Cigarette Smoke Extract–Exposed Methicillin-Resistant *Staphylococcus aureus* Regulates Leukocyte Function for Pulmonary Persistence

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Abstract

Cigarette smoke (CS) predisposes exposed individuals to respiratory infections not only by suppressing immune response but also by enhancing the virulence of pathogenic bacteria. As per our observations, in methicillin-resistant *Staphylococcus aureus* strain USA300, CS extract (CSE) potentiates biofilm formation via the down-regulation of quorum-sensing regulon accessory gene regulator. Because accessory gene regulator is a global regulator of the staphylococcal virulome, in the present study we sought to identify the effects of CS exposure on staphylococcal gene expression using RNAseq. Comparative analysis of RNAseq profiles revealed the up-regulation of important virulence genes encoding surface adhesins (fibronectin- and fibrinogen-binding proteins A and B and clumping factor B) and proteins involved in immune evasion, such as staphylocoagulase, staphylococcal protein A, and nuclease. In concurrence with the RNAseq data, we observed: (1) significant up-regulation of the ability of CSE-exposed USA300 to evade phagocytosis by macrophages and neutrophils, a known function of staphylococcal protein A; and (2) twofold higher ($P < 0.001$) number of CSE-exposed USA300 escaping neutrophil extracellular

trap-mediated killing by neutrophils as a result of CS-mediated induction of nuclease. Importantly, in three different mouse strains, C57BL6/J, Balb/C, and A/J, we observed significantly higher pulmonary bacterial burden in animals infected with CSE-exposed USA300 as compared with medium-exposed control USA300. Taken together, these observations indicate that bioactive chemicals in CS induce hypervirulence by augmenting the ability of USA300 to evade bactericidal functions of leukocytes, such as phagocytosis and neutrophil extracellular trap-mediated killing.

Keywords: cigarette smoke; *Staphylococcus aureus*; USA300; virulence; nuclease

Clinical Relevance

Exposure of bacteria to cigarette smoke extract induces hypervirulent strains of methicillin-resistant *Staphylococcus aureus*, which evade bactericidal function of leukocytes.

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The microbiota of the human upper respiratory tract is comprised of a variety of commensal microbes and potentially deadly pathogens, such as *Staphylococcus aureus* (1, 2). *S. aureus* persistently colonizes the nasopharyngeal mucosa in 20–30% of healthy adults and, importantly, nasal carriage is causally linked with staphylococcal infections that could be life threatening in the immunocompromised, children, and the elderly (2–5). The molecular determinants of nasal carriage include staphylococcal factors involved in colonization and evasion of innate immune responses in the respiratory tract (6). Furthermore, epidemiological studies have established a significant correlation between environmental factors, such as secondhand smoke, and staphylococcal nasal carriage with an increased incidence of respiratory infections in infants, children, and adults (7–10). Thus, the deleterious effects of cigarette smoke (CS) bioactive chemicals may well extend beyond the human host to the alteration of the pathogenic microbes inhabiting the nasopharyngeal microbiota.

Exposure to CS extract (CSE) rapidly induces biofilm formation and epithelial cell adhesion by methicillin-resistant *S. aureus* (MRSA) strain, USA300, in an oxidant radical-dependent manner (11). At the molecular level, exposure to CSE induces transcription of oxidoreductases and surface adhesins, while suppressing the expression of the accessory gene regulator (*agr*) regulon (11). The *agr* regulon is a quorum-sensing system that globally regulates expression of staphylococcal virulence genes. Thus, we hypothesized that exposure to CS would induce global perturbations in gene expression, which may promote virulence factor expression and facilitate the ability of staphylococci to evade innate immune responses. In this article, we present a comprehensive analysis of RNAseq profiles showing that exposure to CSE induced significant ($P < 0.05$, false discovery rate < 0.05) changes in the expression of 344 staphylococcal genes, including those encoding virulence factors, surface adhesins, and effectors involved in immune evasion. To our knowledge, this is the first report describing the global transcriptional changes in *S. aureus* USA300 after exposure to CS. In addition, using an *in vivo* mouse model of acute pneumonia, we establish the hypervirulence of CSE-exposed USA300 in three distinct inbred mouse strains, including C57BL6/J,

Balb/C, and A/J. At the cellular and molecular levels, we specifically show that CSE-mediated induction of nuclease (*nuc*) augments the ability of CSE-USA300 to evade entrapment in neutrophil extracellular traps (NETs) and avoid subsequent killing by the bactericidal compounds present in NETs.

A recent publication reported that CSE exposure modifies the surface charge of USA300 and induces bacterial resistance to killing by antimicrobial peptides and macrophages, which results in a hypervirulence phenotype, in a CD-1 mouse model of pneumonia (12). In our present article, we extend the observations reported by McEachern and colleagues (12) and further delineate novel molecular mechanisms by which CS-exposed staphylococci may become hypervirulent in a vertebrate host.

Materials and Methods

Bacteria, Cell Lines, and Reagents

S. aureus strains, USA300 (13), Newman, and different Newman gene knockout mutants, were grown in tryptic soy medium without antibiotic selection. A detailed analysis of *S. aureus* USA300 genome indicates the presence of arginine catabolic mobile element and methicillin resistance determinant (*mec*) type IV element (14). Cell culture media and supplements were purchased from Life Technologies (Carlsbad, CA).

Bacterial Exposure to CSE

Fresh CSE was prepared for each experiment by bubbling smoke from three Marlboro cigarettes (Philip Morris International Inc., New York, NY) into 20 ml tryptic soy broth (100% CSE) (11). The dose of three cigarettes in 20 ml of culture medium approximates heavy, direct exposure to CS, and is consistent with doses used elsewhere in the literature (15, 16). We used Marlboro cigarettes in place of research cigarettes to maintain consistency with our earlier published work (11). Overnight bacterial cultures were resuspended in medium or 100% CSE and then incubated at 37°C without shaking for 6 hours, and are henceforth referred to here as control and CSE-USA300, respectively. The bacteria were washed in sterile PBS three times before their use in experiments. In separate experiments, CSE was treated

with 25 mM N-acetyl cysteine (NAC; Sigma, St. Louis, MO) or vehicle control (DMSO) for 30 minutes, as previously described (11, 17). USA300 were exposed to CSE treated with either NAC or DMSO.

Mouse Model of Acute Pneumonia by Intranasal Instillation of Bacteria

Animal studies were approved by the Louisiana State University Institutional Animal Care and Use Committee (Baton Rouge, LA). For these studies, 8- to 10-week-old female mice were intranasally inoculated with 50 μ l of sterile PBS containing 5×10^7 CFU of bacteria ($\sim 25 \mu$ l in each naris). After 18 hours, animals were killed (CO₂ asphyxiation followed by cervical dislocation) and bronchoalveolar lavage fluid (BALF), lungs, and spleens were collected. Bacterial CFUs in BALF, lungs (right upper lobe), and spleens were enumerated by dilution plating. A detailed description of BALF collection, BALF cell counting and tissue processing for myeloperoxidase assay, ELISA, and quantitative RT-PCR is provided in the online supplement.

RNASeq Analysis

Bacterial RNA was isolated using Ribopure RNA extraction kit (Ambion, Foster City, CA) as per the manufacturer's instructions. The RNAseq was performed at SeqWright Inc., GE Healthcare (Chicago, IL). The detailed analysis pipeline for RNAseq data is described in the supplemental information.

Statistical Analysis

Results from biological replicates from parametric data are represented as average ($\pm$ SD) and were analyzed using two-way ANOVA followed by Tukey's multiple comparisons test. For nonparametric CFU data, scatter plots with median values are presented and analyzed using Mann-Whitney *U* test. The statistical analysis was performed using GraphPad Prism 6 (La Jolla, CA); a *P* value less than 0.05 was considered significant.

Results

Hypervirulence of CSE-Exposed USA300 in a Mouse Model of Acute Pneumonia

To assess the effects of CSE on the virulence of MRSA strain USA300, we initiated acute pneumonia in age-matched female mice

by instilling 50 μ l suspension containing 5×10^7 CFU (which corresponds to 10^8 CFU/ml) of control or CSE-USA300 into the nares of each mouse ($\sim 25 \mu$ l/naris). At 20 hours postinfection (PI), we observed a significantly higher number of bacterial CFUs in the lung homogenates (control = 4×10^5 CFU/ml, CSE-USA300 = 3.75×10^6 CFU/ml; $P = 0.006$; Figure 1A) and BALF samples

(control = 4.2×10^3 CFU/ml, CSE-USA300 = 1.85×10^6 CFU/ml; $P = 0.029$; Figure 1B) from C57BL6/J mice infected with CSE-USA300 as compared with control-infected mice. To establish that CSE-induced hypervirulence is not limited to only one inbred mouse strain, we also assessed virulence in Balb/C and A/J. At 20 hours PI, there were significantly higher bacterial CFUs in lung tissue and BALF

from both Balb/C (control = 2.65×10^5 CFU/ml, CSE-USA300 = 1.45×10^6 CFU/ml; $P = 0.0002$ in lungs [Figure 1C], and control = 1.4×10^4 , CSE-USA300 = 4.6×10^4 ; $P = 0.0104$ in BALF [Figure 1D]) and A/J (control = 3.9×10^4 CFU/ml, CSE-USA300 = 2.2×10^5 CFU/ml; $P = 0.029$ in lungs [Figure 1E], and control = 655 CFU/ml, CSE-USA300 = 1.1×10^5 CFU/ml; $P = 0.029$ in BALF [Figure 1F]) mice infected with CSE-USA300 compared with control-infected mice. In agreement with our previous reports (11, 17), treatment with the antioxidant, NAC, abrogated the ability of CSE to induce hypervirulence in USA300 (Figures 1A and 1B). These results establish that CSE-USA300 bacteria are significantly better at survival in the lungs of inbred mice and also confirm that the reactive oxygen species present in CSE play a central role in the induction of virulence in USA300.

Infection with CSE-Exposed USA300 Does Not Affect Pulmonary Neutrophil Recruitment

Next, we assessed pulmonary recruitment of neutrophils after infection with either control or CSE-USA300 and found recruitment of a similar number of total white blood cells (Figure 2A) and neutrophils (Figure 2B). The measurement of myeloperoxidase enzyme activity, which is the major determinant of the microbicidal efficacy of neutrophils, is a well established method for determining the presence of neutrophils in the lungs (18–21). We did not observe a significant difference in the level of myeloperoxidase activity in lung homogenates between the medium-treated USA300 and CSE-USA300-infected mice (Figure 2C). Consistent with these results, both groups of mice similarly expressed chemokines, keratinocyte chemoattractant (KC) and macrophage inflammatory protein-2 (MIP2), the main neutrophil chemoattractants produced by lung tissue in response to bacterial infection, at both the mRNA (quantitative RT-PCR, Figures 2D and 2E) and protein (ELISA, Figures 2F and 2G) levels. Thus, the increased pulmonary bacterial burden in CSE-USA300-infected mice is not caused by gross impairments in phagocyte recruitment. These observations led us to compare the bactericidal efficacy of macrophages against control and CSE-USA300.

Figure 1. Increased pulmonary burden in mice infected with cigarette smoke (CS) extract (CSE)-USA300. Mice were intranasally inoculated with 50- μ l suspension containing 5×10^7 CFU (which corresponds to 10^8 CFU/ml) of CSE- or medium-exposed USA300. At 20 hours postinfection (PI), significantly higher CFUs were recovered from the homogenized lung tissues (A, C, and E) and bronchoalveolar lavage fluid (BALF) (B, D, and F) samples obtained from C57BL6/J (four biological replicates, $n = 17$ for control and 16 for CSE-USA300), Balb/C (two biological replicates, $n = 8$ mice), and A/J (one biological replicate, $n = 4$ mice) mice infected with CSE-exposed USA300. Treatment with 25 μ M N-acetyl cysteine (NAC) abrogated CSE-mediated induction of virulence in C57BL6/J mice (A and B; $n = 8$ mice). Inoculum indicated by a dotted line (A and B). Statistical significance determined by Mann-Whitney U statistic. P values for this and subsequent figures are denoted as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; NS, not significant ($P > 0.05$).

Figure 2. Mice infected with CSE-USA300 do not show significantly increased pulmonary neutrophil recruitment. C57BL6/J mice were intranasally inoculated with 10^8 CFUs of CSE- or medium-exposed USA300. At 20 hours PI, the control and CSE-USA300-infected lung tissues showed significant increases in total white blood cells (WBCs) (A, four biological repeats) and differential neutrophil (B, three biological repeats) counts and myeloperoxidase (MPO) activity (C, three biological repeats) compared with the PBS-inoculated control; however, there was no significant difference between the control-treated USA300 and CSE-USA300-infected groups. The total number of mice is superimposed over each histogram. There was no significant difference in the levels of neutrophil chemoattractants keratinocyte chemoattractant (KC) or macrophage inflammatory protein-2 (MIP2) proteins (D and F) estimated by ELISA or mRNA (E and G) determined by quantitative RT-PCR (qRT-PCR) between control-treated USA300 and CSE-USA300-infected lung tissue samples ($n = 3-8$ from two biological repeats). RQ, relative quantification.

CSE Exposure Enhances USA300 Survival Outside and Inside the Phagocytes

Staphylococci resist the bactericidal activities of phagocytes either by evading phagocytic uptake or by surviving inside the phagosome. To test the effects of CSE exposure on the ability of staphylococci to evade phagocytic uptake, we infected murine bone marrow neutrophils or human alveolar macrophages (AMs) with control or CSE-USA300 *in vitro*. We observed significantly higher CFUs in the cell-free supernatants from CSE-USA300-infected wells compared with control (Figures 3A and 3B). This observation indicates that CSE exposure augments the ability of

staphylococci for extracellular survival in the presence of murine neutrophils as well as human AMs.

In separate experiments, the enumeration of intracellular (gentamicin-protected) CFUs over 6 hours PI revealed significantly increased intracellular survival of CSE-USA300 in human AMs (Figure 3C). These observations indicate that CSE exposure enhances the ability of USA300 to survive intracellular killing by macrophages.

CSE Exposure Induces Global Changes in USA300 Gene Expression

To delineate the molecular pathways underlying CSE-induced USA300

hypervirulence in a mouse model of acute pneumonia, we analyzed global changes in gene expression of control-treated USA300 and CSE-USA300 using RNAseq. The *S. aureus* RNAseq data were sequenced using Illumina HiSeq (San Diego, CA) and the reads were aligned and quantified using the edgePro software package (22). The RNA reads from the alignment were then counted to ascertain which genes were differentially regulated using the Bioinformatics tool, DESeq2 (23). This analysis showed that CSE exposure significantly altered ($P < 0.05$) transcription of 344 genes, 204 of which were up-regulated twofold or more and 140 were down-regulated twofold, with a false

Figure 3. CSE-USA300 exhibits significantly improved evasion of phagocytosis and intracellular survival. Murine bone marrow neutrophils (A) or human alveolar macrophages (AMs) (B) were infected with control or CSE-USA300 at multiplicity of infection 10, and the CFUs present in the cell-free supernatants were enumerated at 30 and 60 minutes PI. At 60 minutes PI, there was a two- to fourfold increase in CSE-USA300 in the supernatants, indicating improved evasion of phagocytosis. Enumeration of gentamicin-protected intracellular bacteria revealed significantly improved intracellular survival at 3 and 6 hours PI in human AMs (C). Each plot indicates triplicate results from one representative experiment of two or three biological repeats * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

discovery rate threshold of P less than 0.05 (see Tables 1 and 2). These genes were divided into categories based on function/annotation as defined by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Genes encoding hypothetical proteins were the single largest group detected, with 92 genes differentially transcribed as a result of CSE exposure (47 induced and 45 suppressed by CSE exposure).

To assess which pathways are involved in this process, we binned the filtered genes into known molecular pathways (Figure 4A). These results indicated strong trends suggesting that CSE exposure affects the transcription of genes involved in several important metabolic pathways and in virulence. To formally assess which pathways exhibit significantly altered gene expression, we used the Partek GS Pathway tool (St. Louis, MO). This analysis showed that several important virulence genes are

up-regulated in the CSE-USA300 strain compared with the control USA300 (see Table 3 and Figure 4B).

CSE Exposure Induces USA300 Genes Encoding Virulence Factors

Consistent with our observation that CSE exposure induces emergence of hypervirulent bacteria in the mouse model of acute pneumonia, we observed up-regulation of nine genes encoding staphylococcal virulence proteins as annotated in the KEGG database (Table 3 and Figure 4B). Of these, fibronectin- and fibrinogen-binding proteins (*fnbA*, *fnbB*) and clumping factor B mediate adherence to a variety of host extracellular matrix proteins, such as fibrinogen, fibronectin, and collagen (24); staphylocoagulase interacts with host effectors to subvert hemostasis and induce the formation of fibrin clots and abscesses, inside which staphylococci are protected from phagocytic clearance (25).

Staphylococcal protein A (*spa*) binds to the Fc γ portion of IgG and both blocks the opsonophagocytic killing of staphylococci and stimulates B lymphocyte death (26, 27), in turn aiding CSE-exposed staphylococci in evading phagocytic uptake by neutrophils and macrophages, as described previously here (Figure 3). CSE exposure also up-regulated *nuc*, which degrades the extracellular DNA matrix of NETs and aids bacterial escape from killing by antimicrobial effectors embedded in the NETs (28).

CSE-Induced *nuc* Augments the Ability of *Staphylococcus* to Escape NET-Mediated Killing

Our observation that CSE exposure induces approximately threefold induction of *nuc* expression (Figure 5A) further confirms RNAseq results showing CSE-mediated induction of *nuc* expression (Table 3). This led us to compare the survival of control and CSE-USA300 using an *in vitro* phagocytosis-independent, NET-mediated killing assay. As described in the online supplement, murine bone marrow neutrophils were cotreated with phorbol myristate acetate (to stimulate NET formation) and cytochalasin D (to block actin polymerization and phagocytosis) and infected with control or CSE-USA300. At 60 minutes PI, we detected twofold more CFUs in neutrophils infected with CSE-USA300 as compared with those infected with the control (Figure 5B). To unequivocally confirm the role of *nuc*, we decided to explore whether the augmentation of the ability of CSE-USA300 to escape NET-mediated killing may be abrogated in a *nuc* deletion mutant (Δ *nuc*).

For this set of experiments, we compared the survival of *S. aureus* Newman wild-type (WT) and Δ *nuc* strains after exposure to CSE or medium alone in a phagocytosis-independent, NET-mediated killing assay. The use of *S. aureus* Newman in place of USA300 was driven mainly by the availability of Δ *nuc* deletion mutant in Newman background. *S. aureus* Newman is a human clinical isolate, which exhibits phylogenetic closeness with USA300, especially with regard to the presence of specific virulence genes and their organization within genomic islands (29). Similar to our

Table 1. USA300 Genes Up-Regulated by Exposure to Cigarette Smoke Extract

Annotation	Log ₂ (Fold Change)	Gene Name
SAUSA300_0113	5.1	<i>spa</i>
SAUSA300_0174	4.6	Hypothetical protein
SAUSA300_0176	4.5	ABC transporter permease
SAUSA300_2511	4.5	Hypothetical protein
SAUSA300_0175	4.4	Putative lipoprotein
SAUSA300_2453	4.4	ABC transporter ATP-binding protein
SAUSA300_0177	4.3	Hypothetical protein
SAUSA300_2454	4.3	Membrane spanning protein
SAUSA300_2364	3.9	<i>sbi</i> IgG-binding protein SBI
SAUSA300_0173	3.8	Hypothetical protein
SAUSA300_1997	3.8	Hypothetical protein
SAUSA300_1998	3.6	Hypothetical protein
SAUSA300_1052	3.5	Fibrinogen-binding protein
SAUSA300_2489	3.5	Antibiotic transport-associated protein-like protein
SAUSA300_0386	3.4	<i>xpt</i> xanthine phosphoribosyltransferase
SAUSA300_0776	3.4	<i>nuc</i>
SAUSA300_0387	3.4	<i>pbuX</i> xanthine permease
SAUSA300_0202	3.3	Peptide ABC transporter permease
SAUSA300_0203	3.2	RGD-containing lipoprotein
SAUSA300_2440	3.1	<i>fmbB</i> fibronectin binding protein B
SAUSA300_0018	3.1	tRNA-Glu
SAUSA300_0448	3.0	treP PTS system, trehalose-specific IIBC component
SAUSA300_0224	3.0	<i>coa</i> staphylocoagulase
SAUSA300_0019	2.9	tRNA-Asp
SAUSA300_0524	2.9	<i>rplJ</i> 50S ribosomal protein L10
<i>narZ</i>	2.9	
SAUSA300_0987	2.9	Cytochrome D ubiquinol oxidase, subunit II
SAUSA300_1029	2.8	Iron transport associated domain-containing protein
SAUSA300_0349	2.8	Hypothetical protein
SAUSA300_0986	2.8	Cytochrome D ubiquinol oxidase, subunit I
SAUSA300_0200	2.8	Peptide ABC transporter ATP-binding protein
SAUSA300_2379	2.7	Putative transporter protein
SAUSA300_0435	2.7	ABC transporter ATP-binding protein
<i>rplL</i>	2.7	
SAUSA300_0437	2.7	NLPA lipoprotein
SAUSA300_2321	2.6	Hypothetical protein
SAUSA300_1252	2.6	Amino acid carrier protein
SAUSA300_0215	2.6	Hypothetical protein
SAUSA300_0798	2.6	ABC transporter substrate-binding protein
SAUSA300_1675	2.6	<i>tyrS</i> tyrosyl-tRNA synthetase
SAUSA300_0436	2.6	ABC transporter permease
SAUSA300_2565	2.5	<i>clfB</i>
SAUSA300_0354	2.5	<i>ltrA</i> hypothetical protein
SAUSA300_0848	2.5	Hypothetical protein
SAUSA300_1730	2.5	<i>metK</i> S-adenosylmethionine synthetase
SAUSA300_0056	2.4	Hypothetical protein
SAUSA300_0388	2.4	<i>guaB</i> inosine-5'-monophosphate dehydrogenase
SAUSA300_0230	2.4	Hypothetical protein
SAUSA300_pUSA030027	2.4	Hypothetical protein
SAUSA300_2007	2.4	<i>ilvB</i> acetolactate synthase large subunit
SAUSA300_2117	2.4	tRNA
SAUSA300_2610	2.4	<i>hisC</i> histidinol-phosphate aminotransferase <i>hisC</i>
SAUSA300_0223	2.4	Hypothetical protein
SAUSA300_0085	2.4	Hypothetical protein
SAUSA300_0347	2.4	Sec-independent protein translocase TatC
SAUSA300_0389	2.4	<i>guaA</i> GMP synthase
SAUSA300_1811	2.4	tRNA
SAUSA300_2538	2.3	Amino acid permease family protein
SAUSA300_0432	2.3	Sodium dependent transporter
SAUSA300_0011	2.3	Hypothetical protein
SAUSA300_1055	2.3	<i>efb</i> fibrinogen-binding protein
SAUSA300_0577	2.3	Hypothetical protein
SAUSA300_0433	2.3	<i>cysM</i> cysteine synthase/cystathionine beta-synthas

(Continued)

Table 1. (Continued)

Annotation	Log ₂ (Fold Change)	Gene Name
SAUSA300_0348	2.3	Twin arginine-targeting protein translocase
SAUSA300_1056	2.3	Hypothetical protein
SAUSA300_2441	2.3	<i>fmbA</i> fibronectin binding protein A
SAUSA300_2010	2.3	<i>leuA</i> 2-isopropylmalate synthase
SAUSA300_0576	2.3	Putative pyridine nucleotide-disulfide oxidoreductase
SAUSA300_2116	2.2	tRNA
SAUSA300_1920	2.2	<i>chs</i> chemotaxis-inhibiting protein CHIPS
SAUSA300_2118	2.2	tRNA
SAUSA300_2619	2.2	Hypothetical protein
SAUSA300_0547	2.2	<i>sdrD</i> sdrD protein
SAUSA300_2528	2.2	Hypothetical protein
SAUSA300_2009	2.2	<i>ilvC</i> ketol-acid reductoisomerase
SAUSA300_0407	2.2	Superantigen-like protein
SAUSA300_2012	2.1	<i>leuC</i> isopropylmalate isomerase large subunit
SAUSA300_0434	2.1	<i>metB</i> cystathionine gamma-synthase
SAUSA300_0010	2.1	Hypothetical protein
SAUSA300_1300	2.1	<i>brnQ</i> branched-chain amino acid transport system II carrier protein
SAUSA300_0365	2.1	Hypothetical protein
SAUSA300_0797	2.1	ABC transporter permease
SAUSA300_0683	2.1	DeoR family transcriptional regulator
SAUSA300_0804	2.0	Putative transcriptional regulator
SAUSA300_2210	2.0	<i>glcU</i> glucose uptake protein
SAUSA300_2521	2.0	Hypothetical protein
SAUSA300_0204	2.0	γ -glutamyltranspeptidase
SAUSA300_0083	2.0	Hypothetical protein
SAUSA300_2008	2.0	<i>ilvN</i> acetolactate synthase 1 regulatory subunit
SAUSA300_2006	2.0	<i>ilvD</i> dihydroxy-acid dehydratase
<i>leuD</i>	2.0	
SAUSA300_0084	2.0	Hypothetical protein
SAUSA300_0310	2.0	<i>pfoR</i> perfringolysin O regulator protein
SAUSA300_0438	2.0	CHAP domain-contain protein
SAUSA300_0107	2.0	Na/Pi cotransporter family protein
SAUSA300_1726	1.9	<i>crcB</i> family protein
SAUSA300_0846	1.9	Na ⁺ /H ⁺ antiporter family protein
SAUSA300_2537	1.9	SAUSA300_2537 L-lactate dehydrogenase
SAUSA300_2436	1.9	Putative cell wall surface anchor family protein
SAUSA300_0796	1.9	ABC transporter ATP-binding protein
SAUSA300_0207	1.9	Hypothetical protein
SAUSA300_2389	1.9	Putative drug transporter
SAUSA300_0360	1.9	Cys/Met metabolism PLP-dependent enzyme
SAUSA300_2609	1.9	<i>hisB</i> imidazoleglycerol-phosphate dehydratase
SAUSA300_0557	1.9	HAD family hydrolase
SAUSA300_1814	1.9	tRNA
SAUSA300_2618	1.9	Hypothetical protein
SAUSA300_2282	1.9	Hypothetical protein
SAUSA300_0641	1.9	Putative lipase/esterase
SAUSA300_0057	1.9	Hypothetical protein
SAUSA300_1238	1.9	Hypothetical protein
SAUSA300_0490	1.8	<i>hslO</i> Hsp33-like chaperonin
SAUSA300_0146	1.8	Hypothetical protein
SAUSA300_2526	1.8	<i>pyrD</i> dihydroorotate dehydrogenase 2
SAUSA300_1570	1.8	U32 family peptidase
SAUSA300_0256	1.8	Murein hydrolase regulator LrgA
SAUSA300_2532	1.8	<i>panD</i> aspartate alpha-decarboxylase
SAUSA300_2172	1.8	<i>rplM</i> 50S ribosomal protein L13
<i>hisZ</i>	1.8	
SAUSA300_2011	1.8	<i>leuB</i> 3-isopropylmalate dehydrogenase
SAUSA300_0036	1.8	Hypothetical protein
SAUSA300_0805	1.8	Pathogenicity island protein
SAUSA300_1571	1.8	O-methyltransferase family protein
SAUSA300_2212	1.8	Hypothetical protein
SAUSA300_1004	1.8	Hypothetical protein
SAUSA300_2086	1.8	Hypothetical protein
SAUSA300_2087	1.8	Putative peptidase

(Continued)

Table 1. (Continued)

Annotation	Log ₂ (Fold Change)	Gene Name
SAUSA300_0717	1.8	nrdF ribonucleotide-diphosphate reductase subunit beta
SAUSA300_0370	1.8	Putative enterotoxin
SAUSA300_1883	1.8	putP high affinity proline permease
SAUSA300_1819	1.7	tRNA
SAUSA300_0808	1.7	Hypothetical protein
SAUSA300_0077	1.7	ABC transporter ATP-binding protein
SAUSA300_1569	1.7	U32 family peptidase
SAUSA300_1264	1.7	trpD anthranilate phosphoribosyltransferase
SAUSA300_2561	1.7	phoB alkaline phosphatase
SAUSA300_2080	1.7	Hypothetical protein
SAUSA300_2014	1.7	ilvA threonine dehydratase
SAUSA300_2534	1.7	panB 3-methyl-2-oxobutanoate hydroxymethyltransferase
SAUSA300_0359	1.7	trans-sulfuration enzyme family protein
SAUSA300_2384	1.7	Putative Na ⁺ /H ⁺ antiporter
SAUSA300_0850	1.7	mnhF putative monovalent cation/H ⁺ antiporter subunit F
SAUSA300_0708	1.7	hisC histidinol-phosphate aminotransferase
SAUSA300_1568	1.7	udk uridine kinase
SAUSA300_0201	1.7	Peptide ABC transporter permease
SAUSA300_2611	1.7	hisD histidinol dehydrogenase
SAUSA300_0082	1.7	Hypothetical protein
SAUSA300_1549	1.7	ComE operon protein 1
SAUSA300_2456	1.7	Hypothetical protein
SAUSA300_1919	1.7	Hypothetical protein
SAUSA300_2533	1.7	panC pantoate-beta-alanine ligase
SAUSA300_2265	1.7	Putative amino acid permease
SAUSA300_1283	1.7	pstS phosphate ABC transporter phosphate-binding protein PstS
hisG	1.7	
SAUSA300_2407	1.7	Oligopeptide ABC transporter ATP-binding protein
SAUSA300_0629	1.7	pbp4 penicillin-binding protein 4
SAUSA300_0902	1.7	pepF oligoendopeptidase F
SAUSA300_1287	1.6	asd aspartate semialdehyde dehydrogenase
SAUSA300_1813	1.6	tRNA
SAUSA300_1823	1.6	tRNA
SAUSA300_1066	1.6	tRNA
SAUSA300_0449	1.6	treC alpha,alpha-phosphotrehalase
SAUSA300_1818	1.6	tRNA
SAUSA300_2417	1.6	Putative transporter
SAUSA300_2273	1.6	Na ⁺ /H ⁺ antiporter family protein
SAUSA300_2171	1.6	rpsL 30S ribosomal protein S9
SAUSA300_0991	1.6	def peptide deformylase
SAUSA300_0487	1.6	Hypothetical protein
SAUSA300_0849	1.6	mnhG putative monovalent cation/H ⁺ antiporter subunit G
SAUSA300_0851	1.6	mnhE putative monovalent cation/H ⁺ antiporter subunit E
SAUSA300_2348	1.6	Hypothetical protein
SAUSA300_2520	1.6	Transporter gate domain-containing protein
SAUSA300_0488	1.6	hpt hypoxanthine phosphoribosyltransferase
SAUSA300_1816	1.6	tRNA
SAUSA300_2173	1.6	truA tRNA pseudouridine synthase A
SAUSA300_2408	1.5	Oligopeptide ABC transporter ATP-binding protein
SAUSA300_1259	1.5	ImpB/MucB/SamB family protein
SAUSA300_2623	1.5	pcp pyrrolidone-carboxylate peptidase
SAUSA300_0358	1.5	Bifunctional homocysteine S-methyltransferase/5,10-methylenetetrahydrofolate reductase protein
SAUSA300_0852	1.5	mnhE putative monovalent cation/H ⁺ antiporter subunit E
SAUSA300_0012	1.5	Putative homoserine O-acetyltransferase
SAUSA300_2235	1.5	Iron compound ABC transporter iron compound-binding protein
SAUSA300_1817	1.5	tRNA
SAUSA300_1567	1.5	greA transcription elongation factor GreA
SAUSA300_0789	1.5	Putative thioredoxin
SAUSA300_2174	1.5	Cobalt transport family protein
SAUSA300_0541	1.5	Deoxynucleoside kinase family protein
SAUSA300_1286	1.5	Aspartate kinase
SAUSA300_0586	1.5	Hypothetical protein
SAUSA300_0503	1.5	Transcriptional regulator, gntR family protein

(Continued)

Table 1. (Continued)

Annotation	Log ₂ (Fold Change)	Gene Name
cbiO	1.4	
SAUSA300_2507	1.4	Hypothetical protein
SAUSA300_1891	1.4	Hypothetical protein
SAUSA300_0322	1.4	Oye family NADH-dependent flavin oxidoreductase
SAUSA300_0889	1.4	oppD oligopeptide ABC transporter ATP-binding protein
SAUSA300_2175	1.4	cbiO cobalt transporter ATP-binding subunit
SAUSA300_0076	1.4	ABC transporter ATP-binding protein
SAUSA300_2617	1.4	Putative cobalt ABC transporter ATP-binding protein
SAUSA300_2492	1.3	Acetyltransferase family protein
SAUSA300_2638	1.3	Hypothetical protein
SAUSA300_0206	1.3	Azoreductase
SAUSA300_0784	1.3	LysE/YggA family protein
SAUSA300_2326	1.3	Transcription regulatory protein
SAUSA300_0075	1.3	opp-3C oligopeptide permease, channel-forming protein
SAUSA300_0952	1.3	Aminotransferase, class I
SAUSA300_0747	1.3	trxB thioredoxin-disulfide reductase
SAUSA300_0121	1.3	Putative drug transporter
SAUSA300_0454	1.3	recR recombination protein RecR
SAUSA300_0446	1.3	gltD glutamate synthase subunit beta
SAUSA300_0875	1.2	Hypothetical protein
SAUSA300_0853	1.2	mnhD putative monovalent cation/H ⁺ antiporter subunit D
SAUSA300_2409	1.2	Oligopeptide ABC transporter permease
SAUSA300_2266	1.2	Hypothetical protein
SAUSA300_0945	1.1	Isochorismate synthase family protein
SAUSA300_0074	1.1	opp-3B oligopeptide permease, channel-forming protein
SAUSA300_0073	1.1	Peptide ABC transporter peptide-binding protein
SAUSA300_0430	1.1	Hypothetical protein
SAUSA300_0429	1.0	Hypothetical protein

Definition of abbreviations: ABC, ATP-binding cassette; CHAP, cysteine, histidine-dependent amidohydrolases/peptidases; CHIPS, chemotaxis inhibiting protein; *clfB*, clumping factor B; *coa*, staphylocoagulase; *fmb*, fibronectin binding protein; HAD, haloacid dehalogenase; NADH, nicotinamide adenine dinucleotide; *nuc*, nuclease; PLP, pyridoxal 5'-phosphate; RGD, Arg-Gly-Asp; *spa*, staphylococcal protein A; tRNA, transfer RNA.

earlier observations, we detected two- to threefold more CFUs of CSE-WT *S. aureus* compared with the control-treated WT *S. aureus* at 60 minutes after exposure to phagocytosis-blocked, phorbol myristate acetate-treated neutrophils (Figure 5C). Interestingly, CSE exposure did not enhance escape/survival of Δ *nuc* Newman *S. aureus* from NET-mediated killing (Figure 5C), further confirming the importance of staphylococcal *nuc* in this phenomenon.

Discussion

The upper respiratory tract is inhabited by a diverse microbiome that constitutes pathogens, such as *S. aureus*, which, according to our hypothesis may be subjected to exposure to the toxic, bioactive chemicals from CS. CS exposure will, in turn, promote the emergence of hypervirulent strains of staphylococci. In this regard, others and we have shown that CS exposure induces biofilm formation in the respiratory bacterial pathogens

S. aureus, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and oral pathogens, such as *Porphyromonas gingivalis* (11, 30–32). We previously showed that, at the molecular level, the reactive oxygen species found in CS down-regulate the expression of a quorum-sensing regulon, *agr*, while inducing genes encoding a variety of surface proteins involved in biofilm formation and host cell adherence (11). The *agr* regulon is a redox sensor that regulates global transcription, including the regulation of the staphylococcal virulome and biofilm dispersal (33, 34). Based on these observations, we sought to establish CSE-induced hypervirulence of the USA300 strain in a mouse model of acute pneumonia and to identify CSE-mediated alterations in genome-wide transcription using RNAseq. After intranasal infection of C57BL/6/J mice, the resulting strain, CSE-USA300, exhibited increased pulmonary CFUs compared with control-infected mice. Although the genetic uniformity within an inbred animal strain is often

advantageous because it minimizes the variability in outcomes within an experimental cohort, and thus increases the power and experimental sensitivity, this experimental design inherently overlooks the importance of genotypic variation (35). This is why we confirmed that CSE exposure induces hypervirulence in USA300 using several inbred mouse strains, including A/J and Balb/C. Furthermore, the resulting observations are similar to those from an outbred CD-1 mouse model, as previously reported (12). Taken together, these results confirm that, compared with the control, CSE-USA300 is hypervirulent in mice, regardless of their genetic background, as exhibited by increased pulmonary CFUs at 24 hours PI.

In response to USA300 infection, respiratory epithelial cells and AMs secrete cytokines and chemokines to induce inflammation (TNF- α , IL-1 β) and recruit neutrophils (KC, MIP2) between 2 and 8 hours PI (36, 37). Once recruited, neutrophils are the main source of

Table 2. USA300 Genes Down-Regulated by Exposure to Cigarette Smoke Extract

Annotation	log ₂ (Fold Change)	Gene Name
SAUSA300_0162	-1.1	Capsular polysaccharide biosynthesis protein Cap5K
SAUSA300_1219	-1.1	Putative sensor histidine kinase
SAUSA300_pUSA030018	-1.2	tral transfer complex protein Tral (DNA topoisomerase III)
SAUSA300_0519	-1.2	Hypothetical protein
SAUSA300_1221	-1.2	Hypothetical protein
SAUSA300_1220	-1.2	LuxR family DNA-binding response regulator
SAUSA300_0046	-1.2	Hypothetical protein
SAUSA300_2323	-1.2	cobI CorA family protein
SAUSA300_0566	-1.3	Amino acid permease
SAUSA300_1257	-1.3	msrR peptide methionine sulfoxide reductase regulator MsrR
SAUSA300_0993	-1.3	pdhA pyruvate dehydrogenase E1 component, alpha subunit
SAUSA300_2446	-1.3	Hypothetical protein
SAUSA300_2340	-1.3	narI respiratory nitrate reductase, gamma subunit
SAUSA300_0603	-1.3	Hypothetical protein
SAUSA300_pUSA030012	-1.4	traC membrane protein TraC
SAUSA300_2627	-1.4	2-oxoglutarate/malate translocator
SAUSA300_0725	-1.4	Hypothetical protein
SAUSA300_1967	-1.4	Hypothetical protein
SAUSA300_pUSA030014	-1.4	traE transfer complex protein TraE
SAUSA300_pUSA030015	-1.4	traF transfer complex protein TraF
SAUSA300_pUSA030013	-1.4	traD hypothetical protein
SAUSA300_1926	-1.4	phi77 ORF044-like protein
SAUSA300_1529	-1.4	dgkA diacylglycerol kinase
SAUSA300_0939	-1.4	Glycosyl transferase, group 1 family protein
SAUSA300_1953	-1.4	phiPVL ORF051-like protein
SAUSA300_0101	-1.5	Tandem lipoprotein
SAUSA300_1391	-1.5	phiSLT ORF527-like protein
SAUSA300_0289	-1.5	Hypothetical protein
SAUSA300_1928	-1.5	phi77 ORF002-like protein, phage minor structural protein
SAUSA300_1480	-1.5	Putative traG membrane protein
SAUSA300_1927	-1.5	phi77 ORF109-like protein
SAUSA300_0153	-1.5	Capsular polysaccharide biosynthesis protein Cap5B
SAUSA300_1803	-1.6	Hypothetical protein
SAUSA300_1230	-1.6	Hypothetical protein
SAUSA300_1961	-1.6	phiPVL ORF41-like protein
SAUSA300_0160	-1.6	Capsular polysaccharide biosynthesis protein Cap5I
SAUSA300_0844	-1.6	Hypothetical protein
SAUSA300_2353	-1.6	Hypothetical protein
SAUSA300_1524	-1.6	CBS domain-containing protein
SAUSA300_2592	-1.6	Hypothetical protein
SAUSA300_2352	-1.6	Addiction module antitoxin
SAUSA300_2167	-1.6	Hypothetical protein
SAUSA300_2168	-1.6	Hypothetical protein
SAUSA300_1766	-1.6	epiB lantibiotic epidermin biosynthesis protein EpiB
SAUSA300_pUSA030010	-1.6	traA transfer complex protein TraA
SAUSA300_0154	-1.7	Capsular polysaccharide biosynthesis protein Cap5C
SAUSA300_1956	-1.7	Hypothetical protein
SAUSA300_1581	-1.7	Hypothetical protein
SAUSA300_0793	-1.7	Hypothetical protein
nusA	-1.7	
SAUSA300_1160	-1.7	Hypothetical protein
SAUSA300_2156	-1.7	Lactose phosphotransferase system repressor
SAUSA300_0100	-1.7	Tandem lipoprotein
SAUSA300_1941	-1.7	phi77 ORF003-like protein, phage terminase, large subunit
SAUSA300_1161	-1.7	Ribosomal protein L7Ae
SAUSA300_1339	-1.7	Hypothetical protein
SAUSA300_1930	-1.7	phi77 ORF001-like protein, phage tail tape measure protein
SAUSA300_1764	-1.8	epiD lantibiotic epidermin biosynthesis protein EpiD
SAUSA300_1765	-1.8	epiC lantibiotic epidermin biosynthesis protein EpiC
SAUSA300_1478	-1.8	Putative lipoprotein
SAUSA300_0105	-1.8	M20/M25/M40 family peptidase
SAUSA300_1479	-1.8	Hypothetical protein
SAUSA300_0264	-1.8	Ribose transporter RbsU

(Continued)

Table 2. (Continued)

Annotation	log ₂ (Fold Change)	Gene Name
SAUSA300_2036	-1.8	kdpE DNA-binding response regulator, KdpE
SAUSA300_0794	-1.8	TOPRIM domain-containing protein
SAUSA300_0106	-1.8	Putative drug transporter
SAUSA300_2041	-1.8	Hypothetical protein
SAUSA300_2035	-1.8	kdpD sensor histidine kinase, KdpD
SAUSA300_2138	-1.9	Hypothetical protein
SAUSA300_1929	-1.9	phi77 ORF004-like protein phage tail component
SAUSA300_2137	-1.9	Hypothetical protein
SAUSA300_0232	-1.9	Hypothetical protein
SAUSA300_0395	-1.9	Superantigen-like protein
SAUSA300_1158	-1.9	Hypothetical protein
SAUSA300_2360	-2.0	Multidrug resistance protein
SAUSA300_0172	-2.0	Hypothetical protein
SAUSA300_2540	-2.0	Fructose-1,6-bisphosphate aldolase
SAUSA300_2418	-2.0	Hypothetical protein
SAUSA300_1940	-2.0	Phage portal protein
SAUSA300_1303	-2.0	Hypothetical protein
SAUSA300_1063	-2.0	arcC carbamate kinase
SAUSA300_1653	-2.0	Metal-dependent hydrolase
SAUSA300_1214	-2.1	Hypothetical protein
SAUSA300_0152	-2.1	Capsular polysaccharide biosynthesis protein Cap5A
SAUSA300_1180	-2.1	Hypothetical protein
SAUSA300_1062	-2.1	argF ornithine carbamoyltransferase
SAUSA300_0781	-2.1	Hypothetical protein
SAUSA300_0231	-2.1	ABC transporter substrate-binding protein
SAUSA300_0318	-2.1	N-acetylmannosamine-6-phosphate 2-epimerase
SAUSA300_1374	-2.1	Hypothetical protein
SAUSA300_0792	-2.1	Hypothetical protein
SAUSA300_0949	-2.1	sspC cysteine protease
SAUSA300_0017	-2.2	Adenylosuccinate synthetase (EC:6.3.4.4)
SAUSA300_1943	-2.2	phi77 ORF040-like protein
SAUSA300_0950	-2.2	sspB cysteine protease
SAUSA300_2486	-2.2	Putative ATP-dependent Clp proteinase
SAUSA300_1990	-2.2	agrD accessory gene regulator protein D
SAUSA300_0976	-2.2	purD phosphoribosylamine-glycine ligase
SAUSA300_2525	-2.2	Hypothetical protein
SAUSA300_1939	-2.3	phi77 ORF015-like protein protease
SAUSA300_1182	-2.3	Pyruvate ferredoxin oxidoreductase, alpha subunit
SAUSA300_1760	-2.3	epiG lantibiotic epidermin immunity protein F
SAUSA300_0951	-2.3	sspA V8 protease
SAUSA300_1440	-2.3	Hypothetical protein
purH	-2.3	
SAUSA300_1761	-2.3	epiE lantibiotic epidermin immunity protein F
SAUSA300_1183	-2.3	2-oxoglutarate ferredoxin oxidoreductase subunit beta
SAUSA300_0973	-2.3	purM phosphoribosylaminoimidazole synthetase
SAUSA300_1762	-2.3	epiF lantibiotic epidermin immunity protein F
SAUSA300_0974	-2.3	purN phosphoribosylglycinamide formyltransferase
SAUSA300_1931	-2.4	phi77 ORF100-like protein
SAUSA300_2315	-2.4	Hypothetical protein
SAUSA300_1950	-2.4	Hypothetical protein
SAUSA300_1932	-2.4	Hypothetical protein
SAUSA300_0795	-2.4	Hypothetical protein
SAUSA300_0183	-2.4	Hypothetical protein
SAUSA300_0972	-2.5	purF amidophosphoribosyltransferase
SAUSA300_1934	-2.5	phi77 ORF020-like protein, phage major tail protein
SAUSA300_1767	-2.5	epiA lantibiotic epidermin biosynthesis protein EpiA
SAUSA300_1935	-2.6	phi77 ORF029-like protein
SAUSA300_1936	-2.6	Hypothetical protein
SAUSA300_1395	-2.6	phiSLT ORF116b-like protein
SAUSA300_2415	-2.7	Hypothetical protein
SAUSA300_0770	-2.7	Hypothetical protein
SAUSA300_0067	-2.7	Universal stress protein
SAUSA300_1937	-2.7	phi77 ORF045-like protein
SAUSA300_2567	-2.8	arcC carbamate kinase

(Continued)

Table 2. (Continued)

Annotation	log ₂ (Fold Change)	Gene Name
SAUSA300_0971	−2.8	purL phosphoribosylformylglycinamide synthase II
SAUSA300_2573	−2.8	isaB immunodominant antigen B
SAUSA300_2632	−2.8	Hypothetical protein
SAUSA300_1938	−2.8	phi77 ORF006-like protein capsid protein
SAUSA300_2566	−2.9	arcR Crp/FNR family transcriptional regulator
SAUSA300_0970	−3.0	purQ phosphoribosylformylglycinamide synthase I
SAUSA300_0969	−3.0	purS phosphoribosylformylglycinamide synthase
SAUSA300_0786	−3.0	OsmC/Ohr family protein
SAUSA300_2097	−3.1	Hypothetical protein
SAUSA300_0968	−3.1	purC phosphoribosylaminoimidazole-succinocarboxamide synthase
SAUSA300_2568	−3.3	arcD arginine/ornithine antiporter
SAUSA300_1712	−3.4	ribH 6,7-dimethyl-8-ribityllumazine synthase
SAUSA300_0967	−3.4	purK phosphoribosylaminoimidazole carboxylase ATPase subunit
SAUSA300_1713	−3.5	ribBA riboflavin biosynthesis protein
SAUSA300_1714	−3.5	ribE riboflavin synthase subunit alpha
SAUSA300_1715	−3.5	ribD riboflavin biosynthesis protein
SAUSA300_0966	−3.6	purE phosphoribosylaminoimidazole carboxylase, catalytic subunit
SAUSA300_0883	−4.2	Putative surface protein
SAUSA300_2569	−4.4	arcB ornithine carbamoyltransferase
SAUSA300_2570	−5.3	arcA arginine deiminase

Definition of abbreviations: ABC, ATP-binding cassette; agr, accessory gene regulator protein; CBS, cystathione β-synthase; Clp, caseinolytic protease; nusA, N (phage lambda protein) utilization substance; ssp, stringent starvation protein; TOPRIM, topoisomerase-primase domain.

proinflammatory cytokines and, thus, the sustained presence of activated neutrophils plays a central role in the development of acute lung injury (38). Therefore, we investigated whether increased pulmonary bacterial loads in CSE-USA300-infected mice corresponded with changes in pulmonary neutrophil recruitment. To our surprise, neither the levels of the neutrophil chemoattractant chemokines, KC and MIP2, nor the numbers of recruited neutrophils in the lungs of CSE-USA300 and control-infected mice were significantly different. These results directed us to assess whether CSE exposure might augment the ability of USA300 to escape/evade the innate immune responses elaborated by macrophages and neutrophils.

The highlight of this study is the comparison of RNA profiles between untreated bacteria and CSE-USA300. These findings indicate that CSE exposure triggers global changes in the expression of genes involved in metabolism, the environmental stress response, two-component systems, virulence, and immune evasion. Furthermore, the significant increase in expression of *spa*, *nuc*, clumping factor B, *fnbA*, *fnbB*, staphylocoagulase, and chemotaxis inhibitory protein (*chs*) in CSE-USA300 provides a genetic basis for the

CSE-mediated induction of staphylococcal host cells and extracellular matrix adherence; as well as the evasion of phagocytosis and NET-mediated killing. In addition, the up-regulation of staphylococcal surface adhesins correlates with the earlier observation that CSE exposure induces the adherence of USA300 to respiratory epithelial cells and extracellular matrix proteins, such as fibrinogen and fibronectin (11). Although adherence to host cells may play an important role in staphylococcal virulence, here we focused on the effects of CSE exposure on staphylococcal survival in the face of phagocytosis and NET-mediated killing, as *spa* and *nuc* are the primary virulence effectors that aid evasion from these two immune mechanisms, respectively.

Pulmonary phagocytes recognize and phagocytose invading pathogens, such as *S. aureus*, which are then killed by the action of cationic antimicrobial peptides, reactive oxygen radicals (respiratory burst), and lysosomal enzymes. *S. aureus* surface protein *spa* binds Fcγ, thus capturing host IgG molecules in an inverted position and blocking phagocytosis (39). Using *in vitro* assays, we compared the phagocytosis and intracellular killing of control and CSE-USA300 in murine bone marrow

neutrophils and human AMs. In both cell types, CSE-USA300 exhibited significantly improved abilities to evade phagocytic uptake and intracellular killing, as judged by the enumeration of unphagocytosed extracellular CFUs in the cell-free supernatants or gentamicin-protected intracellular CFUs, respectively. Because CSE exposure up-regulates staphylococcal biofilm formation, which plays a role in the evasion of phagocytosis and the subsequent bacterial persistence in an immunocompetent host (11, 40), we propose that CSE-mediated induction of *spa* gene expression is yet another mechanism underlying this improved evasion of phagocytosis by CSE-USA300.

Activated neutrophils extrude genomic and mitochondrial DNA to form NETs in which antimicrobial peptides, proteases, and histones are embedded (41, 42). Bacteria entrapped in the NETs are subsequently killed by the activity of antimicrobial effectors. NETs play an important role in pulmonary inflammation. For example, in response to *S. aureus* infection neutrophils activate a rapid, oxidant-independent mechanism for NET production (43, 44). To evade NET-mediated killing, *S. aureus* produces *nuc* to degrade the extracellular DNA matrix and escape killing by

Figure 4. RNAseq data. The heatmap shows log₁₀-normalized read counts for control and treated experimental groups for *Staphylococcus aureus* genes, with *off-white* indicating lower counts, and *darker red* indicating higher counts. Gene names, or locus tag if no gene name was present in the annotation file, are shown (A). The pathways for *S. aureus* and the resulting Gene Ontology Enrichment Analysis (GO analysis) for a subset of genes that were expressed significantly differently between treatment and control groups are represented as treated versus untreated (B). For the GO analysis, the score is an indication of the significance of association between the genes and the pathway. *P* values for this panel are indicated as follows: **P* < 0.05; ***P* < 0.01. The expression of staphylococcal virulence genes significantly altered by exposure to CSE as detected by RNAseq is shown (C). The histograms represent log₂ (fold difference) over the medium-exposed control.

Table 3. Staphylococcal Virulence Genes Induced by Exposure to Cigarette Smoke Extract

Annotation	log ₂ (Fold Change)	Gene Name
SAUSA300_0113	5.1	<i>spa</i>
SAUSA300_1052	3.5	Fibrinogen-binding protein
SAUSA300_0776	3.4	<i>nuc</i>
SAUSA300_2440	3.1	<i>fnbB</i> fibronectin binding protein B
SAUSA300_0224	2.9	<i>coa</i> staphylocoagulase
SAUSA300_2565	2.5	<i>clfB</i>
SAUSA300_2441	2.3	<i>fnbA</i> fibronectin binding protein A
SAUSA300_1920	2.2	<i>chs</i> chemotaxis-inhibiting protein CHIPS
SAUSA300_0407	2.1	Superantigen-like protein

Definition of abbreviations: CHIPS, chemotaxis inhibiting protein; *clfB*, clumping factor B; *coa*, staphylocoagulase; *nuc*, nuclease; *spa*, staphylococcal protein A.

antimicrobial effectors (28). Prompted by RNAseq results showing CS-mediated induction of *nuc* transcription in CSE-USA300, we performed *in vitro* assays to

compare NET-mediated killing of control and CSE-USA300. Consistent with the RNAseq results, we observed that, in comparison with control

USA300, CSE-USA300 exhibited significantly improved ability to evade phagocytosis-independent NET-mediated killing.

In the 50 years since the publication of the U.S. Surgeon General's original report (45), sustained research efforts have elucidated the adverse immunophysiological effects of CS in active smokers as well as in those exposed to secondhand smoke (7). These scientific observations have paved the way for public health initiatives to raise awareness about the health risks associated with CS exposure and for legislative bans to reduce the exposure of nonsmokers to secondhand CS. Despite these advances, 18% of adults in the United States continue to smoke and, in turn, expose a disproportionately large number of nonsmokers to secondhand smoke. Thus, cigarette smoking continues to be a menace to society and is responsible for 480,000 deaths every year, with an annual monetary burden of approximately \$300 billion (direct medical care costs as well as lost productivity due to smoking-related illness) in the United States alone (46, 47).

In recent years, growing experimental evidence has supported the idea that exposure to chemicals in CS may alter the physiology of colonizing microbiota in a manner detrimental to human health. In this article, we establish the importance of phagocytosis evasion and *nuc*-mediated escape of neutrophil NETs in the CS-induced hypervirulence of MRSA strain USA300. Furthermore, the RNAseq analysis provides evidence for pleiotropic changes in USA300 gene expression, including genes encoding multiple virulence factors. Future studies are warranted to identify effects of CS exposure on the virulence of other colonizing pathogens in the upper respiratory tract, such as *S. pneumoniae* and *Haemophilus influenzae*. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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Figure 5. CSE-mediated up-regulation of nuclease (*nuc*) enhances survival of CSE-USA300 in the face of neutrophil extracellular trap (NET)-mediated killing. Using qRT-PCR, we confirmed the RNAseq results showing that a 6-hour-long exposure to CSE induces expression of the *nuc* gene, which encodes *nuc* (A). In accordance with these results, we observed a roughly twofold increase in the survival of CSE-USA300 in the NET-mediated killing assay (B). The role of *nuc* in this phenomenon was further confirmed through use of a Δ *nuc* deletion mutant strain of *S. aureus* in which CSE exposure does not induce survival in a NET-mediated killing assay, similar to what is seen with the wild-type (WT) strain (C). * $P < 0.05$; and ** $P < 0.01$.

References

- Frank DN, Feazel LM, Bessesen MT, Price CS, Janoff EN, Pace NR. The human nasal microbiota and *Staphylococcus aureus* carriage. *PLoS One* 2010;5:e10598.
- von Eiff C, Becker K, Machka K, Stammer H, Peters G; Study Group. Nasal carriage as a source of *Staphylococcus aureus* bacteremia. *N Engl J Med* 2001;344:11–16.
- Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, Nouwen JL. The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect Dis* 2005;5: 751–762.
- van Belkum A, Melles DC, Nouwen J, van Leeuwen WB, van Wamel W, Vos MC, Wertheim HF, Verbrugh HA. Co-evolutionary aspects of human colonisation and infection by *Staphylococcus aureus*. *Infect Genet Evol* 2009;9:32–47.
- Peacock SJ, de Silva I, Lowy FD. What determines nasal carriage of *Staphylococcus aureus*? *Trends Microbiol* 2001;9:605–610.
- Cole AM, Tahk S, Oren A, Yoshioka D, Kim YH, Park A, Ganz T. Determinants of *Staphylococcus aureus* nasal carriage. *Clin Diagn Lab Immunol* 2001;8:1064–1069.
- Strachan DP, Cook DG. Health effects of passive smoking. 1. Parental smoking and lower respiratory illness in infancy and early childhood. *Thorax* 1997;52:905–914.
- Bogaert D, van Belkum A, Sluijter M, Luijendijk A, de Groot R, Rümke HC, Verbrugh HA, Hermans PW. Colonisation by *Streptococcus pneumoniae* and *Staphylococcus aureus* in healthy children. *Lancet* 2004;363:1871–1872.
- Melles DC, Bogaert D, Gorkink RF, Peeters JK, Moorhouse MJ, Ott A, van Leeuwen WB, Simons G, Verbrugh HA, Hermans PW, et al. Nasopharyngeal co-colonization with *Staphylococcus aureus* and *Streptococcus pneumoniae* in children is bacterial genotype independent. *Microbiology* 2007;153:686–692.
- Arcavi L, Benowitz NL. Cigarette smoking and infection. *Arch Intern Med* 2004;164:2206–2216.
- Kulkarni R, Antala S, Wang A, Amaral FE, Rampersaud R, Larussa SJ, Planet PJ, Ratner AJ. Cigarette smoke increases *Staphylococcus aureus* biofilm formation via oxidative stress. *Infect Immun* 2012;80: 3804–3811.
- McEachern EK, Hwang JH, Sladewski KM, Nicatia S, Dewitz C, Mathew DP, Nizet V, Crotty Alexander LE. Analysis of the effects of cigarette smoke on staphylococcal virulence phenotypes. *Infect Immun* 2015;83:2443–2452.
- Tenover FC, Goering RV. Methicillin-resistant *Staphylococcus aureus* strain USA300: origin and epidemiology. *J Antimicrob Chemother* 2009;64:441–446.
- Planet PJ, LaRussa SJ, Dana A, Smith H, Xu A, Ryan C, Uhlemann AC, Boundy S, Goldberg J, Narechania A, et al. Emergence of the epidemic methicillin-resistant *Staphylococcus aureus* strain USA300 coincides with horizontal transfer of the arginine catabolic mobile element and speG-mediated adaptations for survival on skin. *MBio* 2013;4:e00889–e13.
- Downs CA, Montgomery DW, Merkle CJ. Age-related differences in cigarette smoke extract-induced H₂O₂ production by lung endothelial cells. *Microvasc Res* 2011;82:311–317.
- Mercer BA, Kolesnikova N, Sonett J, D'Armiento J. Extracellular regulated kinase/mitogen activated protein kinase is up-regulated in pulmonary emphysema and mediates matrix metalloproteinase-1 induction by cigarette smoke. *J Biol Chem* 2004;279: 17690–17696.
- Kulkarni R, Rampersaud R, Aguilar JL, Randis TM, Kreindler JL, Ratner AJ. Cigarette smoke inhibits airway epithelial cell innate immune responses to bacteria. *Infect Immun* 2010;78:2146–2152.
- Werner U, Szelenyi I. Measurement of MPO activity as model for detection of granulocyte infiltration in different tissues. *Agents Actions* 1992;36:C101–C103.
- Jeyaseelan S, Young SK, Fessler MB, Liu Y, Malcolm KC, Yamamoto M, Akira S, Worthen GS. Toll/IL-1 receptor domain-containing adaptor inducing IFN- β (TRIF)-mediated signaling contributes to innate immune responses in the lung during *Escherichia coli* pneumonia. *J Immunol* 2007;178:3153–3160.
- Bradley PP, Priebe DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* 1982;78:206–209.
- Chapman AL, Hampton MB, Senthilmohan R, Winterbourn CC, Kettle AJ. Chlorination of bacterial and neutrophil proteins during phagocytosis and killing of *Staphylococcus aureus*. *J Biol Chem* 2002;277: 9757–9762.
- Magoc T, Wood D, Salzberg SL. EDGE-pro: estimated degree of gene expression in prokaryotic genomes. *Evol Bioinform Online* 2013;9: 127–136.
- Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15: 550.
- Foster TJ, Geoghegan JA, Ganesh VK, Höök M. Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. *Nat Rev Microbiol* 2014;12:49–62.
- Cheng AG, McAdow M, Kim HK, Bae T, Missiakas DM, Schneewind O. Contribution of coagulases towards *Staphylococcus aureus* disease and protective immunity. *PLoS Pathog* 2010;6:e1001036.
- Peterson PK, Verhoef J, Sabath LD, Quie PG. Effect of protein A on staphylococcal opsonization. *Infect Immun* 1977;15:760–764.
- Goodyear CS, Silverman GJ. Staphylococcal toxin induced preferential and prolonged *in vivo* deletion of innate-like B lymphocytes. *Proc Natl Acad Sci USA* 2004;101:11392–11397.
- Berends ET, Horswill AR, Haste NM, Monestier M, Nizet V, von Kückritz-Blickwede M. Nuclease expression by *Staphylococcus aureus* facilitates escape from neutrophil extracellular traps. *J Innate Immun* 2010;2:576–586.
- Baba T, Bae T, Schneewind O, Takeuchi F, Hiramatsu K. Genome sequence of *Staphylococcus aureus* strain Newman and comparative analysis of staphylococcal genomes: polymorphism and evolution of two major pathogenicity islands. *J Bacteriol* 2008; 190:300–310.
- Antunes MB, Chi JJ, Liu Z, Goldstein-Daruech N, Palmer JN, Zhu J, Cohen NA. Molecular basis of tobacco-induced bacterial biofilms: an *in vitro* study. *Otolaryngol Head Neck Surg* 2012;147:876–884.
- Bagaitkar J, Daep CA, Patel CK, Renaud DE, Demuth DR, Scott DA. Tobacco smoke augments *Porphyromonas gingivalis*-*Streptococcus gordonii* biofilm formation. *PLoS One* 2011; 6:e27386.
- Mutepe ND, Cockran R, Steel HC, Theron AJ, Mitchell TJ, Feldman C, Anderson R. Effects of cigarette smoke condensate on pneumococcal biofilm formation and pneumolysin. *Eur Respir J* 2013;41:392–395.
- Boles BR, Horswill AR. agr-Mediated dispersal of *Staphylococcus aureus* biofilms. *PLoS Pathog* 2008;4:e1000052.
- Sun F, Liang H, Kong X, Xie S, Cho H, Deng X, Ji Q, Zhang H, Alvarez S, Hicks LM, et al. Quorum-sensing agr mediates bacterial oxidation response via an intramolecular disulfide redox switch in the response regulator agrA. *Proc Natl Acad Sci USA* 2012;109: 9095–9100.
- Festing MF. Warning: the use of heterogeneous mice may seriously damage your research. *Neurobiol Aging* 1999;20:237–244; discussion 245–236.
- Jacqueline C, Broquet A, Roquilly A, Davieau M, Caillon J, Altare F, Potel G, Asehnoune K. Linezolid dampens neutrophil-mediated inflammation in methicillin-resistant *Staphylococcus aureus*-induced pneumonia and protects the lung of associated damages. *J Infect Dis* 2014;210:814–823.
- Lee MH, Arrecubieta C, Martin FJ, Prince A, Borczuk AC, Lowy FD. A postinfluenza model of *Staphylococcus aureus* pneumonia. *J Infect Dis* 2010;201:508–515.
- Abraham E. Neutrophils and acute lung injury. *Crit Care Med* 2003;31 (4 suppl):S195–S199.
- Kobayashi SD, DeLeo FR. *Staphylococcus aureus* protein A promotes immune suppression. *MBio* 2013;4:e00764–e13.
- Thurlow LR, Hanke ML, Fritz T, Angle A, Aldrich A, Williams SH, Engebretsen IL, Bayles KW, Horswill AR, Kielian T. *Staphylococcus aureus* biofilms prevent macrophage phagocytosis and attenuate inflammation *in vivo*. *J Immunol* 2011; 186:6585–6596.

41. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. Neutrophil extracellular traps kill bacteria. *Science* 2004;303:1532–1535.
42. Wartha F, Beiter K, Normark S, Henriques-Normark B. Neutrophil extracellular traps: casting the NET over pathogenesis. *Curr Opin Microbiol* 2007;10:52–56.
43. Pilsczek FH, Salina D, Poon KK, Fahey C, Yipp BG, Sibley CD, Robbins SM, Green FH, Surette MG, Sugai M, *et al.* A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *J Immunol* 2010;185: 7413–7425.
44. Zawrotniak M, Rapala-Kozik M. Neutrophil extracellular traps (NETs)—formation and implications. *Acta Biochim Pol* 2013;60:277–284.
45. U.S. Department of Health and Human Services. The health consequences of smoking: 50 years of progress. A Report of the Surgeon General. Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health, 2014. Printed with corrections, January 2014.
46. Blackwell DL, Lucas JW, Clarke TC. Summary health statistics for U.S. adults: national health interview survey, 2012. *Vital Health Stat* 10 2014;(260):1–161.
47. Xu X, Bishop EE, Kennedy SM, Simpson SA, Pechacek TF. Annual healthcare spending attributable to cigarette smoking: an update. *Am J Prev Med* 2015;48:326–333.