

## Surface acoustic waves increase the susceptibility of *Pseudomonas aeruginosa* biofilms to antibiotic treatment

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Bacterial urinary tract infections resulting from prolonged patient catheterization have become a major health problem. One of the major issues is bacterial resistance to antibiotic treatments due to biofilm formation inside the catheters, thus enhancing the search for alternative treatments. In the present study, a device containing a piezo element capable of transmitting low-frequency surface acoustic waves (SAW) onto the indwelling catheter was used. The SAW were able to eradicate biofilm-residing bacteria by > 85% when applied simultaneously with an antibiotic in three clinically relevant species, viz. *Escherichia coli*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. Moreover, transcriptome analysis revealed that SAW can alter the transcription pattern of *P. aeruginosa*, suggesting that this signal can be specifically sensed by the bacterium.

**Keywords:** biofilm; surface acoustic waves; transcriptome analysis; antibiotics

### Introduction

Bacteria thrive in nature in two major physiological states, viz. as free-living (ie planktonic) bacteria or in matrix-embedded complex structures termed biofilms (Hall-Stoodley et al. 2004). Biofilm represents a protected mode of growth that allows bacteria to withstand harsh environmental conditions. The ability of bacteria to colonize virtually any surface and form biofilms has made them a major cause of medical infections. It has been estimated that >65% of the bacterial infections treated in hospitals are caused by bacterial biofilms, most of which are associated with indwelling medical devices (Bryers 2008; Donlan 2008). One of the hallmarks of the biofilm lifestyle is its increased resistance to the host-immune system and antibiotic-killing compared to the planktonic mode of growth (Davies 2003; del Pozo and Patel 2007). There are two main obstacles towards eradicating biofilm-residing bacteria using antibiotics: the extracellular matrix and the physiological state of the bacteria within the biofilm (Lopez et al. 2010). As noted above, bacteria residing within biofilms are encapsulated in an extracellular matrix, which consists of several components including polysaccharides, proteins and DNA (Branda et al. 2005). This extrapolymeric matrix acts as a diffusion barrier between embedded bacteria and the environment thus retarding penetration of, among other things, antibacterial agents (Hall-Stoodley and Stoodley 2009). Additionally, it has been suggested that

due to limited nutrient accessibility, the physiological state of the biofilm-residing bacteria is featured by low metabolism and dormancy (ie persister cells) increasing their resistance towards antibiotic agents (Lewis 2010).

All of the above have led researchers to search for novel approaches to enhance antibiotic efficacy in an attempt to successfully treat biofilm-associated infections. One promising approach utilized ultrasound combined with antibiotic treatment. The principle behind ultrasound treatment is based on generation of ultrasound longitudinal and/or shear waves through the material with linear particle motion in a cavitation range. This approach, mostly led by Pitt and colleagues, demonstrated that ultrasonic energy increased bacterial susceptibility to antibiotics (Rediske et al. 1998, 1999a; Qian et al. 1999). Moreover, researchers have shown that the use of ultrasound significantly reduced the antibiotic concentrations required for the inhibition of bacterial growth and also minimized spontaneous antibiotic resistance that enhances bacterial fitness (Qian et al. 1999; Rediske et al. 2000; Carmen et al. 2005). Most of the *in vitro* studies were aimed at eradication of bacterial biofilms that occurred on implants and utilized sonication baths, having either water or air as the conductor (Rediske et al. 1999a). Alternatively, in the *in vivo* studies the antibiotics were administered systemically and local ultrasound treatment was applied (Rediske et al. 1999b; Carmen et al. 2005). Major disadvantages of

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this approach for clinical application are that these systems are fairly large, require operation of professional staff, they are limited in application time and cannot be utilized with indwelling devices. More recently, a different approach was described that used low-energy acoustic waves that were transmitted directly to an indwelling medical device (ie urinary catheters) using a small portable transmitter (Hazan et al. 2006). As opposed to the previous studies, here low-energy surface acoustic waves (SAW) were spread from an actuator to the catheter surface (Hazan et al. 2006). The energy transmission occurs through the interface between the layers of the exposed materials. This leads to dispersion of the acoustic energy on the entire surface of the catheter creating an elliptical movement and chaotic microstreaming in the medium. The SAW approach was shown to be effective in the prevention of biofilm formation of different bacterial pathogens. However, no data exist regarding the effect of SAW on existing an biofilm or antibiotic treatment (Hazan et al. 2006). Furthermore, the overall bacterial response to acoustic treatment has never been examined. Thus, in the present study the effect of SAW, transmitted directly onto a urinary catheter, on the antibiotic susceptibility of a *Pseudomonas aeruginosa* biofilm was further investigated. *P. aeruginosa* is a well-studied bacterium, mostly known for its ability to form drug-resistant biofilm associated infections in the lungs of cystic fibrosis patients (Hoiby et al. 2010). However, *P. aeruginosa*, as well as other pathogenic bacteria, have been implicated in urinary tract infections associated with prolonged catheterization (Mittal et al. 2009). The present results revealed an increase in antibiotic susceptibility of biofilm-residing *P. aeruginosa*, *Escherichia coli* and *Staphylococcus epidermidis* when treated with SAW and antibiotics simultaneously compared with antibiotic treatment alone. Moreover, a transcriptome analysis of *P. aeruginosa* biofilm suggested that exposure to SAW triggers a specific cellular response, which may well influence bacterial resistance and virulence.

## Materials and methods

### Vibration generating device

A device capable of generating and transmitting SAW onto a catheter was provided by NanoVibronix Corp. An electronic driver sends periodic electrical pulses to an actuator harboring a ceramic piezo element. The frequency of the vibrations generated on the piezo element is  $100 \text{ kHz} \pm 10\%$  and at on/off frequency of 30Hz; the acoustic intensity was  $0.4 \text{ W cm}^{-2}$  and an amplitude of  $2 \mu\text{m}$ . The acoustic energy on the inner surface of the catheter is  $0.3 \text{ mW cm}^{-2}$  with a wave amplitude of 0.2–2 nm. In the Epsilon meter test

experiments the acoustic intensity and amplitude were preserved through a decrease in actuator energy by 10-fold.

### Strains

*E. coli* 1313 and *P. aeruginosa* PAO1 were grown on tryptone soya broth (TSB) medium, while *S. epidermidis* was grown on TSB supplemented with 0.2% glucose (Holloway et al. 1979; Girshevitz et al. 2008). For flow cell experiments 10% TSB medium supplemented with 0.2% casamino acids was used. All bacteria were grown at  $37^\circ\text{C}$  for indicated times.

### Epsilon meter test (E-test)

To determine the minimal inhibitory concentration (MIC) the Epsilon meter test (E-test) method (Brown DFJ and Brown L 1991) was utilized. An E-test gentamicin strip (Bio merieux, France) was placed on a Muller Hinton Broth (MHB) media plate and swabbed with  $\sim 10^8$  viable cells. The plate was incubated for 20 h at  $37^\circ\text{C}$  with a SAW actuator attached to the bottom. Following the incubation, the MIC was determined according to the manufactures guidelines and compared to the untreated SAW control. To rule out increase diffusion through the agar in the SAW treated samples, a reversed experiment was designed in which the plate was first incubated with the antibiotic E-test strip for 20 h with and without SAW to allow diffusion of the antibiotic throughout the plate. Following this, the antibiotic strip and SAW actuator were removed and bacterial cells were plated and incubated for 20 h and the MIC was measured.

### Flow cell experiments

The experimental system was composed of a medium container connected through silicon tubes to a 10 cm long Foley catheter segment (16Ch/Fr5/10ml/2cc, standard Latex, Siliconized). Catheters were incubated with 1 ml of  $1.5 \times 10^8$  bacteria  $\text{ml}^{-1}$  of inoculum for 1 h. Following the incubation the medium flow was initiated and sustained by peristaltic pump at flow rate of  $10 \text{ ml h}^{-1}$  for 48 h at  $37^\circ\text{C}$ . At this point the indicated treatment was applied. For antibiotic treatment  $25 \mu\text{g ml}^{-1}$  gentamicin were administered. For the combined treatment or SAW alone the actuator was activated and incubation was prolonged for an additional 24 h. (For a schematic representation of the system see Supplementary information Figure S1. [Supplementary material is available via a multimedia link on the online article webpage]) At the end of the experiment biofilm cells were removed from the catheter and the bacteria were plated and counted.

For flow cell microscope examination a similar setup as described above was utilized, except that the biofilm was grown in a flow cell chamber rather than in a catheter (Banin et al. 2006). Biofilms of *P. aeruginosa* were grown for 4 days, to achieve maturation, and then exposed for an additional 24 h to the various treatments (gentamicin, SAW and combined gentamicin and SAW treatment). Following this the biofilms were stained with the Live/Dead<sup>®</sup> BacLight<sup>™</sup> bacterial viability stain (Invitrogen, Carlsbad, CA) and visualized using a laser confocal scanning microscope (Leica SPE-TCS) as described previously (Glick et al. 2010). After acquisition, images were processed using Volocity (Improvision, Lexington, MA) software.

### RNA purification and transcriptome analysis

The flow cell experiment was performed using catheters as described above. Gentamicin was used at a concentration of 10  $\mu\text{g ml}^{-1}$  in order to obtain enough cells for RNA extraction. SAW were applied for 2 h and bacteria were harvested and resuspended in RNA protect solution (Qiagen). The biofilm was dispersed by sonication and following cell lysis RNA was extracted using the RNeasy Kit (Qiagen). RNA purity was assessed using a Bioanalyzer. The gene expression pattern was determined using the *P. aeruginosa* GeneChip genome arrays (Affymetrix) in the Genome research center at the Hebrew University, Jerusalem. Three biological repeats were combined on one GeneChip and two duplicates were analyzed.

### Transcriptome data analysis

The feature extraction was performed with GeneSpring GX 10.0.1 software (Agilent). The GCRMA algorithm had been used for probe summarization. The algorithm uses quantile normalization. The potential batch effect (separate treatment of biological samples in repeated experiments) was minimized using ComBat R script. For the statistical analysis of differentially regulated gene expression Cyber-T software was utilized. Cluster and TreeView for cluster analysis was obtained from M. Eisen's laboratory (University of California, Berkeley). The hierarchical clustering with a similarity matrix based on correlation coefficient was performed on the gene set filtered by the maximal expression level  $\geq 5$  and max-min  $\geq 5$ . The expression values were centered and normalized for each gene.

### RealTime PCR quantitative analysis

Flow cell experiments and RNA purification were performed as described for the transcriptome analysis. cDNA was synthesized from 2  $\mu\text{g}$  of RNA using the

SuperScript II Synthesis System and random hexamers (Invitrogen, Carlsbad, CA). Real-time PCR (RT-PCR) was performed using a StepOnePlus<sup>™</sup> Real-Time PCR System (Applied Biosystems, Foster City, CA). The 20  $\mu\text{l}$  PCR reactions included 1 ng of cDNA, 1  $\times$  Fast SYBR Green master mix (Applied Biosystems) and 0.4  $\mu\text{M}$  of each primer. PA1684 was used as housekeeping gene control based on the fact that it did not change in any of the microarray tested conditions examined or in those previously published (Balasubramanian and Mathee 2009). The reactions were performed in Optical 8-Tube Strips at 95°C for 20 s followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. Data were collected after each cycle. Following the PCR reaction a melting curve was performed from 60°C to 95°C. A standard curve was performed for each primer pair to check the efficiency of the reactions. The primers used for RT-PCR are listed in Table 1.

## Results

### Influence of SAW on antibiotic efficacy

In the present study a device that generates low energy SAW of practically non-thermal range from electrically activated piezo ceramic elements (Hazan et al. 2006) was used. The device is capable of transmitting the vibration directly to the surface (eg an indwelling device). Based on previous findings the optimal frequency was adjusted to 100 kHz and intensities of 0.3  $\text{mW cm}^{-2}$ . The first goal was to evaluate the ability of SAW to enhance antibiotic activity on bacterial cultures of *P. aeruginosa* growing on agar plates. The modified version of the E-test in which agar plates were exposed to SAW was used. The bacteria were plated on agar plates with a gentamicin E-test strip in the

Table 1. Primers used for RT-PCR experiments.

Primer name	Sequence (5'-3')
MexR-36F	GCTGATGGCGGTCTTCCA
MexR-147R	TTGTTTCGTCGATAAGCTTCAATACAT
PopN-93F	CGAGCGCGTCCACTACGT
PopN-163R	AAAAGGCGAAGGTCAGTCTCT
ExoY-158F	TGGCCAGGCAGACGAATAC
ExoY-238R	TCGGGAAACCCTCTTCGAT
PA3035-46F	CGCCTGTTCTCTCCCTCA
PA3035-185R	AGTTGCAGCTCGCCGTCT
PA3133-147F	GAGCAAGGATGCGCTGATG
PA3133-246R	ACGCTCCTTTCCCGACAAT
PA4825-2053F	CTGGAGGAGGGCGTTATCAA
PA4825-2133R	GAAGTTGGAGCTGGCAGTCAT
NalC-436F	GCGGTCTCCTCACATG
NalC-519R	GAGGAACAGGTCGGCCTTG
PA1684-2F	TGAGCAGCCTTACCGTCTATCA
PA1684-88R	CCAGGGTCTGAAGCGATGT

presence or absence of SAW and the MIC was determined. The MIC of the untreated control was  $1.70 \pm 0.23 \mu\text{g ml}^{-1}$  while the SAW treated bacteria had an MIC of  $0.45 \pm 0.22 \mu\text{g ml}^{-1}$  ( $P < 0.001$ ). Thus the results show that the combined treatment increased the susceptibility of *P. aeruginosa* by approximately 3.7 fold. To rule out the possibility that SAW increased the diffusion of the antibiotic through the agar the following experiment was carried out. The plates with the gentamicin E-test strip were first incubated for 20 h without bacteria, but with or without SAW in order to allow diffusion of the antibiotic through the plate. Only then were the bacteria plated onto the plates without SAW and the MIC were measured 20 h later. No differences in the MIC concentration were observed between the plates that were pre-exposed to SAW compared to those that were not. This demonstrates that an increase in diffusion of the antibiotic through the agar is most likely not the cause for the enhanced susceptibility, but rather the result of the bioacoustic effect on the bacteria directly.

#### Influence of SAW on antibiotic efficacy in biofilms

Previous work by Hazan et al. (2006) showed that application of SAW onto urinary catheters can prevent biofilm formation. This was tested using increasing bacterial inocula. The results demonstrated that SAW were effective in reducing *P. aeruginosa* biofilm formation in catheters challenged with bacterial inoculums as high as  $10^6 \text{ cells ml}^{-1}$ , but were less effective at higher bacterial concentrations (Figure 1).

Next the effect of SAW on existing *P. aeruginosa* biofilm formed on the catheter surface was assessed.

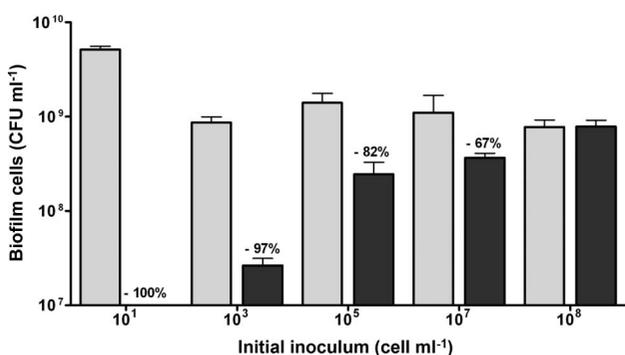


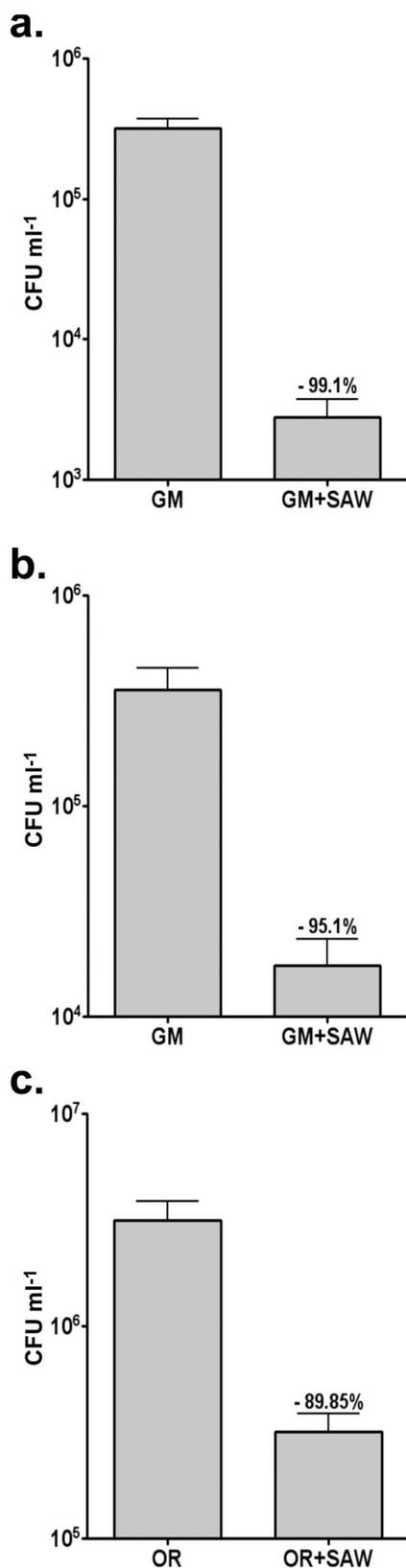
Figure 1. Prevention of biofilm formation using SAW. Catheters were challenged with different *P. aeruginosa* inoculum concentrations with and without SAW. Presented are CFU ml<sup>-1</sup> obtained following incubation for 3 days in catheters in the presence (black) or absence (grey) of SAW. The bars are an average of at least three independent experiments; the error bars represent SD. The percentages above the bars represent the effectiveness of prevention of biofilm formation.

The biofilm was allowed to form over a period of 48 h inside the catheters using the flow cell system (see Supplementary information Figure S1 for the system design [Supplementary material is available via a multimedia link on the online article webpage]) and only then exposed to SAW for an additional 24 h. Following this treatment, the biofilm cells were removed from the catheter and plated for viable counts. No significant change in bacterial burden was observed between treated and untreated catheters, suggesting that SAW treatment alone did not disperse the biofilm (data not shown). Next a study was carried out to determine the combined SAW and antibiotic treatment is effective against *P. aeruginosa* biofilms residing on the catheter. The flow through system was again used, but this time the existing biofilm was treated with the antibiotic in the presence and absence of SAW. The combined SAW and gentamicin treatment resulted in a reduction of two orders of magnitude in the CFU of *P. aeruginosa* compared to the antibiotic treated control (Figure 2a). The analysis was then extended to another Gram-negative pathogen, *E. coli*, and a Gram-positive bacterium, *S. epidermidis*. For both of these species >90% reduction in the biofilm burden was observed in the combined treatment of antibiotic and SAW (Figure 2b and c).

The effect of these treatments on biofilm was next observed using confocal microscopy. For this purpose, following biofilm formation on slides for 4 days the resulting mature biofilms were exposed either to gentamicin, SAW or the combined SAW and gentamicin treatment. Treatment proceeded for 24 h followed by application of Live/Dead staining to determine the efficacy of the treatment (Figure 3). The results clearly showed that the gentamicin treatment only killed the cells in the outer layer (stained red) while the cells in the center of the biofilm remained viable (stained green). The SAW-treated biofilms remained unaffected, in agreement with the catheter experiments (Figure 3). The combined SAW and antibiotic treatment had a much more pronounced effect on the cells inside the biofilm, evident by the increase in red staining in the center. Taken together, these results emphasize that the combined treatment was able to increase the efficacy of antimicrobial agents against biofilms.

#### Impact of SAW on gene expression

The response to SAW treatment could be a result of mechanical and/or physical stimuli or due to a specific bacterial bioacoustic response. To begin addressing this possibility a transcriptome analysis of *P. aeruginosa* biofilms exposed to four different conditions was carried out: (i) untreated control; (ii) gentamicin; (iii) SAW and (iv) SAW and gentamicin as a combined



treatment. Since the previous results showed a substantial reduction in bacterial counts following the combined treatment, the experiment was slightly adjusted to obtain enough cells for gene expression analysis. Exposure to SAW resulted in differential expression of 173 genes (95 were down-regulated and 78 up-regulated). Of these genes 125 were common to both the SAW alone and the combined SAW with gentamicin treatments, highlighting, most likely, the core SAW regulon (Figure 4). RealTime-PCR experiments confirmed GeneChip data for several down-regulated and up-regulated genes (Table 2).

#### *Genes down-regulated in the presence of SAW*

Genes associated with biosynthesis of coenzymes, membrane proteins, transport of small molecules, secreted factors and protein secretion showed down-regulation upon exposure to SAW (Figure 5a and Supplementary information Table 2 [Supplementary material is available *via* a multimedia link on the online article webpage]). SAW caused a clear decrease in expression of several virulence factors such as the Type III secretion system (TTSS), the major virulence system in *P. aeruginosa* (Hauser 2009), and *prpL* and *lasB* genes, extracellular proteases implicated in virulence (Wilderman et al. 2001; Cowell et al. 2003). The operon required for production and regulation of the quorum sensing signal PQS (*pqsA-E*) was also down-regulated upon exposure to SAW. The PQS signal and its precursor molecule HHQ have been shown to reduce the innate immune response and *pqsA* mutant showed reduced dissemination in the lung tissue compared with the wild-type strain in a mouse *in vivo* intranasal infection model, suggesting that HHQ and PQS may play a role in the pathogenicity of *P. aeruginosa* (Kim et al. 2010). SAW also caused down-regulation of the *narI-G* operon coding for the membrane-bound nitrate reductase, which is required for anaerobic respiration with N-oxides as terminal electron acceptors (Stover et al. 2000). This result may suggest a switch from anaerobic to aerobic respiration upon exposure to SAW. There is also a link to virulence. Van Alst et al. (2007) reported that *narGH* mutants were avirulent in a *Caenorhabditis elegans* infection model and were also defective in

Figure 2. Viability of cells within the biofilm after antibiotic exposure with and without SAW. Mature biofilms were grown in 10 cm Foley catheters for 48 h without any treatment. The established biofilms were then treated with antibiotics with and without SAW for another 24 h. (a) *P. aeruginosa* biofilm treated with 25  $\mu\text{g ml}^{-1}$  gentamicin; (b) *E. coli* biofilm treated with 5  $\mu\text{g ml}^{-1}$  gentamicin; (c) *S. epidermidis* biofilm treated with 5  $\mu\text{g ml}^{-1}$  orbenil (OR). The numbers above each bar indicate the additional change in percentage as an outcome of the addition of SAW.

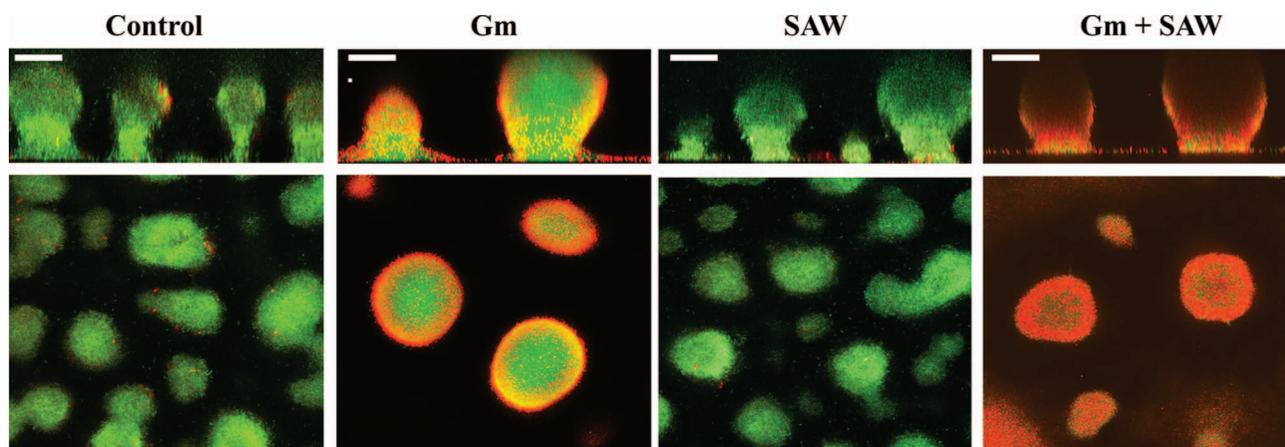


Figure 3. Effect of combined SAW and antibiotic activity on established biofilms of *P. aeruginosa*. Mature *P. aeruginosa* biofilms grown in flow cells for 4 days were exposed for an additional 24 h to gentamicin ( $25 \mu\text{g ml}^{-1}$ ), SAW and a combined gentamicin ( $25 \mu\text{g ml}^{-1}$ ) and SAW treatment. Survival of the bacterium following treatment was measured by Live-Dead staining. Dead cells are stained red and live cells green. The top images are sagittal reconstructions, the middle images represent horizontal sections. Bar = 40  $\mu\text{m}$ .

Table 2. Validation of gene expression levels in different treatments as determined by RT-PCR

Locus tag	Gene name	Gm	SAW	Gm + SAW
PA0424	MexR	1	3.35	4.08
PA1698	PopN	1.53	-5.8	-2.5
PA2191	ExoY	1.48	-6.11	-2.5
PA3035		1.03	183.5	108
PA3133		1.8	66.7	128.3
PA4825		8.3	3.5	21
PA3721	NalC	2	2.8	4.7

Note: Values represent fold change in gene expression compared to untreated sample. Gm = gentamicin.

biofilm formation compared to the wild type strain. SAW also reduced the expression of genes involved in pyochelin uptake and synthesis. Pyochelin is one of two endogenous siderophores produced by *P. aeruginosa* in response to iron starvation (Cornelis 2010).

#### *Genes up-regulated in the presence of SAW*

SAW treatment specifically induced expression of 78 genes. The most significant effect of SAW was observed on two genes residing in an operon PA3132 (probable hydrolase) and PA3133 (probable transcription regulator) (Supplementary information Table 1 [Supplementary material is available *via* a multimedia link on the online article webpage]). Since one of these genes is a probable transcriptional regulator (PA3133) further work will be required to examine its role in the bioacoustic effect. Moreover, the SAW treatments induced the expression of as many as 28 genes associated with transcription regulation (Figure 5a). These included the BifS/R two-component system that

is essential for regulating the transition to irreversible attachment in the initial process of biofilm formation. This two-component system was also shown to be important in maintaining biofilm structure as inactivation of this cluster in mature biofilms resulted in biofilm dispersal (Petrova and Sauer 2010). As mentioned above exposure to SAW did not result in dispersion of mature biofilms, suggesting that over-expression of the BifS/R system might be one of the mechanisms by which the cells enhance their attachment to the surface. Additional regulators induced under SAW included the MexR and NalC regulators involved in antibiotic resistance (see details below) and the PrtN regulator involved in pyocin expression. Balasubramanian and Mathee (2009) compared 23 different transcriptome analyses that were carried out with *P. aeruginosa* exposed to various induced and physiological conditions (eg low iron, and biofilm *vs* planktonic). This wide comparison allowed the authors to extract core genes that are differentially expressed under all tested conditions and specific genes expressed under a certain set of conditions. It is interesting to note that 16 of 28 of the transcription regulators up-regulated upon exposure to SAW were not differentially regulated under any of the 23 conditions described. This may suggest that SAW provides a new signal that is detected by a set of genes that have so far not been observed under any other tested condition.

#### *SAW regulated antibiotic resistance response*

The transcriptome analysis did not provide a clear cellular response that can explain the increased susceptibility to the gentamicin treatment. As

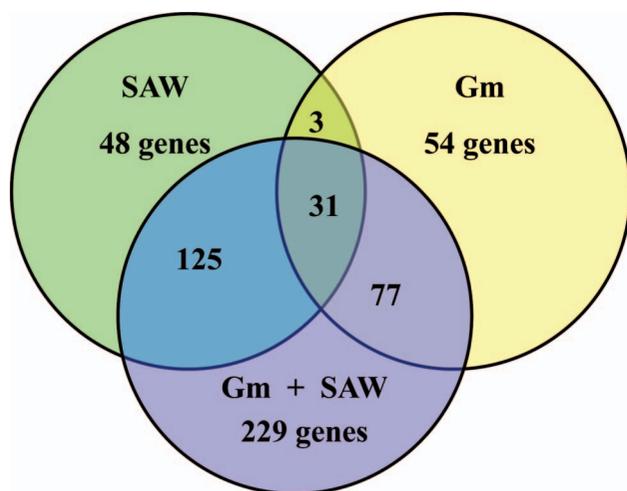


Figure 4. A Venn diagram summarizing the results obtained from *P. aeruginosa* transcriptome analysis. Numbers of genes specifically affected by each treatment (gentamicin [Gm], SAW, or combination of both treatments [Gm+SAW]) are shown in the outer circles. The cross-sections show number of genes that were affected by more than one treatment.

described above two transcription factors involved in antibiotic resistance were upregulated: MexR, which acts as the repressor of the *mexAB-oprM* multidrug efflux pump (Sanchez et al. 2002) and NalC, which is also involved in antibiotic resistance regulation (Cao et al. 2004). It is important to emphasize that the *armR* gene, which acts as an antirepressor of MexR was also induced and overall no change of expression in *mexAB-oprM* was observed (Daigle et al. 2007). When examining the genes that were differentially regulated only by gentamicin (Figure 5b and Supplementary information Tables 3 and 4 [Supplementary material is available *via* a multimedia link on the online article webpage]), ie the surface acoustic treatment did not influence their expression, a few more genes that maybe important to the antibiotic response appear. For example, the efflux pump MexC and the PhoP/Q and PmrB involved in antibiotic resistance (Poole et al. 1996; Macfarlane et al. 2000; McPhee et al. 2003) were induced only in response to gentamicin. In addition, the combined gentamicin and SAW treatment had the largest impact on the bacterial transcriptome when compared to untreated cells with 229 genes that were specifically differentially regulated (122 up-regulated and 107 down-regulated genes) (Figure 5c and Supplementary information Tables 5 and 6 [Supplementary material is available *via* a multimedia link on the online article webpage]). Of these it is interesting to highlight the down regulation of genes involved in biofilm formation including the GacS response regulator that determines the transition to biofilms (Parkins et al. 2001) and genes involved in the synthesis

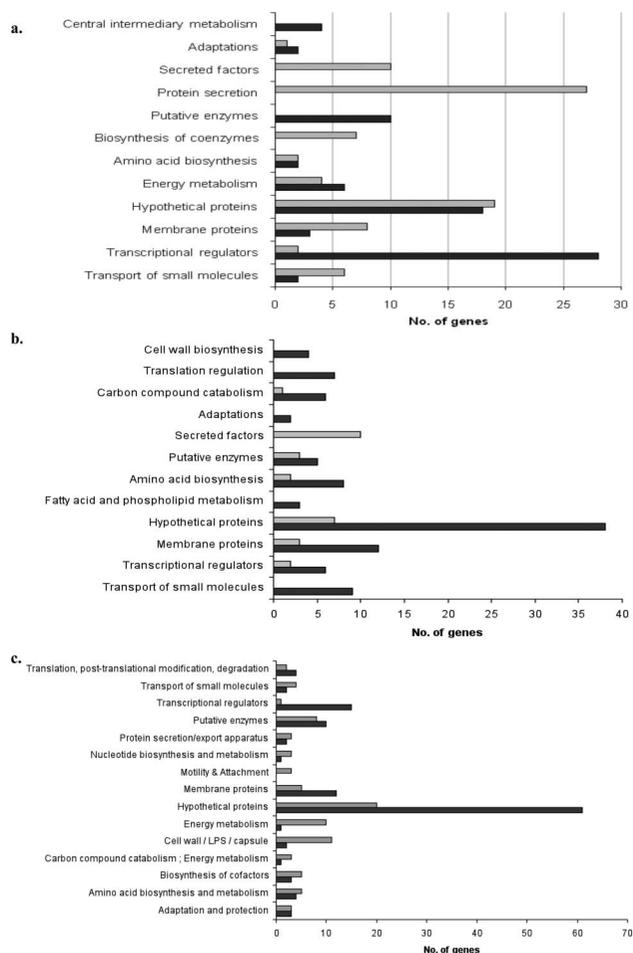


Figure 5. Functional classification of differentially regulated genes made according to the *Pseudomonas* Genome Project (<http://www.pseudomonas.com>). Grey bars represent down-regulated genes. Black bars represent up-regulated genes. (a) genes affected by both application of SAW and SAW + gentamicin; (b) genes affected by gentamicin treatment and combined gentamicin + SAW treatments; (c) genes unaffected by either gentamicin or SAW single treatments, but only during the combined gentamicin + SAW treatment.

of the Psl polysaccharide that is an essential component of the biofilm extracellular matrix (Ryder et al. 2007). Both of these might impact the susceptibility of cells within the biofilm. It may be speculated that one of the main outcomes of this combined treatment is an overall stress response triggered by the exposure to these two stressors (ie antibiotic and surface acoustics) impairing bacterial resistance and rendering the cells more susceptible to the antibiotic treatment.

## Discussion

Bacterial infections present a major health issue especially on implants and indwelling medical devices such as urinary or intravenous catheters. This is mainly

due to the ability of bacteria to form biofilms that are extremely resistant to antibiotic treatments, thus forcing researchers to explore additional methods of coping with this problem. Previous studies have demonstrated that application of ultrasound significantly increases antibiotic efficacy in treatment of bacterial biofilms (Rediske et al. 1998, 1999a, 1999b). The main effort was targeted towards treatment of biofilms formed on implants such as prosthetic hips or bones (Carmen et al. 2004a, 2004b). Thus the application of ultrasound was developed as a complementing treatment for systemically administered antibiotics. However, the ultrasound-generating device utilized in these studies cannot be used with indwelling medical devices. In the present study a small novel device that works on a bending vibration mode was applied and this was designed such that when attached to the urinary catheter surface it excited SAW waves on the catheter inner and outer surfaces. Using this SAW-generating device the efficacy of acoustic energy on antibiotic treatment of bacterial biofilms was tested. Acoustic energy creates stable cavitation and microstreaming which, theoretically, allows better drug penetration and acceleration of bacterial metabolism through active transportation of oxygen and nutrients (Qian et al. 1999). The acoustic energy levels used in the present experimental setups were much lower. SAW propagates the acoustic energy in non-cavitation power intensity ranges, eg these power levels are 3 orders of magnitude lower than the thresholds beyond which cavitation is produced (for ultrasound frequencies of 100 kHz, cavitation is generated at acoustic intensities in the range of  $0.5\text{--}2 \times 10^3 \text{ mW cm}^{-2}$ , while the intensities generated by SAW are  $0.3 \text{ mW cm}^{-2}$ ). This vibration was previously demonstrated to have the ability to prevent biofilm formation (Hazan et al. 2006), and this is also supported by the present results. Further proceeding with these findings, it was also demonstrated that a combined SAW and antibiotic treatment is capable of effectively treating biofilm of several clinically relevant bacterial species, such as *P. aeruginosa*, *S. epidermis* and *E. coli*.

There is still a question of whether the effects observed were due to mechanical interference or perhaps the bacteria sensing the ultrasound signal. This sensation might influence the bacterial physiological state making them more susceptible to antibiotic treatment. To address this, transcriptome analysis of *P. aeruginosa* was performed, which allowed us to gain insight into the bacterial physiological state upon exposure to SAW. The data suggests that SAW is most likely sensed by the bacteria, thus affecting the gene expression pattern. Nevertheless, the possibility that expression of some genes is altered due to

environmental changes that occur as the result of SAW application cannot be ruled out. For example, down-regulation of *narI-G* operon, that marks a switch from anaerobic to aerobic metabolism, might be due to increased oxygen penetration that occurs due to microstreaming produced by SAW (Pitt and Ross 2003). Down-regulation of the PQS operon, which regulates quorum sensing signaling, was also observed. Previously Hazan et al. (2006) suggested that chaotic microstreaming in the medium, which occurs due to SAW application prevents formation of consistent gradient of quorum sensing molecules. This disruption of signal could lead to the negative feedback loop observed as a reduction in gene expression. Nevertheless, these examples are not in conflict with the possibility that bacteria directly sense the ultrasound. Up-regulation of numerous putative regulators that were previously undetected under various physiological and environmental conditions tested suggests that some of them might be responsible for specific responses (Balasubramanian and Mathee 2009). Given that 10% of the *P. aeruginosa* genome is presumed to code for regulatory proteins not all the possible response loops and conditions to which these regulators react are known (Stover et al. 2000).

One of the main goals in performing the transcriptome analysis on bacteria exposed to combined treatment of SAW and gentamicin was to attempt to reveal the mechanisms that allow better antibiotic efficacy. However, given that under the combined treatment many genes that were unaffected by a single treatment (ie SAW or gentamicin) were up- and down-regulated more than two-fold implies that perhaps bacteria experience a general stress response, which makes them more susceptible to antibiotics. Previous *in vitro* work on *E. coli* and *P. aeruginosa* showed that gentamicin is capable of better penetration through biofilm in the presence of ultrasound (Carmen et al. 2004a). This enhanced penetration is also true for nutrients and oxygen causing a change in the bacterial metabolic state, which can also be concluded from the transcriptome data (Pitt and Ross 2003). The confocal images clearly show that there is increased killing of bacteria inside the biofilm. However, it is difficult to distinguish whether this is due to better antibiotic penetration, the bacterial metabolic state or a physiological change inflicted by the acoustic energy. Most likely it is the combination of all the above that leads to the observed effect.

To conclude, the results clearly demonstrate that exposure to SAW can enhance antimicrobial activity. Although the fact that SAW can enhance the diffusion through the biofilm or through the cell membrane cannot be ruled out, the transcriptome analysis suggests there is also a bioacoustic cellular response upon

exposure to SAW. Future work will be required to further characterize how bacteria sense this signal and the nature of the physiological role and interplay between the genes associated with this response.

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