGXX®, or GOX-AGXX® fibres for 0, 30, 120, and 180 min, 2650 genes in *S. aureus* 04-02981 were differentially expressed in total. The numbers of differentially expressed genes per sample and time-point are shown in Fig. 3.

Some unexpected but interesting results are apparent from Fig. 3. Cellulose hugely impacted gene expression of MRSA at all time-points (Fig. 3A). Since we did not expect cellulose to significantly change gene expression of *S. aureus* 04-02981, we looked into genes possibly associated with cellulose degradation or utilisation and checked their expression pattern in the RNA-seq data, namely those of aryl-phospho- β -D-glucosidase (*bglA*), 6-phospho- β -galactosidase (*lacG*), and some other

Fig. 2. Box plot of normalised signals. The majority of the log2 normalised gene count values of each sample and experiment are presented as bars. The experiment names indicate the sample and the time (in min) for which the culture was exposed to the particular material. The experiment AGXX*0 represents the *S. aureus* 04-02981 culture exposed to AGXX® fibres for 0 min, AGXX*120 represents the *S. aureus* 04-02981 culture exposed to AGXX® fibres for 120 min, and the same scheme applies to all the experiments. Outliers are represented by dots and the mid-point as a horizontal line.

Fig. 3. Number of differentially expressed genes in *S. aureus* 04-02981. Total number of up-regulated (black bars) and down-regulated (grey bars) genes in *S. aureus* 04-02981 on exposure for different times (X-axis) to A) Cellulose (uncoated), B) GOX, C) AGXX®, D) GOX-AGXX®.

Table 1
Differential expression of genes putatively associated with cellulose degradation in *S. aureus* 04-02981.

Gene ID	Gene/product	Description	Sample	Fold change
SA2981_0266	<i>bglA</i>	6-Phospho-beta-glucosidase activity	GOX at t0	2.9
			GOX-AGXX® at t0	3.9
			Cellulose at t30	4
			Cellulose at t120	3.2
			Cellulose at t180	2.7
SA2981_2127	<i>lacG</i>	6-Phospho-beta-galactosidase activity	GOX-AGXX® at t0	3.3
			Cellulose at t30	8.7
			AGXX® at t30	2.2
			Cellulose at t120	2.8
			GOX at t120	2.5
			AGXX® at t120	2.6
			Cellulose at t180	−3

This table only shows the samples and time-points at which the respective genes were differentially expressed in RNA-seq.

genes. The gene products of *bglA* and *lacG* belong to the glycoside hydrolase (GH) 1 family. These enzymes hydrolyse β -glycosides in various carbohydrates. β -D-glucosidase hydrolyses cellulose with the help of the phosphotransferase system by converting cellobiose to glucose [30]. Differential expression of these genes is presented below in Table 1.

3.3. GOX, AGXX® and their combination fibres repress genes associated with biofilm formation in *S. aureus* 04-02981

We checked the effect of the different fibres (uncoated cellulose, GOX, AGXX®, and GOX-AGXX®) on the expression of genes involved in biofilm formation and those that are essential for survival of MRSA in biofilms. Additionally, the effect on virulence-associated genes was investigated. Several genes associated with biofilm formation, survival in biofilms, and virulence were indeed differentially expressed (see Fig. 4 for an overview). Supplementary Table 4 gives the gene ID, names, and differential expression, as fold changes, of the genes shown in Fig. 4.

Most of the genes depicted in Fig. 4 were differentially expressed at all time-points of exposure to at least one of the antimicrobials. The

genes of the two-component system SaeRS impact biofilm formation by modulating the synthesis of extracellular proteases. Uncoated cellulose fibres at t180 had the highest impact on expression of *saeS*, encoding the sensor histidine kinase and *saeR*, specifying the response regulator of the system [31]. Both were down-regulated by 3 and 4.5 fold, respectively. Extracellular fibrinogen gene *efb*, an important virulence factor in *S. aureus* [32], was affected the most on exposure to AGXX® at t0 while the cell-wall anchored protein gene *sasD* [33] was highly affected at t180 in the presence of GOX. These two genes were down-regulated 4.7 and 3.6-fold, respectively. No differential expression was observed at t120 for any of these four genes upon exposure to all different fibres.

Accessory gene regulator (*agr*) genes are essential for biofilm formation in *S. aureus*. The gene *agrC*, specifying a member of the AgrCA two-component system serves as the sensor histidine kinase, while *agrD* is required for production of autoinducer peptide [31,34]. Stage V sporulation gene *G spoVG* is involved in antibiotic resistance and the synthesis of virulence factors in *S. aureus* [35]; it was only differentially expressed at t0, and only in the presence of GOX-AGXX®. The three genes *agrC*, *agrD* and *spoVG* were down-regulated 2.2, 3.5, and 2.4-fold,

Fig. 4. Biofilm and virulence-related genes differentially expressed in *S. aureus* 04-02981 upon exposure to GOX, AGXX®, or GOX-AGXX® fibres. The genes are clustered as indicated by the dendrogram on the left of the heatmap. Cluster 1 (red) contains the *saeS*, *efb*, *sasD*, and *saeR* genes. Cluster 2 (purple) represents *rsbU*, *agrD*, and *ftsL*. Cluster 3 (green) groups *agrC* and *sarA**. *spoVG*, and the genes for the Veg protein and protein MSA are individually represented by blue, yellow, and orange colours, respectively. ‘*’, gene selected for validation through RT-qPCR. The experiment names at the bottom of the heatmap indicate the time-point (Tx) followed by the specific sample. E.g., the experiment T00_AGXX represents the culture exposed to AGXX® fibres for 0 min, T30_AGXX represents the culture exposed to AGXX® fibres for 30 min, etc. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

respectively. GOX-AGXX® at t0 also had the highest impact on staphylococcal accessory gene regulator *sarA*, a virulence regulator, cell division protein gene *ftsL* [36], and on the gene encoding the Veg protein, which stimulates biofilm formation by inducing extracellular matrix genes [37]. These genes were down-regulated 5.6, 14.5, and approximately 20-fold, respectively.

3.4. GOX, AGXX® and their combination fibres repress genes critical for survival of MRSA in biofilms

The genes for arginine metabolism *arcA*, *arcB*, *arcC*, and *arcD* are essential for MRSA to survive in biofilms [38,39]. Their expression upon exposure to GOX, AGXX® or GOX-AGXX® fibres is presented in Fig. 5 and Supplementary Table 5.

The products of *arcABC* catalyse the conversion of arginine to ornithine and are related to intermediary metabolism; ArcD enables arginine uptake and ornithine export. All four genes were most differentially expressed (down-regulated) in the presence of GOX-AGXX®. On one hand, *arcB*, *arcC*, and *arcD* were down-regulated at t0 by 4.8, 4.4, and 4.8 fold, respectively. On the other hand, GOX-AGXX® at t120 had the highest impact on *arcA*, repressing the gene 4 fold. All these genes were repressed in all the samples except *arcC*, which was up-regulated 2.6-fold upon exposure to GOX-AGXX® at t180. Previous studies have reported induced expression of the *arc* genes in *S. aureus* biofilms compared to planktonic cultures [38,40]. Interestingly, the *arc* genes were down-regulated here as well as several genes associated with biofilm formation namely, *agrD*, *saeRS*, *sarA*, *efb*, and those of the proteins Veg, MSA, and FtsL (see Fig. 4, Supplementary Table 4). Combining these data on genes associated with biofilm formation and virulence, and those essential for survival in biofilms one might speculate that GOX, AGXX®, and GOX-AGXX® assist in reducing biofilm formation in *S. aureus* 04-02981.

3.5. GOX, AGXX® and their combination fibres affect the expression of potassium transport (*kdp*) genes in *S. aureus* 04-02981

The RNA-seq data revealed a strong effect of the cellulose-based fibres on the expression levels of the *kdp* genes (Fig. 6 and

Supplementary Table 6).

When *S. aureus* 04-02981 was exposed to cellulose, GOX, AGXX®, or GOX-AGXX® fibres, all five *kdp* genes were differentially expressed at some of the time-points (0, 30, and 120 min).

kdpA was remarkably differentially expressed in the presence of cellulose fibres at t180 (187-fold up-regulated). It was up-regulated in the presence of all the fibre materials at t180 but down-regulated at the earlier time-points, e.g. in the presence of GOX-AGXX® at t0 (by approximately 5 fold) and in the presence of cellulose at t30 (9 fold). A similar pattern of differential gene expression was observed for *kdpC* and *kdpD* where the cellulose fibres at t180 had the strongest effect on expression. The genes were down-regulated at earlier time-points such as t0, and t30, while they were up-regulated at later time-points such as at t120, and t180. The transcription of *kdpB* and *kdpE* was most affected in the presence of GOX-AGXX® at t180 with up-regulation by 17, and 97-fold, respectively. There is a link between the KdpDE system and bacterial stress response which makes its association with survival within a host clear and relevant [41]. Since K⁺ ions stimulate the induction of the *kdp* genes [41] it is no surprise that salt shock also affects the transcription of these genes. Additionally, *kdpDE* is also linked to antimicrobial and oxidative stress response [41]. In our experiments, we used growth medium (TSB) devoid of salt which may be one of the reasons why the expression of these genes was affected. We also suspect that the antimicrobial and oxidative stress imposed by the ROS generated in the presence of AGXX® leads to loss of ions from the cell (due to the damage caused to the bacterial membranes) and hence *S. aureus* 04-02981 up-regulates the expression of *kdpABCDE* to recover from the loss of K⁺, for its survival.

In addition to genes associated with biofilm formation and virulence (Fig. 4), biofilm survival (Fig. 5), and potassium transport (Fig. 6), other highly affected groups of genes were those involved in antibiotic resistance (Supplementary Table 7), and siderophore biosynthesis (*sbh* genes) which are associated with iron homeostasis (Supplementary Table 8).

Considering that GO and AGXX® have both previously been shown to produce ROS [16,22], the expression of the oxidative stress genes was not surprising. The genes encoding the alkyl hydroperoxidase subunit F (*ahpF*), catalase (*katA*), and thioredoxin A (*trxA*) are all

Fig. 5. MRSA genes crucial for survival in biofilm. Arginine-ornithine antiporter gene *arcD*, and the ornithine transcarbamylase gene *arcB* are present in cluster 1 (blue colour), while carbamate kinase gene *arcC*, and the arginine deiminase gene *arcA* are present in red and green clusters, respectively. “*”, gene selected for validation by RT-qPCR. For further explanation of the experiment names, see the legend to Fig. 4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. Heatmap of the expression of potassium transport genes in *S. aureus* 04-02981 upon exposure to GOX, AGXX® or GOX-AGXX® fibres. Differential expression of the genes encoding potassium transport ATPase A (*kdpA*), ATPase B (*kdpB*), and ATPase C (*kdpC*). KdpDE is a two-component system where *kdpD* is the sensor histidine kinase and *kdpE* the response regulator. For the coding of the experiments at the bottom of the figure, see the legend to Fig. 4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

induced in the presence of AGXX® and/or GOX-AGXX® fibres (see Supplementary Table 9).

3.6. Validation of RNA-sequencing data

We used RT-qPCR to validate the transcriptomic data obtained by RNA-sequencing. Four genes, namely *bglA*, *sarA*, *arcC*, and *kdpE* were selected as representatives of the groups of genes that were highly affected in MRSA upon exposure to GOX, AGXX®, or GOX-AGXX® fibres. Gene *bglA* was chosen to confirm the response of MRSA to cellulose since this sugar polymer unexpectedly seemed to interfere with gene expression in this organism. The gene for *SarA* - a virulence regulator that enables biofilm formation in MRSA, *arcC* - necessary for survival in biofilms, and *kdpE* - a potassium transporter gene also involved in virulence regulation in MRSA, were the other selected genes. Figs. 7 and 8 summarize the RT-qPCR results and compare them with the RNA-seq data.

The trend of differential gene expression was similar between both techniques. Some differences observed in RNA-seq and RT-qPCR were as follows i) expression of *bglA* was highly induced in all the samples in

RT-qPCR at t0 especially in the presence of cellulose (41 fold), ii) *sarA* was differentially expressed in all the samples in RT-qPCR while in RNA-seq, only in the presence of GOX, and GOX-AGXX®, and iii) *kdpE* was induced in the presence of cellulose in RT-qPCR (4 fold) but was not differentially expressed in RNA-seq. All gene expression values in terms of fold change are presented in detail in Supplementary Table 10.

We also compared the data of both techniques for the selected genes at t120 (see Fig. 8, Supplementary Table 11).

In the presence of cellulose, both methods showed an up-regulation of *bglA*, RNA-seq data revealed a 3 fold, and RT-qPCR data a 4 fold induction, the change of gene expression in the presence of GOX-AGXX® in RT-qPCR was also around 3 fold (*bglA* = 3, *sarA* = 2, *kdpE* = 4, and *arcC* = -2fold).

Although the two techniques, RNA-seq, and RT-qPCR cannot be directly compared in terms of absolute gene expression values, the data obtained using both these techniques matched well for some samples, especially for GOX at t0, and GOX, and AGXX® at t120. The expression difference of some of the significantly differentially expressed genes as revealed by RT-qPCR was under the threshold value of 2 fold as obtained by RNA-seq (these genes were not expressed OR not

Fig. 7. Differential expression of four selected genes upon exposure to GOX, AGXX®, or GOX-AGXX® fibres at time-point zero min. Expression patterns of the selected genes by RT-qPCR (left panel) and by RNA-seq (right panel). Error bars indicate standard deviation. Asterisks indicate p-values showing statistical significance (****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05; n.s., not significant). The samples indicate the antimicrobial or cellulose and the time (in min) that *S. aureus* 04-02981 was exposed to the particular substance.

Fig. 8. Differential expression of four validation genes at time-point 120 min after exposure to GOX, AGXX®, or GOX-AGXX® fibres. Expression patterns of the selected genes using RT-qPCR (left panel) and RNA-seq (right panel). For further explanation, see legend to Fig. 7.

differentially expressed in RNA-seq) which is the cut-off value of this technique. Examples are the changes of expression of *sarA* (−1.7 fold) in the presence of AGXX®, and that of *arcC* (−1.9fold) in the presence of GOX (Fig. 8).

4. Discussion

Multi-drug resistant pathogens such as MRSA cause lethal nosocomial infections. Thus, it is imperative to find solutions to fight these organisms as well as to control their spread. We tested the effect of two different antimicrobial materials - GOX, and AGXX® - coated on cellulose fibres as well as the combination of both materials on the growth and transcriptome of the clinical MRSA strain *S. aureus* 04-02981.

The RNA-seq data revealed that all the cellulose-based fibres had a remarkable effect on the transcriptome of *S. aureus* 04-02981. Surprisingly, uncoated cellulose fibres also markedly impacted gene expression in *S. aureus* 04-02981 at all time-points examined. This strain was influenced most when exposed to uncoated cellulose fibres for 30 min (1007 genes differentially expressed), with the number of genes going gradually down after longer exposure times. These results were intriguing since uncoated cellulose fibres did not markedly influence the growth of *S. aureus* 04-02981 in the microbiological tests. Since the main aim here is to get a deeper understanding of the mechanism of the two materials GOX, and AGXX® and to investigate their synergistic effect only brief information on the effect of uncoated cellulose is included.

Cellulose is a linear chain of β-1, 4-glucose monomers linked by β-1, 4-glycosidic bonds [42,43]. Bacteria degrade it using three types of enzymes: endoglucanases and cellobiohydrolases, which synergistically depolymerize cellulose to cellobiose, followed by β-glucosidases, which hydrolyse cellobiose to glucose [42,44,45]. The action of the β-glucosidases is the rate-limiting factor in cellulose degradation, making the study of the expression of these genes imperative to understand cellulose degradation [44]. Hence, we examined the expression of *bglA*, and *lacG*, and found them highly upregulated with uncoated cellulose fibres at t30 and t120. The effect of uncoated cellulose on the transcriptome of *S. aureus* 04-02981 in general, and particularly the upregulation of these genes is noteworthy and need to be investigated in detail in follow-up studies.

Among the genes whose expression was impacted by the antimicrobials were those associated with biofilm formation and virulence. Agr and Sae are among the most studied and best characterized regulators in *S. aureus* with AgrCA and SaeRS two-component systems crucial for virulence in *S. aureus*. Agr is a quorum-sensing system specified by four genes *agrA*, *agrB*, *agrC*, and *agrD*. Agr positively regulates virulence factors and is linked to virulence by the effector molecule RNAIII [39,46]. In our study, the sensor histidine kinase *agrC* and the autoinducing peptide *agrD* are both generally down-regulated. AgrCA and SaeRS positively regulate expression of extracellular proteins Eap

and Emp required for biofilm formation in *S. aureus*. Like AgrCA, SaeRS is also critical for biofilm formation [31,46]. Induction of SaeRS increased biofilm formation in *S. aureus* [47]. The expression of both *saeR* and *saeS* is down-regulated in our study.

Another global regulator in *S. aureus*, SarA, affects transcription of staphylococcal virulence genes and is critical for biofilm formation [46,48]. It increases the transcription of *agrBDCA* and RNAIII by binding to the P2 and P3 promoters, respectively, in the *agr* locus [46,49,50]. A study of the biofilm formation characteristics of eight wild type strains of *S. aureus* and their respective *sarA* and *agr* mutants revealed that deletion of *sarA* reduced biofilm formation in six out of eight of the strains [51]. Repression of *agrCD*, *saeRS*, and *sarA* along with other virulence factors such as *rsbU*, and genes encoding protein MSA, and cell division proteins such as Efb, SasD, FtsL, Veg and SpoVG in the presence of GOX, AGXX®, or GOX-AGXX® (Fig. 4 and Supplementary Table 3) suggests that the antimicrobial materials may have a negative effect on biofilm formation in *S. aureus* 04-02981.

Agr encodes RNAIII which is linked to biofilm formation and virulence, and is also associated with biofilm survival. The auto inducer RNAIII-activating protein induces phosphorylation of RNAIII-activating protein (TRAP). Korem et al. [52] demonstrated that TRAP increases the expression of genes essential for survival in biofilms such as the *arcABCD* genes [52]. *arcABC* are part of the arginine deiminase pathway and catalyse the conversion of arginine to ornithine, ammonia and carbon dioxide. Bacterial cells also require these genes to survive under stress conditions [53]. The *arcABCD* genes were generally down-regulated in our data. Interestingly, they were repressed in the samples in which the expression of genes associated with biofilm formation was also diminished. This suggests the potential of GOX, AGXX® and their combination fibres in attenuating biofilm formation in *S. aureus* 04-02981, especially when combined with the fact that these fibres inhibit bacterial growth, hence reducing the population density in the culture. We have previously shown reduction of biofilm formation in *S. aureus* 04-02981 in the presence of AGXX® [10].

Another group of genes that were highly differentially expressed were those encoding the Kdp system. These are essentially potassium transporters, but they also act as virulence regulators. K⁺ is a vital cation for bacterial growth and survival [54]. The K⁺ concentration is crucial for maintaining the turgor of the cells, regulating pH and for the infectious status of *S. aureus* [54,55]. KdpDE, first characterized in *E. coli*, is an important virulence regulator in *S. aureus* [55,56]. The primary role of KdpFABC and KdpDE in *S. aureus* NCTC8325 was proposed not to be in K⁺ transport but in the regulation of transcription of virulence genes [55]. KdpE can directly bind to the promoters of virulence genes and alters their transcription [55]. *kdpDE* mutants show very low survival rates, indicating that *kdpDE* is essential for survival of *S. aureus* [55]. KdpDE also influences the expression of biofilm and virulence regulator genes like *arcA*, *hisB*, Veg protein gene, *ahpC*, *kdpB*, *kdpC*, *agrC*, *agrD*, *lrgA*, *aur*, *spa*, *capA*, and thioredoxin genes [55].

We observed that at t0, t30, and at t120, the *kdp* genes were downregulated. The opposite trend was seen at t180, where the genes were highly upregulated. One reason behind this trend might be the fact that the transcription of the *kdp* genes is increased under K⁺ deficient conditions and in the post-exponential phase in *S. aureus* [55]. Since KdpDE responds to population density to decide whether or not to elicit a response, the downregulation at earlier time-points such as at t0 and t30 where the population density was lower than at t120 and t180 where these genes were up-regulated is at least partially justified. Up-regulation at t180 especially in the presence of uncoated cellulose fibres further explains this since uncoated cellulose fibres did not inhibit bacterial growth in the growth inhibition experiments.

Iron is another vital nutrient required for growth of many bacteria [57]. Some strains of *S. aureus* produce siderophores under iron-limited growth conditions; production of siderophores is crucial to virulence of the bacteria [57,58]. A nine-gene operon called *sbm* is responsible for siderophore production in *S. aureus* [57]. In our study, all the *sbm* genes were up-regulated in the presence of all the materials, at all tested time-points. The highest up-regulation was observed in the presence of uncoated cellulose at t30 (for *sbmA*, *sbmB*, and *sbmE* genes), at t120 (for *sbmC*, *sbmD*, *sbmF*, and *sbmH*), and in the presence of GOX-AGXX at t0 (for *sbmG* and *sbmI* genes). Since the expression of the *sbm* operon enables prolonged survival of *S. aureus* in the environment [57], we speculate that the increased expression of these genes in *S. aureus* 04-02981 is a response to overcome the stress imposed by the antimicrobial materials, a strategy necessary for the survival of the bacterium.

Further, we confirmed the RNA-seq data by performing RT-qPCR on four selected genes, *bglA*, *sarA*, *arcC*, and *kdpE* at t0, and t120. *gyrB* was used as the house-keeping gene. The data obtained were normalised to *gyrB* and fold changes were calculated with respect to the untreated control of the respective time-points. The differences in gene expression measured via RT-qPCR and RNA-seq were similar in case of GOX, and GOX-AGXX at t0 and GOX, AGXX at t120 (Figs. 7 and 8). We also observed some differences between RNA-seq and RT-qPCR data. *bglA*, *sarA*, and *arcC* were differentially expressed in RT-qPCR in presence of AGXX at t0 but not in RNA-seq data. In RT-qPCR, all the selected genes (*bglA*, *kdpE*, *sarA*, and *arcC*) were differentially expressed in presence of uncoated cellulose at t0 and GOX-AGXX at t120, whereas these genes were not differentially expressed in the RNA-seq data. However, in case of GOX-AGXX at t120, the fold changes of *bglA*, *sarA*, and *arcC* in RT-qPCR were close to the threshold of 2, hence the difference between RT-qPCR and RNA-seq for these genes is not remarkable. The data obtained via RNA-seq and RT-qPCR cannot be quantitatively compared due to different normalisation and calculation approaches used in both techniques. Thus, with an exception of uncoated cellulose and AGXX at t0, we could confirm the trends in gene expression obtained in RNA-seq, via RT-qPCR.

Several studies have examined the combination of GO and metals like silver. A nanocomposite with chitosan, GO and zinc oxide nanoparticles was tested against *E. coli* and *S. aureus*. The nanocomposite had a strong inhibitory effect on both organisms, mediated by production of ROS [59]. GO was also used in combination with silver nanoparticles (AgNPs) coated on polyurethane foil against *E. coli*, *S. aureus*, *S. epidermidis*, and the pathogenic yeast *Candida albicans* [60]. The combination proved to be far more efficient than GO or Ag-NPs alone. The antimicrobials had a stronger effect on Gram-negative than on Gram-positive bacteria [60]. In a similar study, GO-AgNPs conditioned with sodium borohydride were tested against *E. coli*, and *S. aureus* [61]. Transmission electron microscopy revealed that GO attached to *S. aureus* cells and wrapped the bacteria. On the other hand, AgNPs penetrated the bacterial cells and damaged the cell membrane, leading to cell death [61]. Severe growth inhibition was seen in both *E. coli* and *S. aureus* [61]. Surface-modified materials in combination with biocidal substances can be efficient in fighting multidrug resistant bacteria [60]. The work presented here also shows this potential.

5. Conclusion

AGXX® and the combination of GOX and AGXX® act as effective antibacterial agents against *S. aureus* 04-02981. These materials seem to affect the ability of *S. aureus* 04-02981 to form and survive in biofilms. They also affect the transcription of *kdp*, a system that is crucial for intracellular survival and pathogenesis of MRSA. In addition, the cellulose-based fibres influence the expression of siderophore genes suggesting that they impose stress on the bacterial cells and create iron-deficient conditions. GOX, AGXX®, and their combination have numerous potential applications in medical equipment, as novel biocides, and in agriculture.

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Declaration of competing interest

No conflict of interest declared.

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Authors contributions

AV performed all the microbiological and molecular experiments, analysed the RNA-sequencing data and drafted the manuscript. RA, OW and RH developed the antimicrobial fibres and RA and OW generated them. AdJ conducted the bioinformatic analyses of RNA sequences and prepared the RNA-seq data for deposition in NCBI. JK edited the manuscript and gave insightful suggestions towards the discussion of the data. EG designed the project, supervised all the experiments and edited the manuscript. All authors discussed and revised the manuscript.

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