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Molecular Basis of Tobacco-Induced Bacterial Biofilms: An In Vitro Study

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Abstract

Objective. To evaluate changes in the expression of biofilm-related genes when exposed to tobacco smoke and oxidative stress.

Study Design. Experimental, in vitro.

Setting. Laboratories of Rhinology and Microbiology, University of Pennsylvania.

Subjects and Methods. Bacterial biofilm mass was measured using crystal violet staining and measurement of the optical density. Biofilm-related genes of the *Pseudomonas aeruginosa* PAO1 strain (*pilF, flgK, lasI, lasB, rhlA*, and *algC*) were studied following repetitive exposure to exogenous tobacco smoke and hydrogen peroxide. This was done using a reporter plasmid.

Results. After 1 exposure to smoke, there was no change in biofilm formation. However, after 2 and 3 exposures, the biofilm formed had an increased mass ($P < .05$). With respect to oxidative stress in the form of H_2O_2, bacterial cultures demonstrated a dose- and time-dependent induction of biofilm formation compared with control conditions. Gene expression following repetitive smoke exposure demonstrated an increase in expression of *pilF, flgK, algC*, and *lasI* genes ($P < .05$); a decrease in *rhlA* ($P < .05$); and no significant change in the *lasB* gene ($P = 0.1$). Gene expression following H_2O_2 exposure demonstrated an increase in *pilF* ($P < .05$), whereas the other genes failed to demonstrate a statistical change.

Conclusions. Repetitive tobacco smoke exposure leads to molecular changes in biofilm-related genes, and exposure to oxidative stress in the form of H_2O_2 induces biofilm growth in PAO1. This could represent adaptative changes due to oxidative stress or chemically mediated through any of the several chemicals encountered in tobacco smoke and may explain increased biofilm formation in microbes isolated from smokers.

Keywords

chronic rhinosinusitis, biofilm, tobacco, oxidative stress, quorum sensing, virulence factor

Chronic rhinosinusitis (CRS) is an inflammatory disease of multifactorial etiology. It is estimated that it affects about 5% of the American population, resulting in over 600,000 surgeries in the United States alone with an annual cost that surpasses $8 billion.[1-3] Despite the incredible impact on the health care system and on patients' quality of life,[4] its pathogenesis has not been well defined. Recently, there has been an increase in evidence highlighting the role of biofilm in the persistence of recalcitrant CRS and the necessity for additional surgical intervention.[5-7]

Biofilms are complex sessile 3-dimensional structures of bacteria that are attached to a surface. They are embedded in an extracellular matrix and exhibit a distinct phenotype and genotype when compared with their planktonic counterpart.[8,9] Biofilm formation develops in a stepwise fashion that starts with attachment to a surface. Following the attachment, there is the formation of a microbial microcolony, and once this reaches a certain density, biofilm formation ensues.

Tobacco use is a worldwide epidemic that the World Health Organization (WHO) estimates accounts for 3% of the world's morbidity and mortality at a cost of tens of billions of dollars annually.[10] Tobacco smoke exposure has been suggested as a risk factor for the aggravation of CRS,[11] with studies demonstrating that smokers have a higher prevalence of CRS when compared with nonsmokers.[12,13] Moreover,

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Table 1. Primers Designed for Plasmid Construction

flgK	PA1086-F-KpnI, len 28, 5′-GAGGTACCTGGCCAACTTCGCCAACATC-3′
	PA1086-R-PstI, len 27, 5′-GTCTGCAGTTGGCATGAGCACGCATCG-3′
pilF	PA3805-F-KpnI, len 27, 5′-GAAGGTACCAGGTGTCGGAAGGCCACT-3′
	PA3805-R-EcoRI, len 30, 5′-GAGAATTCAACGGCGTCCGGCTTGATGATG-3′
lasI	PA1432-F-KpnI, len 28, 5′-GTGGTACCTATCTGCAACTGCTCGGAAG-3′
	PA1432-R-EcoRI, len 30, 5′-GTGAATTCTTCGCGCCGACCAATTTGTACG-3′
lasB	PA3724-F-KpnI, len 28, 5′-GTGGTACCGGATCGTCGGCGAGCGTCAC-3′
	PA3724-R-PstI, len 25, 5′-GACTGCAGGGAGTTTGGACACGTCG-3′
rhlA	PA3479-F-KpnI, len 28, 5′-GAGGTACCAGTGTCCTATAAGGACCGTG-3′
	PA3479-R-EcoRI, len 30, 5′-GAGAATTCGCCCTTGCAAACCGATACCAAC-3′
algC	PA5322-F-KpnI, len 30, 5′-GAAGGTACCCTGGACAGCGGCAATCCGAAC-3′
	PA5322-R-EcoRI, len 27, 5′-GTGAATTCTACCACGCCACGGATGTCG-3′

cigarette smoking has also been implicated in worse outcomes following functional endoscopic sinus surgery (FESS).[14-17] Although evidence is mounting linking tobacco smoke exposure and CRS, the pathophysiologic mechanisms are yet to be identified. Recently, it was demonstrated that tobacco smoke exposure induces an increase in biofilm mass in respiratory bacteria and that smoking cessation should revert bacteria back to a smoke-naive phenotype.[18] Because microbes residing in a biofilm state are known to have increased resistance to antibiotics, tobacco-induced biofilm formation may contribute to the refractory nature of many respiratory infections found in smokers. With the premise that tobacco smoke has implications in biofilm formation, the aim of this study was to evaluate changes in the expression of biofilm-related genes when exposed to tobacco smoke and oxidative stress.

Methods

Bacteria

Pseudomonas aeruginosa PAO1 (ATCC BAA-47) was chosen as a model due to 2 factors: (1) this bacteria has been implicated in CRS and other biofilm-related diseases, and (2) the entire genome of the PAO1 strain has been described in the Pseudomonas Genome Database,[19] facilitating genetic manipulation. Our institution does not require institutional review board approval for in vitro studies with bacteria obtained commercially.

Gene Selection

Six genes involved in the biofilm formation cascade were chosen: *pilF, flgK, lasI, lasB, rhlA*, and *algC*.

Plasmid/Promoter Construction

Gene expression was examined by transcriptionally fusing the promoter region of the gene of interest with bacterial luciferase operon *luxCDABE* on a plasmid. Briefly, the promoter sequences were polymerase chain reaction (PCR) amplified using primers designed based on www.pseudomonas.com[19] (**Table 1**) and cloned into pMini-CTX-Lux.[20] The resulting plasmids were introduced into *P aeruginosa* PAO1 by conjugation. Successful transformation was confirmed by Western blot assays and by digital photography with and without the luminescence filter (**Figure 1**).

Tobacco Smoke Exposure

The 96-well plate containing freshly diluted cultures was placed with the lid off into an airtight box with an inflow port at the top center and a diffuser midway between the inflow port and the plate. Cigarettes used for exposure were obtained from the Tobacco and Health Research Institute, University of Kentucky, and tobacco smoke was generated as previously described.[21] Briefly, the standardized research cigarettes 1R5F were ignited in an automated smoking machine (Teague TE-10; Teague Enterprises, Davis, California) that was programmed to take a 2-second, 35-mL puff from the burning cigarette every 60 seconds. A total of 5 cigarettes were burned with the ''inhaled'' smoke being directly pumped into the exposure box. Bacteria were exposed to tobacco smoke for 3 hours and then incubated in a designated 37°C ''smoke'' incubator. Sham-exposed bacteria were placed in similar boxes (albeit never having been used for tobacco smoke exposure) with room air being vented in a similar fashion. After 3 hours, sham-exposed plates were incubated in a separate incubator. Following 20 hours of recovery, plates were processed as described below (Biofilm Mass Measurement) to score them for biofilm formation.

Oxidative Stress Exposure

To determine whether the tobacco-induced biofilm formation was caused by oxidative stress, bacterial cultures were serially exposed to varying concentrations of hydrogen peroxide (H_2O_2) solution (0.3%, 0.03%, 0.003%) at several different time intervals (0, 90, and 180 minutes). Sham-exposed bacteria were exposed to 100% Luria-Bertani (LB) broth instead of H_2O_2 at each of the time intervals. Following 20 hours of recovery, plates were processed as described below.

Gene Expression Measurement

Pseudomonas aeruginosa strains containing the promoter of the gene of interest—*luxCDABE* fusion plasmids—were

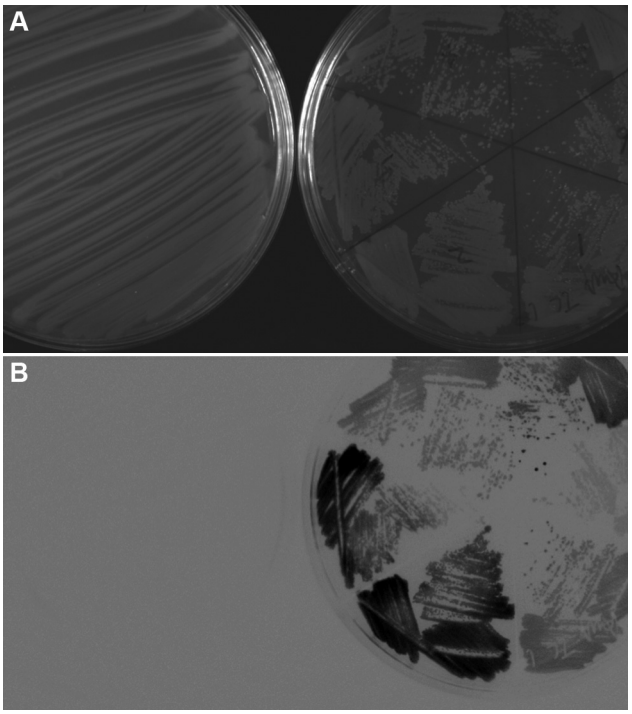

Figure 1. Digital photography (A) without and (B) with a luminescence filter. On the left side of the picture is the negative control (*Pseudomonas aeruginosa* PAO1 without promoter/plasmid) and on the right is the plate containing the *P aeruginosa* PAO1 with the 6 different promoter/plasmid constructs inserted into it.

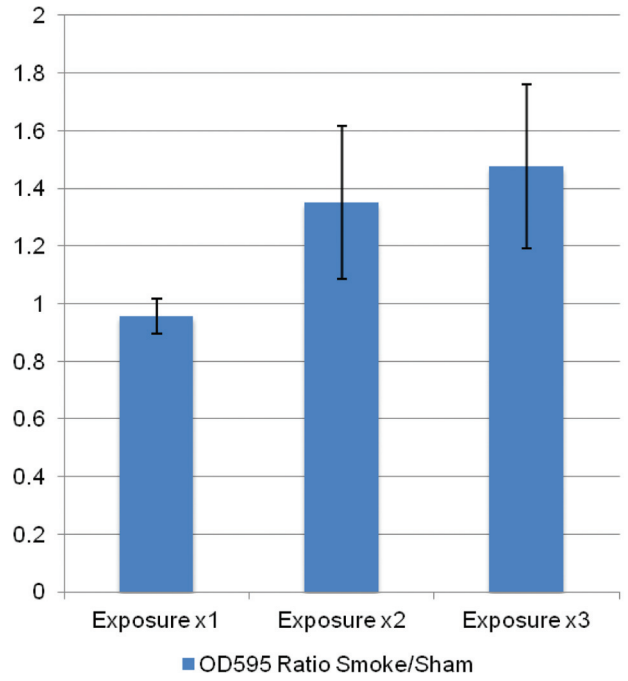

Figure 2. Biofilm mass ratio between bacteria exposed to tobacco smoke and sham groups after 1, 2, and 3 exposures to tobacco smoke (error bars: ±1 SD).

grown in 75% LB broth with tetracycline at 37°C for 20 hours. Bacterial cultures were withdrawn and the luminescence was measured using a BioTek Synergy HT spectrophotometer (BioTek, Winooski, Vermont) and normalized for growth against the optical density at 595 nm (OD595). Lux expression is reported as light units/OD595.

Biofilm Mass Measurement

To determine the biofilm formation, the 6 different modified *P aeruginosa* PAO1 (with the gene promoter/plasmid construct inserted), wild-type *P aeruginosa* PAO1 (the control strains), and non-biofilm-forming mutants (sad-31 [type IV pili] and sad-36 [flagella K] negative controls[5]) were grown and biofilm mass measured as previously described.[18] Following experimental (smoke or H_2O_2) or sham exposure, the plates were incubated for an additional 20 hours at 37°C and biofilm mass again determined.

Data Analysis

Data were expressed as mean ± standard deviation. The luminescence values were converted to ratios in relation to the baseline, and the mean was analyzed using the 1-way analysis of variance (ANOVA) with Bonferroni adjustment. The results were then confirmed using the Kruskal-Wallis test (k value). The biofilm mass values were also converted to ratios and the mean was analyzed using the t test. Differences were considered statistically significant when $P < .05$.

Results

Biofilm Mass in the Presence of Repetitive Smoke Exposure

Bacterial cultures were repetitively exposed to tobacco smoke for 3 consecutive days. Biofilm formation was tested 20 hours following each exposure. Our results are shown in **Figure 2**. Because each culture served as its own control, the data are expressed as a ratio of biofilm formation (OD595 smoke/sham). After 1 exposure to smoke, there was no change in biofilm formation, with a mean (SD) ratio of biofilm formation smoke to sham of 0.95 (0.06) ($P = .1$). After 2 and 3 exposures, the biofilm mass was increased (mean [SD] ratio of 1.35 [0.26], $P = .007$ and 1.47 [0.28], $P = .002$, respectively).

Gene Expression in the Presence of Repetitive Smoke Exposure

Bacterial cultures had luciferase activity measured 20 hours following each exposure, immediately before the cultures were submitted to the biofilm assay. The results are summarized in **Figures 3** to **5**, divided by the type of gene (motility, quorum-sensing, and extracellular matrix genes). The motility genes, *pilF* and *flgK*, had a significant increase in activity ($P = .0009$, $k = 0.002$ and $P = .03$, $k = 0.002$, respectively) after repetitive tobacco smoke exposure. The quorum-sensing genes, *lasI* and *rhlA*, also demonstrated

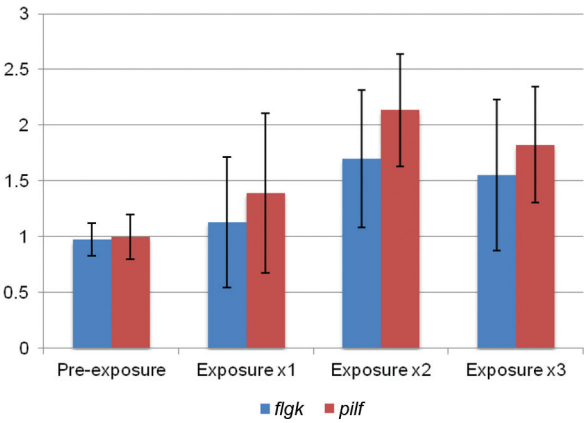

Figure 3. Luminescence ratio in motility genes (*flgK* and *pilF*) between smoke and sham groups after 1, 2, and 3 exposures to tobacco smoke (error bars: ±1 SD).

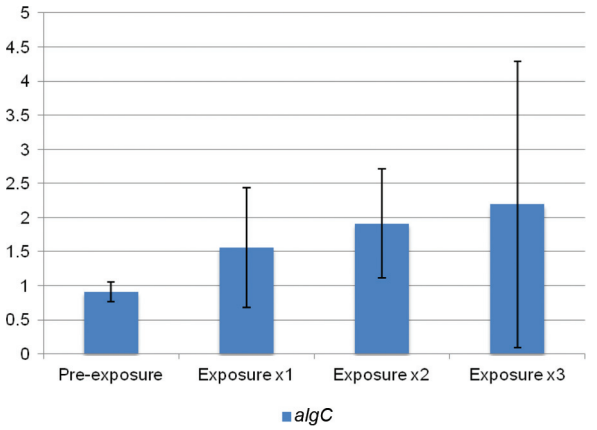

Figure 5. Luminescence ratio in the extracellular matrix gene (*algC*) between smoke and sham groups after 1, 2, and 3 exposures to tobacco smoke (error bars: ±1 SD).

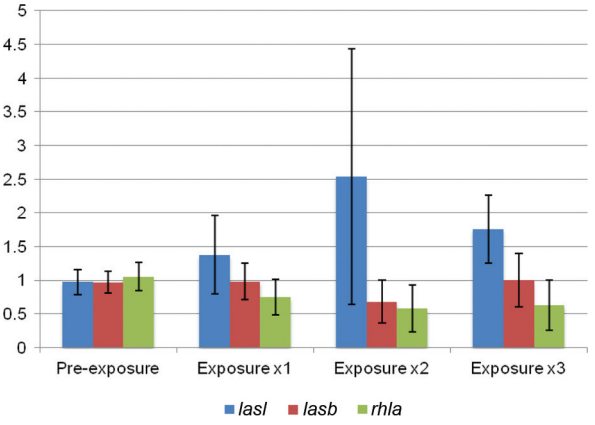

Figure 4. Luminescence ratio in quorum-sensing genes (*lasI*, *lasB*, and *rhlA*) between smoke and sham groups after 1, 2, and 3 exposures to tobacco smoke (error bars: ±1 SD).

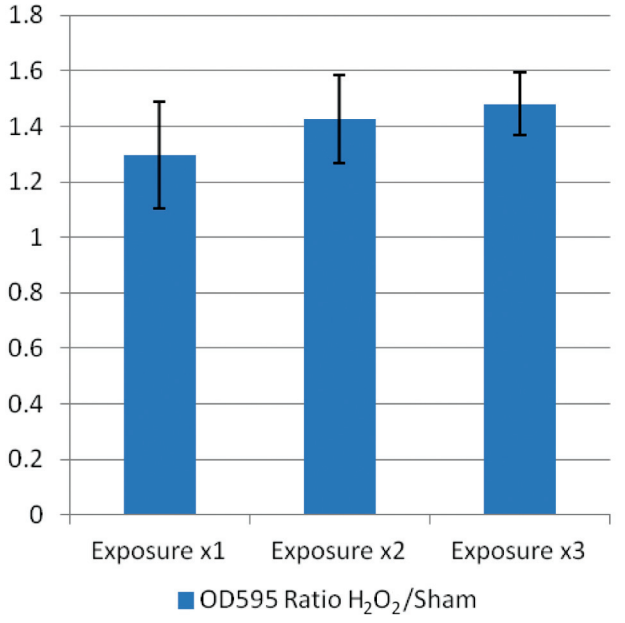

Figure 6. Biofilm mass ratio between bacteria exposed to 0.3% H_2O_2 and sham groups after 1, 2, and 3 exposures to H_2O_2 (error bars: ±1 SD).

significant differences in expression following smoke exposure. However, although the activity on *lasI* was increased (P = .03, k = 0.001), *rhlA* was decreased (P = .02, k = 0.04). The remaining quorum-sensing gene, *lasB*, did not show significant difference in expression with smoke exposure (P = .1, k = 0.1). The extracellular matrix gene, *algC*, demonstrated increased activity following smoke exposure. This was not significant with ANOVA (P = .1) but was significant using the Kruskal-Wallis test (k = 0.002). This could be explained by the presence of a single value that was discrepant with the remaining, elevating the standard deviation.

Biofilm Mass in the Presence of an Acute Oxidative Stress Exposure

Biofilm formation was tested 20 hours following the final exposure. Bacterial cultures demonstrated a dose- and time-dependent induction of biofilm formation compared with control conditions. At 0.3%, all 3 exposures (time 0; time 0 and 90; time 0, 90, and 180) yielded statistically significant (P = .004, P = .0001, P = .000003) increases in biofilm formation compared with control conditions (**Figure 6**), whereas at 0.03%, a statistically significant increase in biofilm formation was not achieved at all 3 exposures (P = .009, P = .07, P = .02).

Gene Expression in the Presence of an Acute Oxidative Stress Exposure

Bacterial cultures had luciferase activity measured 20 hours following each exposure, immediately before the cultures

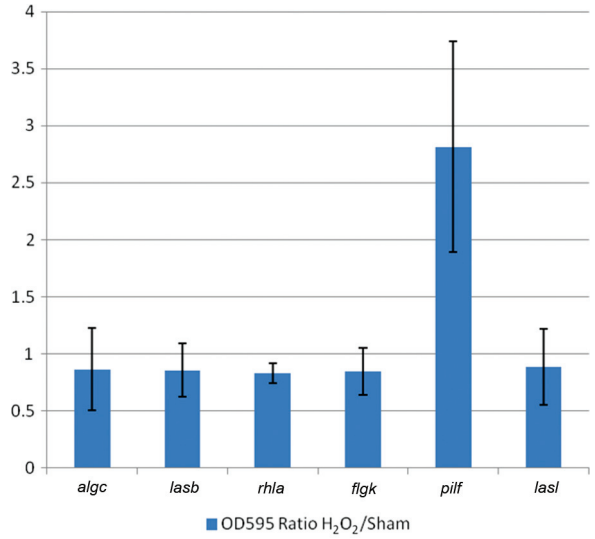

Figure 7. Luminescence ratio comparison between bacteria given an acute exposure to 0.3% H_2O_2 and sham groups (error bars: ±1 SD).

were submitted to the biofilm assay. The motility gene *pilF* had a significant increase in activity (P = .0006), whereas *algC* (P = .38), *lasB* (P = .21), *rhlA* (P = .07), *flgK* (P = .13), and *lasI* (P = .39) did not demonstrate a statistically significant increase in activity in response to an acute exposure to H_2O_2 (**Figure 7**).

Discussion

Tobacco smoke has been implicated in detrimental effects in the respiratory epithelium physiology,[21-24] being suggested as a risk factor for CRS.[11-13] In addition, several studies that evaluated the role of tobacco smoke exposure on outcomes of FESS demonstrated that tobacco is the most significant factor leading to a need for revision surgery,[14,15] diminished postoperative endoscopic improvement,[16] and worse long-term outcomes based on patient-reported symptoms.[17]

Although much is known about the devastating effects of tobacco smoke on the human body, very little is known about its effect on bacteria, specifically those found in the upper respiratory tract. Evidence suggests that smoking uptake and cessation have the ability to alter microbial communities,[22,25] with smokers hosting not only different microbial communities, but those communities being composed of a greater frequency of potential pathogens.[26] Even though the mechanisms have not been elucidated, it has been demonstrated that cigarette smoke can change gene expression in microbial pathogens.[27,28] One study used a microarray representative of the whole genome of *Porphyromonas gingivalis* to determine the effect of cigarette-conditioned media in gene expression of the pathogen. It was demonstrated that cigarette exposure caused differential regulation of 6.8% of the *P gingivalis* genome, including virulence factors, membrane proteins, and regulation of oxidative stress genes.[27] It is likely that other pathogens will demonstrate similar genetic responses to tobacco smoke.

The role of oxidative stress has been studied in planktonic bacterial physiology. Schlag et al[29] demonstrated reduced multicellular aggregation when *Staphylococcus aureus* and *Staphylococcus epidermidis* were grown planktonically under oxygen limitation conditions in the presence of the alternative electron acceptors. They observed that the acidified nitrite derivative NO is involved in the inhibition of biofilm formation, but the precise role of oxidative stress has not yet been elucidated. Miranda et al[30] reported that when *S aureus* was subjected to various conditions of oxidative stress, there were optimal conditions for biofilm formation (time = 18-24 hours, temperature = 37°C, pH <7). Recently, Hoopman et al[31] reported that when *Moraxella catarrhalis* was exposed to oxidative stress, it demonstrated a dose-dependent upregulation in response to the hydrogen peroxide. Oxidative stress plays a role in biofilm formation, both at the extracellular and intracellular levels, with the extracellular levels probably playing a more significant role.[30]

To our knowledge, only 1 study has evaluated the influence of tobacco smoke in biofilm formation,[18] and no study to date has looked at the gene expression in bacterial biofilms exposed to tobacco smoke. To further evaluate the role of tobacco smoke in biofilm formation, more specifically, on the molecular level, we used a luciferase-based reporter plasmid to examine the expression of genes in key steps of biofilm formation: *flgK* and *pilF*, involved in bacterial motility and attachment; *lasI, lasB*, and *rhlA*, involved in bacterial quorum sensing; and *algC*, involved in extracellular matrix biosynthesis.

Flagellar-mediated motility and type IV pili biogenesis have sequential steps in biofilm formation. Flagella mediates cell attachment to the surface, whereas the type IV pili mediates microcolony formation.[32,33] Bacterial motility is key for the planktonic bacteria to encounter a surface. This is promoted mainly through the flagellar protein. Once attached, flagella expression is initially downregulated. In the later stages of biofilm formation, it will again be upregulated as it is important in bacterial detachment. In *P aeruginosa*, similarly to other bacterial species, mutations in the *flgK* gene result in a nonmotile phenotype, as *flgK* is a vital gene involved in flagellar synthesis.[34] The lack of flagella in *P aeruginosa* decreases the magnitude of early biofilm formation but does not prevent biofilm formation completely.[35] The formation of type IV pili enables a diversity of cellular functions, including surface motility, microcolony and biofilm formation, cell-host adhesion, cell signaling, and phage attachment.[36,37] For many pathogens, including *P aeruginosa*, type IV pili is an important virulence factor, as demonstrated by reduced virulence when its assembly is disrupted.[38-41] Although more than 50 proteins are involved in its biosynthesis, *pilF* is part of a subset that is essential for type IV pili biogenesis in *P aeruginosa*.[42,43] A single pilus fiber can be extended out of the cell for several micrometers,[44] and once attached to a surface, it can

mediate a retractile force in excess of 100 pN,[45] making it a very powerful virulence factor. No studies to date have evaluated the effect of tobacco smoke on those virulence factors, *flgK* or *pilF*, or on their products, flagellar protein and type IV pili. Our study demonstrated that the expression of the motility genes *flgK* and *pilF* are significantly increased after repetitive tobacco smoke exposure. This, in turn, supports the finding of our previous study[18] that demonstrated more biofilm formation with increased mass in bacteria exposed to tobacco smoke. With increased expression of those virulence factors, one can infer that more planktonic bacteria will find their way to a surface and initiate the biofilm cascade.

Quorum sensing is a cell density–dependent communication system that is used by many microorganisms to regulate biofilm formation. *Pseudomonas aeruginosa* uses this response to regulate the transcription of hundreds of genes, many of which encode extracellular virulence factors[46-48] that ultimately lead to the biofilm phenotype. These bacteria use 2 major quorum-sensing systems: *las* and *rhl*. These systems operate hierarchically, with *las* regulating *rhl*.[49,50] However, the latter can, under special conditions, be active without input from the *las* system.[51,52] These systems are regulated by molecules produced by the bacteria, named autoinducers. When the bacterial population reaches a certain density, the autoinducer concentration in the media reaches a certain point that activates a complex that regulates gene transcription. The gene *lasI* codes for the enzyme responsible for the synthesis of the autoinducer for the *las* system. An increase in the expression of this gene has been correlated with an increase in its production.[53] When it reaches a critical concentration, this molecule binds to the transcriptional regulatory protein, lasR, which in turn will trigger the expression of several biofilm-related genes, including *lasI*, creating a positive feedback loop.[54] Once *lasI* activates lasR, the *las* system pathway is initiated and triggers the expression of *lasB*, with its subsequent product, elastase.[55,56] Elastase is a zinc metalloprotease virulence factor and is highly toxic, promoting tissue damage and allowing invasion, promoting immunomodulation,[57] and acting intracellularly to initiate bacterial biofilm growth.[58] *lasB* makes biofilms highly resistant to the host's immune response and antibiotics through enzymes within the bacterial cell. Moreover, the inflammatory response raised against the biofilm matrix perpetuates the inflammatory cycle in the host.[59-62] Inhibition of *lasB* has been correlated with a reduction in biofilm formation,[63] and the antibiofilm effect has been shown to be dependent on both the potency and the concentration of the *lasB* inhibitors, validating the key role of *lasB* in biofilm formation. Following activation of the *rhl* system, the gene *rhlA* will be upregulated. It will code for the enzyme rhamnosyltransferase, being responsible for the first step in the synthesis of rhamnolipids, which are biosurfactants. Even though several studies have been performed with rhamnolipids, its exact physiological significance is still a matter of debate. It is likely that this molecule plays more than one role and has been suggested that its importance lies in surface activity and motility, uptake, and biodegradation of poorly soluble substrates,[64,65] virulence factor,[66] and microcolony formation.[67-69] In terms of its role in biofilm development, the rhamnolipids are important in microcolony formation and differentiation of the biofilm structure.[70] They also seem to have a role in cell-cell and cell-substratum interactions mediating the detachment of the bacteria from the biofilm[71] and in maintaining the patency of the water channels in the biofilm structure.[72] *rhlA* expression is modulated by the localization of the bacterial cells in the biofilm structure.[73] No studies have evaluated the role of tobacco smoke in quorum sensing. Our findings were intriguing, with repetitive smoke exposure upregulating *lasI*, downregulating *rhlA*, and having no impact in *lasB* expression. It has been demonstrated that the hierarchical relationship between the *las* and *rhl* systems is dependent on the growth conditions,[53] which could explain our findings of elevation in gene expression in the *las* system and repression in the *rhl* system.

The production of alginate by *P aeruginosa* is not a recent finding.[74] When this microorganism assumes a biofilm phenotype, it embeds itself in an exopolymer matrix composed mainly of alginate.[75] The gene *algC* is a key regulatory point in alginate biosynthesis; it encodes for the enzyme phosphomannomutase. The activities of alginate biosynthetic enzymes are overall low, even in mucoid strains of *P aeruginosa*,[76-78] with maximal activity around 24 hours of incubation for bacteria with biofilm phenotype.[79] The *algC* gene has been shown to be activated by attachment to surfaces, ethanol, nitrogen limitation, high oxygen tension, and environmental signals such as high osmolarity, such as in secretions from patients with cystic fibrosis.[80] This study demonstrates for the first time the presence of tobacco smoke as an environmental stimulant for *algC* activity.

Conclusion

Repetitive tobacco smoke exposure leads to molecular changes in biofilm-related genes that support previously published data that state that tobacco smoke induces an increase in biofilm mass. The exact pathway by which these molecular changes occur is still unclear. Exposure to oxidative stress in the form of H_2O_2 induces biofilm growth in PAO-1. This may explain increased biofilm formation in microbes isolated from smokers. This could represent adaptative changes due to oxidative stress or chemically mediated through any of the several chemicals encountered in tobacco smoke. Furthermore, host defenses that use an oxidative burst may encourage microbial biofilm formation in vivo.

Author Contributions

Marcelo B. Antunes, design, acquisition and interpretation of data, drafting the article, and final approval; **John J. Chi**, design, acquisition and interpretation of data, drafting the article, and final approval; **Zhi Liu**, acquisition and interpretation of data, reviewing

the article, and final approval; **Natalia Goldstein-Daruech**, acquisition and interpretation of data, reviewing the article, and final approval; **James N. Palmer**, interpretation of data, reviewing the article, and final approval; **Jun Zhu**, acquisition and interpretation of data, reviewing the article, and final approval; **Noam A. Cohen**, design, interpretation of data, reviewing the article, and final approval.

Disclosures

Competing interests: None.

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