Cigarette Smoke Increases Staphylococcus aureus Biofilm Formation via Oxidative Stress

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The strong epidemiological association between cigarette smoke (CS) exposure and respiratory tract infections is conventionally attributed to immunosuppressive and irritant effects of CS on human cells. Since pathogenic bacteria such as *Staphylococcus aureus* are members of the normal microbiota and reside in close proximity to human nasopharyngeal cells, we hypothesized that bioactive components of CS might affect these organisms and potentiate their virulence. Using *Staphylococcus aureus* as a model organism, we observed that the presence of CS increased both biofilm formation and host cell adherence. Analysis of putative molecular pathways revealed that CS exposure decreased expression of the quorum-sensing *agr* system, which is involved in biofilm dispersal, and increased transcription of biofilm inducers such as *sarA* and *rhp*. CS contains bioactive compounds, including free radicals and reactive oxygen species, and we observed transcriptional induction of bacterial oxidoreductases, including superoxide dismutase, following exposure. Moreover, pretreatment of CS with an antioxidant abrogated CS-mediated enhancement of biofilms. Exposure of bacteria to hydrogen peroxide alone increased biofilm formation. These observations are consistent with the hypothesis that CS induces staphylococcal biofilm formation in an oxidant-dependent manner. CS treatment induced transcription of *fnbA* (encoding fibronectin binding protein A), leading to increased binding of CS-treated staphylococci to immobilized fibronectin and increased adherence to human cells. These observations indicate that the bioactive effects of CS may extend to the resident microbiota of the nasopharynx, with implications for the pathogenesis of respiratory infection in CS-exposed humans.

*S. aureus* is a Gram-positive pathogen that colonizes the skin and mucosal spaces of human hosts (42, 50), with a population-wide carriage rate estimated at 20 to 32% (59, 72). It causes a wide range of community- and hospital-acquired infections ranging in severity from uncomplicated cellulitis to deep-seated infections such as endocarditis and osteomyelitis (10). The emergence and spread of methicillin-resistant *S. aureus* (MRSA) is a global public health issue (29, 41). Much progress has been made in determining the roles of various staphylococcal virulence factors in the pathogenesis of infection (32). However, the underlying mechanisms of the transition of *S. aureus* from asymptomatic colonizer to human pathogen remain unclear and may involve the concerted activity of host, bacterial, and possibly environmental factors.

Cigarette smoking is another important global health threat. Despite intensive public health interventions, rates of smoking remain high in the United States (11) and worldwide (71). Smoking causes a tremendous health burden, not only in smokers but in those exposed to second-hand cigarette smoke as well (24). The adverse health outcomes associated with cigarette smoke, including carcinogenesis (62), promotion of atherosclerosis (56), and chronic lung disease (74), are generally thought to result from the direct action of components of cigarette smoke (CS) on human cells. CS contains a plethora of bioactive compounds, including oxidant, genotoxic, and immunomodulatory factors (15, 57). CS exposure increases the risk of several infectious diseases (25, 33, 73). We recently demonstrated that CS could impair epithelial innate immune responses to microbial products (37), perhaps setting the stage for overgrowth and invasion.

Because the colonizing microbiota inhabits human mucosal spaces, microbes may share exposure to a variety of environmental stimuli, including CS. We hypothesized that exposure of *S. aureus* to CS might induce pathways relevant to both survival and pathogenesis. We focused on adherence and biofilm formation, which are important determinants of *S. aureus* colonization and disease (32). We observed that CS exposure increased staphylococcal biofilm formation in a rapid, dose-dependent, and oxidant-mediated manner. CS enhanced *S. aureus* transcription of the fibronectin binding protein A gene (*fnbA*), leading to increased staphylococcal binding to human fibronectin and increased adherence to human cells. These observations emphasize the importance of understanding the effects of environmental stimuli, including CS exposure, on the colonizing microbiota rather than solely on host cells.

**MATERIALS AND METHODS**

**Reagents.** Unless otherwise specified, reagents were purchased from Sigma.

**Bacterial strains, cell lines, and preparation of cigarette smoke extract.** *Staphylococcus aureus* strains RN6390 (54), Newman (49), USA300 (65), and 502A (RN6607, NRS149 spa type 230) were grown in tryptic soy broth (TSB) without antibiotic selection. For assays of biofilm formation, dextrose-free TSB supplemented with 0.5% glucose (TSBG) was used. Immortalized human upper airway epithelial cell line A549 (ATCC CCL-
Fresh cigarette smoke extract (CSE) was prepared for each experiment by bubbling smoke from three Marlboro cigarettes into 20 ml TSBG (100% CSE). In the absence of specific dosage data regarding exposure of colonizing microbes to CS, this maximum dose (3 cigarettes/20 ml) was chosen in order to approximate heavy direct CS exposure and is consistent with doses reported to be used elsewhere in the literature (22, 48). Effects were tested over a range of CS concentrations.

**Bacterial exposure to CSE.** Overnight bacterial cultures in TSB were centrifuged, and pellets were resuspended in TSBG. These were exposed to various concentrations of CSE for biofilm formation assay or quantitative real-time PCR (qRT-PCR) analysis as described below. Alternatively, short-term CSE exposure was performed as follows. Overnight *S. aureus* cultures were diluted 1:40 in TSBG and grown to an optical density at 600 nm (OD600) of 1, cultures were centrifuged, and pellets were exposed to 0 or 100% CSE for 1 h. Following short-term stimulation, bacteria were washed to remove residual CSE and tested for biofilm formation, adherence, or gene expression by qRT-PCR as described above.

**Biofilm formation assay.** The protocol for biofilm formation was published earlier (18). In brief, overnight cultures of bacteria were grown to an OD600 of 0.6 to 1.0 in TSB, washed in TSBG, and resuspended at a 1:40 dilution in TSBG with various concentrations of CSE (0, 10, 25, and 50%) or H2O2 (0, 5, and 10 mM) in 96-well plates. These were incubated at 37°C with horizontal rotation (150 rpm) overnight. Planktonic bacteria were discarded, and biofilms were washed gently with 0.9% NaCl solution three times. Biofilm biomass was then fixed by baking the plates at 60°C for 1 h, followed by staining with 0.3% crystal violet for 15 min at room temperature. Excess stain was removed with water. Plates were dried, biofilm-associated stain extracted in a 70% ethanol–10% methanol mixture, and absorbance (590 nm) determined.

For enumerating planktonic and sessile CFU, following overnight incubation as described above, supernatants were collected gently without disturbing the biofilms, following which biofilm-associated biomass was scraped off the bottoms of the wells. Serial dilutions of the supernatant and biofilm fractions were plated on tryptic soy agar and colonies counted after overnight incubation at 37°C.

**qRT-PCR.** Total RNA was extracted from bacteria grown in the presence or absence of CSE for the indicated times using the RiboPure bacterial RNA extraction kit (Ambion) according to the manufacturer’s instructions. RNA was reverse transcribed to cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) was carried out using Power SYBR green master mix in a StepOne Plus thermal cycler (Applied Biosystems). The list of primers used for qRT-PCR is shown in Table 1. Relative quantification (RQ) values were calculated using a comparative threshold cycle (ΔΔCt) program on the StepOne software version 2.0.

**Fibronectin binding assay.** Human fibronectin, reconstituted as a 2.5-mg/ml stock in Dulbecco’s phosphate-buffered saline (D-PBS) and diluted to specified concentrations (20, 10, 5, 2.5, and 1.25 μg/ml), was used to coat 96-well flat-bottom enzyme-linked immunosorbent assay (ELISA) plates overnight at 4°C. *S. aureus* was exposed to 100% CSE or TSBG alone using the short-term exposure protocol described above, washed in sterile D-PBS twice, and resuspended in sterile D-PBS (the final OD600 was adjusted to 0.45) for 1 h at 37°C with horizontal rotation (150 rpm) overnight. Planktonic bacteria were discarded, and biofilms were washed gently with 0.9% NaCl solution three times. Biofilm biomass was then fixed by baking the plates at 60°C for 1 h, followed by staining with 0.3% crystal violet for 15 min at room temperature. Excess stain was removed with water. Plates were dried, biofilm-associated stain extracted in a 70% ethanol–10% methanol mixture, and absorbance (590 nm) determined.

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**Cell culture adherence assay.** A549 cells were grown to confluence in 24-well plates. *S. aureus* was exposed to various concentrations (10, 50, or 100%) of CSE or TSBG alone using the short-term exposure protocol described above, washed in sterile MEM twice, and resuspended in sterile MEM. The final OD600 was adjusted to 0.45 so as to achieve a multiplicity of infection (MOI) of ~10. The inoculum CFU (IC) was enumerated. In order to facilitate the bacterial contact with A549 cells, centrifugation was carried out at 800 × g for 5 min at room temperature. After incubation for 1 h at 37°C in the presence of 5% CO2, wells were washed with sterile D-PBS with 1 mM Ca2+ and 1 mM Mg2+ three times to remove nonadherent bacteria. Adherent bacteria (AB) were enumerated and percent adherence frequency calculated as (AB ÷ IC) × 100.

**Statistical analysis.** The biofilm assay results are expressed as averages of quadruplicate readings ± standard deviations from minimum of 2 different experiments. Data were compared by one-way analysis of variance (ANOVA) followed by Dunnet’s multiple-comparison test. The gene expression results (see Fig. 2) are expressed as averages of values from triplicate readings ± standard deviations from one representative out of three experiments. The gene expression data were compared by the two-tailed t test. Prism 4 software (GraphPad) was used for all statistical analysis. A P value of less than 0.05 was considered significant.

**RESULTS**

**CS exposure induces biofilm formation in multiple strains of *S. aureus.** To determine the effects of CS exposure on biofilm formation, *S. aureus* strains were exposed to various concentrations

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**Table 1: Primers used in this study**

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<th>Primer</th>
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<tr>
<td>16S_R</td>
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185) was maintained in minimal essential medium (MEM) (Gibco) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate (Cellgro), and 10 μg/ml ciprofloxacin (Fisher).
CS induces staphylococcal biofilm formation. S. aureus strains were exposed to various concentrations of CSE (0, 10, 25, or 50%) for 18 h or to 0 or 100% CSE for a short period (1 h), and biofilm formation was quantified. (A) Crystal violet staining of biofilm-associated biomass revealed CS-mediated dose-dependent upregulation of biofilm formation in different strains of S. aureus as indicated. Histograms indicate average values of quadruplicate readings from at least two separate experiments ± standard deviations. (B) This was further confirmed by enumeration of bacterial CFU in sessile and planktonic fractions at the end of 18 h of incubation, which showed significantly more sessile than planktonic CFU. Average percent CFU values from three separate experiments ± standard deviations are shown. (C) CS-induced biofilm formation was observed even when S. aureus Newman, RN6390, and USA300 were exposed to CS for a short period (1 h) Averages of quadruplicate readings from at least two separate experiments ± standard deviations are shown. P values for this and subsequent figures are denoted as follows: *, P < 0.05; **, P < 0.001; ***, P < 0.0001.

(0, 10, 25, or 50%) of CSE for 18 h and biofilm formation quantified. Treatment with CSE led to a dose-dependent increase in biofilm formation in S. aureus strains Newman, RN6390, and 502A (Fig. 1A). For S. aureus USA300, we observed maximum biofilm augmentation following exposure to 10% CSE (Fig. 1A). In order to confirm these results, we plated serial dilutions of planktonic and sessile (biofilm-associated) fractions. For S. aureus strains Newman and RN6390, there was a significantly (P < 0.05) higher number of bacterial CFU in the biofilm fraction than in the planktonic fraction upon treatment with 50% CSE (Fig. 1B and data not shown.) We noted that even a short-term (1-h) exposure to CS was enough to prime increased biofilm formation in S. aureus (Fig. 1C). Short-term exposure to CS did not affect bacterial viability (see Fig. S1 in the supplemental material).

CS exposure rapidly alters transcription of genes relevant to biofilm formation. The quorum-sensing accessory gene regulator (agr) system controls transition between planktonic and sessile forms of S. aureus (52). Bacteria dispersing from biofilms have been demonstrated to exhibit increased agr activity (75). We analyzed whether CS-mediated augmentation of S. aureus biofilms was a result of alterations in agr expression by comparing levels of agrC transcripts in S. aureus cells exposed to CS for 1 h. For Newman (Fig. 2A), RN6390 (Fig. 2B), and USA300 (Fig. 2C), 1 h of exposure to CS led to an approximately 10-fold (P < 0.01) decrease in the level of agrC compared to that in untreated cells.

Staphylococcal accessory regulator A (SarA) is a regulator of the expression of virulence factors such as Fnba adhesin and α-, β-, and δ-toxins (13, 14). It activates biofilm formation by increasing transcription of the ica (intercellular adhesin) operon, which is involved in the production of exopolysaccharide adhesin (12). Another gene, rbf (required for biofilm formation), induces biofilm formation by downregulating the icaR negative regulator (20). Short-term exposure to CSE led to a 10- to 15-fold increase in sarA and rbf transcripts in different S. aureus strains. This effect was not accompanied by induction of icaA or by downregulation of icaR in RN6390 and Newman (see Fig. S2A and B in the supplemental material), while in the case of USA300, we observed an ~100-fold increase in icaA transcript levels following short-term exposure to CSE (see Fig. S2C in the supplemental material).

CS-mediated enhancement of staphylococcal biofilms requires oxidative stress. CS contains many free radicals and reactive oxygen species (such as O2−, NO, OH−, and H2O2) that increase oxidative stress and may contribute to pathology. qRT-PCR analysis of CS-exposed S. aureus Newman revealed statistically significant increases in the transcripts of oxidoreductase genes such as ahpC (encoding alkyl hydroxy peroxidase) and sod (encoding superoxide dismutase) (Fig. 2A). To analyze whether CS-mediated upregulation of S. aureus biofilm is due to the action of reactive oxygen species on bacteria, we pretreated CS with N-acetyl cysteine (NAC). Treatment with 25 mM NAC abrogated the ability of CS to upregulate biofilm formation, consistent with a role for the oxidative stress in this phenomenon (Fig. 3A). This
was further corroborated by observation that exposure to H$_2$O$_2$ alone leads to increased production of biofilms in Newman and USA300 (Fig. 3B) and in RN6390 (data not shown). We also observed reduced transcription of ahpC and sod and increased levels of agr transcripts in USA300 exposed to NAC-pretreated CS compared to levels from bacteria exposed to CS alone (Fig. 3C).

**CS-enhanced biofilms are DNase and proteinase K sensitive.**

The staphylococcal biofilm matrix has been reported to contain extracellular genomic DNA (eDNA), proteins, and polysaccharides (17,19, 58). We observed that addition of either DNase I or proteinase K leads to dispersal of established CS-induced biofilms, reducing them to the level of biofilms formed by untreated staphylococci (Fig. 4). Susceptibility of staphylococcal biofilms to DNase I as well as proteinase K is well established. eDNA release has been described to be dependent on cell death and lysis mediated by the cidA murein hydrolase (58). Notably, we observed a minor (1.7-fold) increase in the level of cidA transcripts in CS-treated S. aureus Newman (see Fig. S2A in the supplemental material), an 8-fold increase in RN6390 (see Fig. S2B in the supplemental material), and a 6-fold increase in USA300 (see Fig. S2C in the supplemental material) over control values.

**CS exposure induces S. aureus binding to fibronectin and adherence to human cells.**

Fibronectin binding enhances S. aureus adherence to human cells (47). Moreover, overexpression of surface adhesin FnbA leads to formation of a staphylococcal biofilm with a proteinaceous matrix (68). We observed that CS exposure led to increased transcription of fnbA compared to that in control bacteria grown in the absence of CS (Fig. 2). CS-exposed S. aureus RN6390 and USA300 adhered to immobilized human fibronectin at significantly higher levels than bacteria grown in CS-free medium (Fig. 5A). In contrast, strain Newman exhibited weaker overall fibronectin binding (Fig. 5A). Prior work has established that S. aureus Newman is weakly adherent to immobilized fibronectin despite robust transcription of fnbA because of a premature stop codon that leads to the production of truncated protein that is not anchored to the bacterial outer membrane (30, 60). Increased binding of CS-exposed Newman to immobilized fibronectin may be attributed to a mechanism such as extracellular adhesion protein (Eap) (31), which functions independently of fibronectin binding proteins (FnBPs), or to other surface-attached MSCRAMM proteins that may bind human fibronectin. CS-treated Newman and USA300 (Fig. 5B) as well as RN6390 (data not shown) adhered to A549 human lung epithelial cells more efficiently than control bacteria.

**DISCUSSION**

The observation that cigarette smokers and individuals exposed to second-hand smoke are more susceptible to upper respiratory tract infections is conventionally attributed to immunomodulatory and other deleterious effects of CS components (61). We hypothesized that CS might have direct effects on members of the human-associated microbiota as well. This theme has been explored by recent publications, including a prospective study showing a strong correlation between CS exposure and increased acquisition of pathogenic bacteria in subgingival plaques (38) and studies showing augmentation of dental plaque-associated biofilms owing to increased production of *Porphyromonas gingivalis* fimbrial protein in the presence of CS (2), altered expression of outer membrane protein leading to suppression of the proinflammatory cytokine response mediated by *P. gingivalis* (3), and CS-
mediated induction of biofilm formation in *Streptococcus pneumoniae* (51) and *Pseudomonas aeruginosa* (1). Results from different epidemiological studies to determine the nature of the association between smoking status and staphylococcal prevalence are inconclusive: Olsen et al. attributed decreased *S. aureus* nasal carriage in smokers to the bactericidal activity of CS components and increased immune activity due to smoke-induced hypoxia (53), Wang et al. found no significant association between smoking and acquisition of community-acquired MRSA (70), and Bogaert et al. and Melles et al. observed that in children (≤18 years of age), exposure to passive (and not active) smoking increases *S. aureus* nasal carriage (8,46). We observed that CS exposure led to increased biofilm formation and enhancement of fibronectin binding in an important human pathogen, *S. aureus*. Biofilms are surface-attached multicellular communities enclosed inside a polymeric matrix, the bacterial residents of which exhibit coordinated behavior (21,52).

*S. aureus* can adhere to a variety of host tissues and establish biofilms that are encased within a matrix composed of extracellular polysaccharides (17), proteins (19), and eDNA (58). In this work, we observed upregulation of biofilm formation when different *S. aureus* strains, including important clinical isolates such as USA300, were exposed to CS. These findings are consistent with the observations made by Goldstein-Daruech et al. (27), who demonstrated that in bacteria isolated from sinonasal cavities of smokers suffering from chronic rhinosinusitis (CRS), biofilm formation is increased upon *in vitro* exposure to CS, while in bacteria derived from a nonsmoking CRS population, CS-mediated upregulation of biofilm formation is absent. We noted that even a short (1-h) exposure followed by removal of CS was enough to commit *S. aureus* to a sessile, biofilm-associated life style.

**FIG 3** CS induces biofilm formation in an oxidant-dependent manner. *S. aureus* Newman and USA300 were exposed to vehicle control or CS with or without NAC pretreatment for 30 min and used in a biofilm assay. (A) Antioxidant treatment abrogated the ability of CS to induce biofilm formation, indicating a role for oxidative stress in this phenomenon (NS, *P* > 0.05). (B) A similar biofilm augmentation phenotype was observed when *S. aureus* Newman and USA300 were exposed to hydrogen peroxide. Averages of four replicate readings from at least two separate experiments ± standard deviations are shown for panels A and B. (C) USA300 cells were used for qRT-PCR analysis as described for Fig. 2. The gene expression pattern in bacteria exposed to NAC-pretreated CS shows significantly reduced transcription of oxidoreductase genes (*ahpC* and *sod*) and increased transcription of *agrC* compared to that in bacteria exposed to CS alone. (***, *P* < 0.0001 compared to medium alone; ###, *P* < 0.001 compared with CS-treated bacteria).

**FIG 4** Extracellular DNA and proteins are important constituents of the CS-induced staphylococcal biofilm matrix. *S. aureus* Newman and USA300 cells were exposed to 100% CSE or medium alone for 1 h, CSE was washed off, and bacteria were grown in 0.5% TSBG for 18 h in 96-well polystyrene plates. Biofilms were treated with DNase I or proteinase K for 3 h and biofilm formation quantified. DNase I as well as proteinase K treatment caused dispersal of CS-induced biofilms. Average *A*590 values of triplicate readings from three separate experiments ± standard deviations are shown.
Two distinct molecular pathways leading to biofilm formation have been described in *S. aureus*. The ica-dependent pathway involves enzymes for the synthesis of exopolysaccharide (poly-N-acetylglucosamine) adhesin (17). In contrast, ica-independent pathways employ surface-anchored protein adhesins such as biofilm-associated protein (Bap) of mastitis-associated staphylococci (19), *H9254*-toxin (69), and other factors that increase membrane hydrophobicity, such as teichoic acids (28), to form proteinaceous biofilms. The ica operon is regulated negatively by icaR (34) and positively by sarA (encoding staphylococcal accessory regulator) (5), sarX (20), and rbf (43). We noted marked upregulation of sarA and rbf transcription in CS-treated bacteria (Fig. 2). The quorum-sensing agr system is another important coordinator of gene expression that plays a crucial role in *S. aureus* biofilm dispersal by controlling the production of surfactants such as phenol-soluble modulins (55, 75). The qRT-PCR analysis of staphylococci treated with CS showed significant increases in the transcript levels of important oxidoreductases such as *ahpC* and *sod*. *S. aureus* employs SodA and SodM to catalyze the conversion of O$_2^-$ to O$_2$ and H$_2$O$_2$ (36); following these reactions, the enzymes catalase and Ahp reduce H$_2$O$_2$ to H$_2$O and O$_2$ (16). Pretreatment of CS with an antioxidant compound (NAC) significantly abrogated induction of staphylococcal biofilm formation. Using qRT-PCR, we also confirmed that in staphylococci exposed to NAC-pretreated CSE, transcript levels of oxidoreductase genes *ahpC* and *sod* were lower than what was seen in bacteria treated with CSE alone. This was accompanied by increased levels of *agrC* transcripts in staphylococci exposed to NAC-pretreated CSE. Moreover, sublethal levels of hydrogen peroxide alone enhanced biofilm formation. The previously reported inhibition of *S. aureus* biofilms by hydrogen peroxide at concentrations of $\geq$2.5% may be attributed to its bactericidal activity, as the 50% inhibitory concentration (IC$_{50}$) of H$_2$O$_2$ for *S. aureus* is $\sim$500 mM (1.7%) (66, 76). We propose that at the substantially lower concentrations used in our studies (5 mM [0.015%] to 10 mM [0.03%]), H$_2$O$_2$
may induce oxidative stress conditions akin to those generated by exposure to CSE without being toxic to S. aureus cells. These observations imply a cross talk between the oxidative stress response and the quorum-sensing agr system resulting in CS-mediated up-regulation of biofilm formation in S. aureus. Indeed, these observations are in accordance with a recently published report by Sun et al. (63) showing that the agr system of Staphylococci has built-in oxidation-sensing mechanism in the form of an intramolecular disulfide switch in AgrA. Oxidative stress induces disulfide bond formation in AgrA, rendering it unable to bind the agr regulator (P2) and in turn reducing the transcription of agrC (63).

Other gene products that are involved in the formation of biofilms include murein hydrolase CidA (58) and Clp proteases (26). This process is further complicated by the fact that biofilm formation and the composition of the biofilm matrix may also be dependent on a variety of environmental signals, which include osmolarity (39), available glucose (39), anaerobiosis (28), and levels of iron, copper (4), nitrates, citrate, and ethanol. Staphylococcal biofilms induced by CS exposure were observed to be susceptible to enzymatic treatment with DNase I and proteinase K, similar to what is seen in bacteria grown in the absence of CS, indicating that eDNA and proteins may be important structural components of CS-induced staphylococcal biofilms. CS exposure also increased fnbA transcription and staphylococcal binding to fibronectin-coated wells in a dose-dependent manner. Fibronectin is an important extracellular matrix protein found in plasma and on cell surfaces. Binding to fibronectin increases S. aureus adherence and invasion and may be relevant to both colonization and invasive disease (23).

Environmental stimuli such as CS may have profound impacts on human cells, with implications for a wide variety of disease processes. As our appreciation for the pleiotropic effects of the human-associated microbiota on health grows (40), it is important to remember that our resident microbes may share our exposure through gastrointestinal, respiratory, or other modes of entry. Such exposures may alter microbial physiology in ways relevant to human health. Because of the prevalence of cigarette smoking and the relevance of S. aureus to health, we explored one such potential interaction. CS exposure markedly alters S. aureus gene expression as well as phenotypic characteristics relevant to pathogenesis. Confirmation of these findings in relevant animal models of colonization and disease is warranted.

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Cigarette Smoke and Biofilms