Electronic Cigarette (E-Cigarette) Vapor Exposure Alters the *Streptococcus pneumoniae* Transcriptome in a Nicotine-Dependent Manner without Affecting Pneumococcal Virulence

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ABSTRACT The effects of electronic cigarette (e-cigarette) vapor (EV) exposure on the physiology of respiratory microflora are not fully defined. We analyzed the effects of exposure to vapor from nicotine-containing and nicotine-free e-liquid formulations on the virulence and transcriptome of *Streptococcus pneumoniae* strain TIGR4, a pathogen that asymptotically colonizes the human nasopharyngeal mucosa. TIGR4 was preexposed for 2 h to nicotine-containing EV extract (EVE^{+NIC}), nicotine-free EV extract (EVE^{-NIC}), cigarette smoke extract (CSE), or nutrient-rich tryptic soy (TS) broth (control). The differences between the treatment and control strains were explored using transcriptome sequencing (RNA sequencing [RNA-Seq]), *in vitro* virulence assays, and an *in vivo* mouse model of acute pneumonia. The analysis of RNA-Seq profiles revealed modest changes in the expression of 14 genes involved in sugar transport and metabolism in EVE^{-NIC}-preexposed TIGR4 compared to the control, while EVE^{+NIC} or CSE exposure altered expression of 264 and 982 genes, respectively, most of which were involved in metabolism and stress response. Infection in a mouse model of acute pneumonia with control TIGR4 or with TIGR4 preexposed to EVE^{+NIC}, EVE^{-NIC}, or CSE did not show significant differences in disease parameters, such as bacterial organ burden and respiratory cytokine response. Interestingly, TIGR4 exposed to CSE or EVE^{+NIC} (but not EVE^{-NIC}) exhibited moderate induction of biofilm formation. However, none of the treatment groups showed significant alterations in pneumococcal hydrophobicity or epithelial cell adherence. In summary, our study reports that exposure to EV significantly alters the *S. pneumoniae* transcriptome in a nicotine-dependent manner without affecting pneumococcal virulence.

IMPORTANCE With the increasing popularity of e-cigarettes among cigarette smoking and nonsmoking adults and children and the recent reports of vaping-related lung illness and deaths, further analysis of the adverse health effects of e-cigarette vapor (EV) exposure is warranted. Since pathogenic bacteria such as *Streptococcus pneumoniae* can colonize the human nasopharynx as commensals, they may be affected by exposure to bioactive chemicals in EV. Hence, in this study we examined the effects of EV exposure on the physiology of *S. pneumoniae* strain TIGR4. In order to differentiate between the effects of nicotine and nonnicotine components, we specifically compared the RNA-Seq profiles and virulence of TIGR4 exposed to vapor from nicotine-containing and nicotine-free e-liquid formulations. We observed that nicotine-containing EV augmented TIGR4 biofilms and altered expression of TIGR4 genes predominantly involved in metabolism and stress response. However, neither nicotine-containing nor nicotine-free EV affected TIGR4 virulence in a mouse model.

KEYWORDS vaping, e-cigarette, *Streptococcus pneumoniae*, biofilms, RNA-Seq, transcriptome, acute pneumonia

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The electronic cigarette (e-cigarette) is a handheld device that electronically heats an e-liquid and generates aerosolized e-cigarette vapor (EV) that is inhaled by the user. Originally, e-cigarettes were marketed as a safer alternative to smoking and as an effective smoking cessation device. Contrary to these claims, emerging research has repeatedly demonstrated the adverse health effects of EV exposure, including recent reports of lung injury and deaths associated with e-cigarette use (vaping) from most of the states in the United States; the numbers of new e-cigarette users (vapers) and cigarette smokers who also use e-cigarettes (dual users) have also increased at a rapid pace in the last decade (1, 2). Currently, the exploding popularity of vaping is threatening the success of various public health campaigns to reduce cigarette smoking. Especially worrisome is the rise in vaping among children and teenagers. In 2018, 4.9% of middle school students and 20.8% of high school students (~3.6 million total) in the United States reported vaping (3). Commercially available e-liquids typically contain three main ingredients: (i) a vehicle mixture of the humectants propylene glycol and/or vegetable glycerin, which determines vapor density and throat hit intensity; (ii) flavoring chemicals such as cinnamaldehyde, diacetyl 2,3-pentanedione, acetoin, and maltol; and (iii) nicotine in concentrations ranging from 0 to 36 mg/ml (4). The aerosolized EV generated by heating the e-liquid contains a number of respiratory irritants and toxicants, such as volatile organic compounds, acrolein, and formaldehyde (5). Emerging experimental evidence indicates that the EV chemicals are cytotoxic, increase the production of mucin, proinflammatory cytokines, and proteases, induce airway hyper-reactivity, and suppress mucociliary clearance (6, 7). These observations implicate EV exposure in the impairment of antimicrobial defenses and destruction of lung tissue. However, the effects of EV exposure on the respiratory microbiota are largely unexplored. Here, we report our research analyzing the effects of EV exposure on the pathogenesis of the Gram-positive bacterium *Streptococcus pneumoniae*, a potentially deadly pathogen that asymptotically colonizes the respiratory tract.

Streptococcus pneumoniae is the most frequent cause of pneumonia in children ≤ 5 years, adults older than 65 years, and the immunocompromised (8, 9). It is known to persist asymptotically as a commensal in the human nasopharynx for months at a time (10). Significantly higher pneumococcal carriage is observed in crowded facilities such as day care centers, schools, military bases, and jails (11). Pneumococcal colonization of the nasopharynx is a necessary precursor for pneumonia (11). Exposure to cigarette smoke (CS), another critical risk factor for pneumonia, is known to facilitate lower respiratory tract infections by affecting the development and function of both the innate and adaptive arms of the immune system. This in turn weakens respiratory immune defenses and damages the airway architecture (12–15). In the last decade, the idea that exposure to environmental irritants such as CS can affect the composition of the respiratory microbiome and the physiology of colonizing microbes has been experimentally explored. These studies have established that CS exposure, in an oxidant-dependent manner, potentiates *Staphylococcus aureus* virulence characteristics such as biofilm formation, lung epithelial adherence, hydrophobicity, and the ability to evade phagocytes and antimicrobial peptides (16–18). Using transcriptome sequencing, we have previously reported that CS exposure induces expression of staphylococcal virulence genes encoding surface adhesins and effectors involved in immune evasion (18). Importantly, CS-exposed staphylococci with augmented virulence have been shown to induce a higher pulmonary bacterial burden and increased mortality in mouse models of acute pneumonia (17, 18). In *S. pneumoniae*, the effects of CS exposure on virulence are limited to the induction of biofilm formation and significant attenuation of the activity of the pore-forming toxin pneumolysin gene (*ply*) (19, 20). At the transcriptomic level, *in vitro*, acute CS exposure is reported to alter the expression of genes encoding factors required primarily for pneumococcal stress response and survival, and to significantly downregulate *ply* expression without affecting the expression of other virulence genes (20, 21). To date, the pathogenesis of CS-exposed pneumococci has not been examined in a mouse model of respiratory tract infection.

Few studies have explored the effects of EV exposure on the physiology of pathogens colonizing the upper respiratory mucosa. Exposure to EV was shown to induce *S.*

aureus biofilm formation, hydrophobicity, and adherence to epithelial cells (22). In another study, EV exposure was shown to facilitate pneumococcal host epithelial adherence via significant upregulation of platelet-activating factor receptor (PAFR) in nasal epithelial cells from vapers, as well as in EV-exposed A549 lung carcinoma cells (23). Notably, the effects of EV exposure on the transcriptome and the pathogenesis of *S. pneumoniae* are largely undefined. Whether the presence of nicotine in e-liquid significantly affects the pneumococcal physiology has also not been explored. To fill these major gaps in our understanding of the effects of vaping on nasopharyngeal colonizers, we exposed the model organism *S. pneumoniae* strain TIGR4 to EV extract (EVE) (generated by bubbling EV into tryptic soy [TS] broth) from e-liquids that either contained nicotine (EVE^{+NIC}) or were nicotine free (EVE^{-NIC}), to CS extract (CSE) (generated by bubbling CS into TS broth [16]), or to TS broth alone. This was followed by the comparison of the effects of nicotine-containing and nicotine-free EV on the transcriptome and pathogenesis of *S. pneumoniae* using transcriptome sequencing and a mouse model of acute pneumonia.

RESULTS

RNA-Seq and read mapping. RNA from three independent biological replicates for control and three test conditions was used to generate 12 libraries by Illumina transcriptome sequencing (RNA sequencing [RNA-Seq]), which resulted in an average of approximately 32 million 150-bp paired-end reads (31 to 35 million) for each library. After adapter trimming and quality filtering, we retained an average of 91% of reads (90.3 to 91.8%) per library. Subsequently, an average of 30 million reads (28 to 32 million) per library was successfully mapped to the *Streptococcus pneumoniae* TIGR4 reference genome (GenBank accession no. [AE005672.3](#)), with an average of only 1.5% of reads (0.5 to 2.2%) mapping to ribosomal genes. The libraries had an average estimated depth of coverage of 4,216-fold (3,967- to 4,478-fold), with each library having 10 or more reads mapping to a minimum of 2,211 genes in the *Streptococcus pneumoniae* TIGR4 reference genome (2,292 genes total). For comparisons of TS-TIGR4 versus CSE-TIGR4 and TS-TIGR4 versus EVE^{+NIC}-TIGR4, Euclidean distances between samples and principal-component analyses revealed that expression patterns in biological replicates of each treatment group were more similar to each other than they were to those in biological replicates of the contrasting treatment group (data not shown). This was not the case for TS-TIGR4 versus EVE^{-NIC}-TIGR4, which is expected as this comparison revealed very few differentially expressed genes (DEGs) (data not shown).

Comparative analysis of differential gene expression among CSE-TIGR4, EVE^{+NIC}-TIGR4, EVE^{-NIC}-TIGR4, and TS-TIGR4. We hypothesized that e-cigarette vapor chemicals, especially nicotine, would affect the global transcriptome of TIGR4 with significant changes (absolute log₂ fold change [log₂FC] of ≥1 and adjusted *P* value [*P*_{adj}] of ≤0.05) in the expression of virulence genes. In comparison to TS-TIGR4, we detected 188 upregulated and 76 downregulated genes (a total of 264 DEGs) in CSE-TIGR4 and 500 upregulated and 482 downregulated genes (a total of 982 DEGs) in EVE^{+NIC}-TIGR4 (Fig. 1). Notably, EVE^{-NIC} exposure had a minimal effect on the TIGR4 transcriptome relative to the CSE-TIGR4 and EVE^{+NIC}-TIGR4 treatments. We detected only 14 modestly (log₂FC ≈ 1.5) upregulated genes in EVE^{-NIC}-TIGR4 relative to the TS-TIGR4 control treatment, and no genes were downregulated (Fig. 1). Tables S1, S2, and S3 in the supplemental material contain the complete lists of all differentially expressed genes for all comparisons. A description of the RNA-Seq results for specific genes that are known to play a role in pneumococcal virulence is provided below.

The pneumococcal two-component systems (TCS) constitute a phosphorelay between the membrane-bound sensory histidine kinase and cytoplasmic response regulator. The histidine kinase senses environmental stimuli and phosphorylates the response regulator, which in turn modulates transcription of virulence and stress response genes (24). Of the 13 annotated TCS in TIGR4, genes encoding LiaS/R

FIG 1 Comparison of differentially expressed genes among different treatments. The numbers of statistically significantly differentially expressed genes are shown for three comparisons: TS-TIGR4 control versus CSE-TIGR4, TS-TIGR4 control versus EVE⁺NIC-TIGR4, and TS-TIGR4 control versus EVE⁻NIC-TIGR4. The results represent analysis of RNA-Seq data from three independent biological replicates. Differential expression was assessed using a threshold of $\log_2$ FC of ≥ 1 and P_{adj} of ≤ 0.05 . Bold numbers indicate the number of genes upregulated in the noncontrol treatment, whereas nonbold numbers indicate the number of genes downregulated in the noncontrol treatment.

(SP_0386/SP_0387) were significantly upregulated in both EVE⁺NIC-TIGR4 and CSE-TIGR4 (Table 1). LiaS/R is crucial for biofilm formation and survival in acidic pH in *Streptococcus mutans* (25) by regulating the expression of the *hrcA-grpE-dnaK-dnaJ* (SP_0515 to SP_0519) operon, which encodes heat shock proteins, all of which were significantly upregulated in CSE-TIGR4 and EVE⁺NIC-TIGR4 but not in EVE⁻NIC-TIGR4 (Table 1). Another TCS, CiaR/H (SP_0798/SP_0799), and its downstream effector *htrA* (SP_2239, Table 2) were upregulated only in CSE-TIGR4. CiaR/H is involved in the oxidative stress response by inducing expression of the HtrA serine protease and by maintaining pneumococcal integrity by regulating genes encoding surface proteins, transport systems, and cell envelope-modifying enzymes (26, 27). We did not observe upregulation of genes encoding TCS11 (SP_2000/SP_2001) in CSE-TIGR4 or in EVE⁺NIC-TIGR4 (Table 1). These results contradict previous observations that CS exposure upregulates transcription of TCS11 genes in pneumococcal serotypes 19F and 23F, indicating the strain-dependent nature of these effects (20, 21).

Oxidative stress induced by free radicals and reactive oxygen species (ROS) (such as O_2^- , NO, OH^- , and H_2O_2) contained in CS is thought to be an important mediator of physiological changes in the human host and in pathogens (16, 28). In CSE-TIGR4 and EVE⁺NIC-TIGR4, we did not observe increased expression of genes encoding superoxide dismutase (*sodA*, SP_0766), thiol peroxidase (*psaD*, SP_1651), and NADH oxidase (*nox*, SP_1469), which form the first line of defense against oxidative stress (29–31). The treatments differed in their upregulation of nonenzymatic proteins involved in the oxidative stress response; non-heme-containing ferritin (*dpr*, SP_1572) was upregulated only in CSE-TIGR4, whereas the glutathione (GSH) transporter (SP_1550) and glutathione reductase (*gor*, SP_0784) were upregulated only in EVE⁺NIC-TIGR4. The *dpr* knock-out mutants of pneumococci are more sensitive to a variety of environmental stresses, including oxidative stress by hydrogen peroxide, and are defective in nasopharyngeal colonization in a mouse model (32). The glutathione transporter (SP_1550) and glutathione reductase (*gor*, SP_0784) regulate glutathione uptake and oxidation to protect pneumococci from damage caused by ROS, reactive nitrogen species (RNS), and divalent metal ions (33).

The highly conserved Clp proteases are involved in ATP-dependent proteolysis of misfolded proteins. Clp proteases have a two-component architecture made of ATPase-activated chaperone and protease subunits (34). The ClpCP chaperone-protease plays a role in TIGR4 thermotolerance, oxidative stress tolerance, and virulence by regulating expression of genes encoding choline-binding protein (CBP) surface adhesins (*cbp*) and pneumolysin (*ply*) (35, 36). We observed upregulation of ClpP (SP_0746) and ClpC

TABLE 1 RNA-Seq results for genes encoding components of two-component systems of signal transduction

TCS ^a	Gene name	Gene ID	Log ₂ FC ^b			GenBank definition
			CSE-TIGR4	EVE ⁺ NIC-TIGR4	EVE ⁻ NIC-TIGR4	
TCS08	<i>saeR</i>	SP_0083	-0.39	-0.21	0.15	DNA-binding response regulator Sensor histidine kinase
	<i>saeS</i>	SP_0084	-0.19	-0.22	0.18	
TCS07	<i>yesN</i>	SP_0156	0.41	-0.11	-0.02	DNA-binding response regulator Putative sensor histidine kinase
	<i>yesM</i>	SP_0155	1.02*	0.88	0.06	
Orphan		SP_0376	0.02	0.17	-0.03	DNA-binding response regulator
TCS03	<i>liaR</i>	SP_0387	2.18*	2.62*	-0.13	DNA-binding response regulator Putative sensor histidine kinase
	<i>liaS</i>	SP_0386	2.36*	3.25*	-0.10	
TCS13	<i>blpR</i>	SP_0526	-0.09	-0.20	0.16	Response regulator BlpR Putative sensor histidine kinase BlpH
	<i>blpH</i>	SP_0527	0.01	-0.12	0.10	
TCS10	<i>vncR</i>	SP_0603	0.27	-0.33	-0.14	DNA-binding response regulator VncR Sensor histidine kinase VncS
	<i>vncS</i>	SP_0604	0.22	-0.53	-0.05	
TCS09	<i>zmpR</i>	SP_0661	0.05	-0.60	-0.15	DNA-binding response regulator Putative sensor histidine kinase
	<i>zmpS</i>	SP_0662	0.11	-0.98	-0.08	
TCS05	<i>ciaR</i>	SP_0798	2.43*	0.05	-0.43	DNA-binding response regulator CiaR Sensor histidine kinase CiaH
	<i>ciaH</i>	SP_0799	2.53*	-0.16	-0.48	
TCS02	<i>vicR</i>	SP_1227	0.26	-0.02	0.13	DNA-binding response regulator Sensory box sensor histidine kinase
	<i>vicK</i>	SP_1226	0.30	0.12	0.13	
TCS01		SP_1633	-0.23	0.13	-0.04	DNA-binding response regulator Sensor histidine kinase
		SP_1632	-0.03	-0.38	0.02	
TCS11	<i>desR</i>	SP_2000	-0.03	-0.83	0.01	DNA-binding response regulator Putative sensor histidine kinase
	<i>desK</i>	SP_2001	-0.07	-0.61	0.02	
TCS04	<i>pnpR</i>	SP_2082	-0.12	-0.36	0.08	Response regulator Sensor histidine kinase PnpS
	<i>pnpS</i>	SP_2083	-0.22	-0.74	0.06	
TCS06	<i>cbpR</i>	SP_2192	0.30	0.50	-0.09	Sensor histidine kinase DNA-binding response regulator
	<i>cbpS</i>	SP_2193	0.40	1.10*	-0.02	
TCS12	<i>comE</i>	SP_2235	-0.11	0.07	-0.02	Response regulator ComE Putative sensor histidine kinase ComD
	<i>comD</i>	SP_2236	0.13	0.95	-0.04	

^aFor each TCS, the gene encoding the DNA response regulator is listed first, followed by the gene encoding the histidine kinase.

^b*, statistically significant ($P_{\text{adj}} < 0.05$). Positive values indicate greater expression in the noncontrol treatment, and negative values indicate greater expression in the control treatment.

(SP_2194) only in EVE⁺NIC-TIGR4, whereas ClpL (SP_0338) and ClpE (SP_0820) were upregulated in both EVE⁺NIC-TIGR4 and CSE-TIGR4.

Expression of PsaR (SP_1638), a transcriptional regulator that plays a role in cation (Mn²⁺ and Zn²⁺) homeostasis and TIGR4 virulence (37, 38), was unchanged in all treatment groups. PsaR suppresses expression of the *psa* operon (*psaABC*, SP_1650/SP_1649/SP_1648) (which encodes the manganese ABC transporter *rlrA* pilus islet [SP_0461 to SP_0468]), *prtA* serine protease (SP_0641), MerR family transcriptional regulator (SP_1856), and *czcD* (SP_1857) (encoding the Zn²⁺ efflux system). While *psaR* expression was unaffected in all treatment groups, the *psa* operon and *prtA* were significantly downregulated in CSE-TIGR4 and EVE⁺NIC-TIGR4. SP_1856 and *czcD* were upregulated in CSE-TIGR4 and EVE⁺NIC-TIGR4. None of the treatments significantly altered the expression of the *rlrA* pilus islet.

TIGR4 expresses many surface-anchored proteins that facilitate nasopharyngeal colonization and virulence by interfering with immune responses and by augmenting pneumococcal adherence to host cells and extracellular matrix proteins (39, 40). Of these, choline-binding protein (CBP) genes *lytA* (cell wall hydrolytic autolysin, SP_1937)

TABLE 2 RNA-Seq results for stress response genes

Protein category and gene name	Gene ID	Log ₂ FC ^a			GenBank definition
		CSE-TIGR4	EVE ^{+NIC} -TIGR4	EVE ^{-NIC} -TIGR4	
Heat shock proteins					
<i>clpL</i>	SP_0338	2.10*	6.04*	0.24	Putative ATP-dependent Clp protease, ATP-binding subunit
<i>hrcA</i>	SP_0515	1.22*	5.60*	0.16	Heat-inducible transcription repressor HrcA
<i>grpE</i>	SP_0516	1.48*	3.89*	0.14	Heat shock protein GrpE
<i>dnaK</i>	SP_0517	1.99*	3.63*	−0.09	DnaK protein
<i>dnaJ</i>	SP_0519	1.63*	3.44*	−0.08	DnaJ protein
<i>clpP</i>	SP_0746	0.73	1.09*	−0.14	ATP-dependent Clp protease, proteolytic subunit
<i>groEL</i>	SP_1906	0.74	3.63*	0.04	Chaperonin, 60 kDa
<i>groES</i>	SP_1907	0.29	3.11*	0.12	Chaperonin, 10 kDa
<i>htrA</i>	SP_2239	1.83*	−1.94*	−0.79	Serine protease
Oxidative stress proteins					
<i>sodA</i>	SP_0766	0.47	−0.07	−0.03	Superoxide dismutase, manganese dependent
<i>gor</i>	SP_0784	0.36	1.11*	−0.21	Glutathione reductase
<i>gshT</i>	SP_1550	0.27	2.48*	0.2	Glutathione S-transferase family protein
<i>dpr</i>	SP_1572	1.09*	−0.38	−0.24	Non-heme-iron-containing ferritin
<i>psaB</i>	SP_1648	−1.92*	−2.13*	−0.13	Manganese ABC transporter, ATP-binding protein
<i>psaC</i>	SP_1649	−1.91*	−1.77*	−0.08	Putative manganese ABC transporter, permease protein
<i>psaA</i>	SP_1650	−1.92*	−1.24*	−0.14	Manganese ABC transporter, manganese-binding adhesion lipoprotein
<i>psaD</i>	SP_1651	0.43	0.08	−0.15	Thiol peroxidase
<i>nox</i>	SP_1469	−0.18	−0.55	−0.04	NADH oxidase

^a*, statistically significant ($P_{\text{adj}} < 0.05$). Positive values indicate greater expression in the noncontrol treatment, and negative values indicate greater expression in the control treatment.

and *cbpD* (murein hydrolase, SP_2201) were upregulated in EVE^{+NIC}-TIGR4 (Table 3), whereas *cbpF* (SP_0391) and *cbpG* (SP_0390) were upregulated in both CSE-TIGR4 and EVE^{+NIC}-TIGR4 (Tables S1 and S2). CbpD and LytA release cytoplasmic virulence factors that help pneumococci interfere with the host immune responses (41, 42). CbpD is also involved in competence-associated fratricide of noncompetent pneumococci, which facilitates interbacterial gene exchange. In response to the cell wall damage by CbpD and LytA, LiaS/R TCS is activated in both CSE-TIGR4 and EVE^{+NIC}-TIGR4, as previously reported (43).

EVE^{+NIC}-TIGR4 showed significant upregulation of *celA* (SP_0954) and the *cgl* operon (SP_2050 to SP_2053), whereas *coiA* (SP_0978) was upregulated in both EVE^{+NIC}-TIGR4 and CSE-TIGR4 (Tables S1 and S2). The activity of *celA*, the *cgl* operon, and *coiA* is linked to the competence and natural transformability of pneumococci (44). We did not observe any alterations in the expression of the *comAB* or *comCDE* operons in the different treatment groups. The expression of genes encoding other surface-anchored virulence factors, such as *eno* (SP_1128) and *pavA* (SP_0966), was significantly downregulated in CSE-TIGR4 and EVE^{+NIC}-TIGR4 as judged by quantitative reverse transcription-PCR (qRT-PCR) (Table 3).

Both CSE-TIGR4 and EVE^{+NIC}-TIGR4 also exhibited altered expression of genes involved in carbon uptake and metabolism (phosphotransferase systems [PTS]), the pneumococcal stress response (Clp proteases, *hrcA*/SP_0515), and transcriptional regulators (*mgrA*/SP_1800, *marR*/SP_1863, and *merR*/SP_1856) (Table 2). Among pneumococcal virulence genes, we noted downregulation of *ply* (encoding pore-forming pneumolysin, SP_1923) in CSE-TIGR4, upregulation of *lytA* (cell wall hydrolytic autolysin, SP_1937) in EVE^{+NIC}-TIGR4, and downregulation of *prtA* (surface serine protease, SP_0641) in both CSE-TIGR4 and EVE^{+NIC}-TIGR4 (Table 3). All differentially expressed genes in EVE^{-NIC}-TIGR4 were modestly ($\log_2\text{FC} \approx 1.5$) upregulated and were those involved in sugar uptake and catabolism (Table S3).

Next, we validated our RNA-Seq results by qRT-PCR analysis of the gene expression. The panel of genes analyzed by qRT-PCR included those encoding virulence factors, transcriptional regulators, and surface proteins involved in stress response and adhesion (Table 3). The relative-quantification (RQ) values from TS-TIGR4 controls were set

TABLE 3 RNA-Seq results for important virulence genes and results from qRT-PCR analysis for the validation of RNA-Seq results

Protein category and gene name	Gene ID	CSE-TIGR4		EVE ^{+NIC} -TIGR4		EVE ^{-NIC} -TIGR4		GenBank definition
		Log ₂ FC ^a	RQ ^b	Log ₂ FC	RQ	Log ₂ FC	RQ	
Virulence proteins								
<i>zmpC</i>	SP_0071	−0.21	ND	−0.16	ND	−0.18	ND	Zinc metalloprotease ZmpC
<i>pspA</i>	SP_0117	−0.19	ND	0.91	ND	−0.24	ND	Pneumococcal surface protein A
<i>pbpX</i>	SP_0336	0.00	ND	0.71	ND	0.08	ND	Penicillin-binding protein 2X
<i>cps4A</i>	SP_0346	−0.46	−1.55*	0.40	−0.95	0.12	−0.97	Capsular polysaccharide biosynthesis protein Cps4A
<i>prtA</i>	SP_0641	−1.27*	ND	−2.22*	ND	−0.31	ND	Serine protease, subtilase family
<i>zmpB</i>	SP_0664	0.09	ND	−0.47	ND	−0.08	ND	Zinc metalloprotease ZmpB
<i>sodA</i>	SP_0766	0.47	ND	−0.07	ND	−0.03	ND	Superoxide dismutase, manganese-dependent
<i>lytB</i>	SP_0965	−0.76	ND	−0.14	ND	−0.35	ND	Endo-beta- <i>N</i> -acetylglucosaminidase
<i>pavA</i>	SP_0966	−0.22	−1.10*	0.08	−1.01*	−0.24	−0.96	Adherence and virulence protein A
<i>zmpA</i>	SP_1154	0.15	−0.92	−0.08	−1.97*	−0.18	−0.67	Immunoglobulin A1 protease
<i>nox</i>	SP_1469	−0.18	ND	−0.55	ND	−0.04	ND	NADH oxidase
<i>lytC</i>	SP_1573	0.21	−0.74	−0.18	−0.61	0.09	0.89	Lysozyme
<i>ddlA</i>	SP_1671	−0.12	ND	−0.34	ND	−0.18	ND	D-Alanine-D-alanine ligase
<i>nanB</i>	SP_1687	0.41	−0.31	−0.68	−3.17*	0.12	−0.60	Neuraminidase B
<i>nanA</i>	SP_1693	1.09*	2.50*	0.34	0.28	0.30	1.11*	Neuraminidase A
<i>plyA</i>	SP_1923	−1.32*	−2.12*	0.31	−0.67	0.01	−0.52	Pneumolysin
<i>lytA</i>	SP_1937	−0.01	−0.44	1.24*	0.45	−0.02	−0.07	Autolysin
Surface proteins								
<i>rrgB</i>	SP_0463	−0.90	−2.09*	−0.34	−1.93*	0.11	−0.45	Cell wall surface anchor family protein
<i>eno</i>	SP_1128	−0.02	−1.16*	−0.41	−2.12*	−0.33	−1.08*	Enolase
<i>psrP</i>	SP_1772	−0.37	−1.07*	−0.94	−3.46*	−0.12	0.35	Cell wall surface anchor family protein
<i>cbpA</i>	SP_2190	0.07	−0.19	−0.47	−1.79*	−0.22	−0.45	Choline-binding protein A
<i>cbpD</i>	SP_2201	0.26	−0.12	1.41*	0.33	−0.08	−0.12	Choline-binding protein D
Regulators								
<i>luxS</i>	SP_0340	0.13	−0.89	0.31	−1.17*	−0.11	−0.83	Autoinducer-2 production protein
<i>codY</i>	SP_1584	−0.25	−1.27*	0.34	−1.11*	0.23	0.00	GTP-sensing transcriptional pleiotropic repressor CodY
<i>mgrA</i>	SP_1800	−1.13*	−1.60*	−2.53*	−4.10*	0.03	−0.47	Putative transcriptional activator
<i>marR</i>	SP_1863	2.10*	1.29*	3.06*	1.92*	0.27	−0.17	Transcriptional regulator, MarR family
<i>comD</i>	SP_2236	0.13	−0.58	0.95	−0.75	−0.04	−1.15	Putative sensor histidine kinase ComD
Membrane transport proteins								
Glyoxalase gene	SP_0073	4.18*	4.82*	8.01*	7.64*	0.69	0.57	Conserved hypothetical protein
<i>psaA</i>	SP_1650	−1.92*	−3.11*	−1.24*	−3.42*	−0.14	−0.51	Manganese ABC transporter, manganese-binding adhesion lipoprotein

^a*, statistically significant ($P_{\text{adj}} < 0.05$). Positive values indicate increased expression in the noncontrol treatment, and negative values indicate greater expression in the control treatment.

^b*, statistically significant ($P < 0.05$ by unpaired *t* test). Positive values indicate increased expression in the noncontrol treatment, and negative values indicate greater expression in the control treatment. ND, not done.

to 1. The differential regulation for a majority of genes in the panel was found to be similar by both RNA-Seq and qRT-PCR (Table 3). Of note, we detected significant upregulation of glyoxalase (SP_0073) and *marR* (SP_2062) and significant downregulation of *mgrA* (SP_1800) and *psaA* (SP_1650) in CSE-TIGR4 and EVE^{+NIC}-TIGR4. The zinc transporter-encoding *czcD* (SP_1857) was significantly upregulated only in EVE^{+NIC}-TIGR4 and not in CSE-TIGR4, while *ply* (SP_1923) was significantly downregulated in CSE-TIGR4 but not in EVE^{+NIC}-TIGR4. Similar to our RNA-Seq observations, CSE^{-NIC}-TIGR4 exhibited minimum alteration in the expression of genes tested by qRT-PCR.

EV exposure activates TIGR4 biofilm formation. We tested the effects of CSE, EVE^{+NIC}, and EVE^{-NIC} exposure on virulence characteristics of *S. pneumoniae* strain

FIG 2 Effects of CSE and EVE exposure on TIGR4 virulence. TS-TIGR4 (control), CSE-TIGR4, EVE^{+NIC}-TIGR4, and EVE^{-NIC}-TIGR4 were assayed for biofilm formation (A), adherence to A549 cells (B), hydrophobicity (C), and H_2O_2 sensitivity (D). Biofilm formation was also assayed after exposure to 2 mg/ml nicotine (A). For panels A, C, and D, the plots show a representative result from ≥ 3 biological replicates, with >3 technical replicates for each. All data are presented as the average $\pm$ standard deviation. For panel B, adherence index values represent the average $\pm$ standard deviation from 8 experiments. The data were analyzed by one-way ANOVA followed by Dunnett's multiple-comparison test. Statistical significance compared to TS-TIGR4 is shown as follows: *, $P < 0.05$; NS, not significant. Statistical significance compared to CSE-TIGR4 is shown in panel A as * ($P < 0.05$). Not-significant differences compared to CSE-TIGR4 are not shown.

TIGR4, such as hydrophobicity, epithelial adherence, and biofilm formation. We observed significant upregulation of biofilm formation in EVE^{+NIC}-TIGR4 and CSE-TIGR4 (Fig. 2A) but not in EVE^{-NIC}-TIGR4 (Fig. 2A). These results suggest the involvement of nicotine in the induction of pneumococcal biofilms, which was further supported by our observation that pretreatment with 2 mg/ml nicotine also significantly increased TIGR4 biofilm formation compared to that of TS-TIGR4 (Fig. 2A). CSE exposure has been previously shown to induce pneumococcal biofilms. (19).

In contrast to its effects on biofilm formation, exposure to EVE^{+NIC}, EVE^{-NIC}, or CSE did not significantly alter the ability of TIGR4 to adhere to human lung epithelial cell line A549 (Fig. 2B), hydrophobicity (Fig. 2C), or pneumococcal sensitivity to hydrogen peroxide (Fig. 2D). This is similar to the previous report by Manna et al. demonstrating that 45 min of CSE exposure does not significantly alter TIGR4 hydrophobicity or adherence to A549 (21).

EV exposure does not affect TIGR4 virulence in a mouse model of acute pneumonia.

To define the effects of acute EV exposure on pneumococcal pathogenesis, we infected four cohorts of anesthetized C57BL6 mice with 5×10^7 CFU of CSE-TIGR4, EVE^{+NIC}-TIGR4, EVE^{-NIC}-TIGR4, or TS-TIGR4 resuspended in 50 μ l phosphate-buffered saline (PBS). Prior to infection, all bacterial cultures were washed twice in sterile PBS to minimize the carryover of EVE or CSE chemicals into the mouse respiratory tract, as previously described (18). Anesthetization of mice results in the aspiration of bacterial suspension into the lungs, inducing acute pneumonia (45). At 18 h postinfection, we compared organ burden and cytokine expression in the respiratory tracts of mice from four different cohorts that were infected with TS-TIGR4 controls, EVE^{+NIC}-TIGR4, EVE^{-NIC}-TIGR4, or CSE-TIGR4. We did not observe significant differences in CFU recovered from the lower respiratory tract (lung and bronchoalveolar lavage fluid [BALF]) (Fig. 3C and D) or from the upper respiratory tract (nasal septum and nasal lavage) (Fig. 3A and B) among these groups. Moreover, we detected among these groups similar levels of total protein in BALF (Fig. 4A) and of the cytokines interleukin-6 (IL-6) and interferon- γ (IFN- γ) in homogenized lung tissue as measured by enzyme-linked immunosorbent assay (ELISA) (Fig. 4B and C). We also did not observe differences in the transcript levels of tumor necrosis factor alpha (TNF- α), IL-6, IFN- γ , CCL2, and CXCL10 in the lung tissues obtained from these four mouse groups (qRT-PCR

FIG 3 Analysis of the virulence of TIGR4 preexposed to CSE or EVE in a mouse model. C57BL6 mice were intranasally inoculated with 5×10^7 CFU of TS-TIGR4 (control), CSE-TIGR4, EVE^{+NIC}-TIGR4 (C), or EVE^{-NIC}-TIGR4. The bacterial burden in the nasal lavage (A), nasal septum (B), lung (C), and bronchoalveolar lavage fluid (D) was determined at 18 h postinfection. The scatter plots depict CFU/ml recovered from each mouse and median values. Statistical significance compared to values for the TS-TIGR4-infected mouse cohort, determined by the Mann-Whitney U statistic, is shown as NS (not significant).

results not shown). Overall, these results indicate that changes in TIGR4 gene expression profiles caused by a 2-h-long preexposure to EVE^{+NIC}, EVE^{-NIC}, or CSE do not significantly affect the virulence of TIGR4 in a mouse model of acute pneumonia.

DISCUSSION

According to the Centers for Disease Control and Prevention, approximately 50,000 deaths in 2017 were attributed to pneumonia, making it a leading cause of infection-related deaths in the United States (46). Exposure to cigarette smoke is a key risk factor for pneumonia because it affects the physiology and immune responses of the respiratory tract and augments the virulence of pathogens colonizing the nasopharyngeal mucosa (12–21). In contrast, the effects of e-cigarette use on the composition and physiology of the nasopharyngeal microflora are relatively unknown. The major objective of our research project was to evaluate the effects of e-cigarette vapor (EV) exposure on the physiology of the respiratory pathogen *S. pneumoniae* strain TIGR4. We analyzed the effects of exposure to nicotine-containing and nicotine-free EV on the TIGR4 transcriptome using RNA sequencing and those on TIGR4 virulence using *in vitro* assays and an *in vivo* mouse model of acute pneumonia.

Due to the commercial availability of thousands of premixed e-liquid flavors with various nicotine concentrations and the advent of custom e-liquid kits, which allow the users to mix chemicals according to their preference, selecting a representative e-liquid formulation for experimental analysis is a major challenge (47). Our choice of

FIG 4 Analysis of the cytokine and chemokine expression in mice infected with TIGR4 preexposed to CSE or EVE. C57BL6 mice were intranasally inoculated with 5×10^7 CFU of TS-TIGR4 (control), CSE-TIGR4, EVE^{+NIC}-TIGR4, or EVE^{-NIC}-TIGR4. At 18 h postinfection, total protein in BALF (A) and cytokines in homogenized lungs tissues (determined by ELISA) (B and C) were analyzed. The histograms show average $\pm$ standard deviation and were analyzed by one-way ANOVA followed by Dunnett's multiple-comparison test. Statistical significance compared to values for TS-TIGR4 is shown as NS (not significant).

strawberry-flavored e-liquid for this project was informed by its highest cytotoxicity among tested flavors (48). To distinguish between the effects of nicotine and nonnicotine components in e-liquid, we selected the same commercial brand of strawberry-flavored e-liquid either with or without 3 mg/ml nicotine. We separately exposed TIGR4 for 2 h to EVE generated by heating strawberry-flavored e-liquid either with or without nicotine (referred to as EVE^{+NIC}-TIGR4 and EVE^{-NIC}-TIGR4, respectively). Because e-cigarettes are touted as a safe alternative for cigarette smoking and even as smoking cessation devices, we compared the transcriptome profiles and virulence of EVE^{+NIC}-TIGR4 and EVE^{-NIC}-TIGR4 with those of TIGR4 preexposed to CSE for 2 h (CSE-TIGR4). TIGR4 cultures maintained in parallel in TS broth (TS-TIGR4) were used as the control. It is important to emphasize that for all experiments, bacteria preexposed to various treatments were washed twice in sterile PBS. This washing step minimizes the carryover and subsequent confounding effects of the chemicals from EVE^{+NIC}, EVE^{-NIC}, or CSE on human cells or a mouse model of acute pneumonia, as previously described (18). Comparison of transcriptome profiles of the treatments with that of control TS-TIGR4 informed our understanding of the effects of EV with or without nicotine and CS on the expression of genes involved in pneumococcal survival, stress response, and virulence. The transcriptome profiling of CSE-TIGR4 served as a control, based on the results published by Manna et al. (21). Importantly, we also compared the virulence of EVE^{+NIC}-TIGR4, EVE^{-NIC}-TIGR4, CSE-TIGR4, and TS-TIGR4 by monitoring disease progression in a mouse model of acute pneumonia as well as using *in vitro* assays of virulence characteristics.

Compared to previous studies analyzing the effects of CS exposure on the pneumococcal transcriptome, the main discrepancy observed was in the expression of genes encoding TCS11. Previous studies have reported that exposure to CS induces upregulation of TCS11 (SP_2000, SP_2001) expression in two different pneumococcal strains (serotypes 19F and 23F) and that TCS11 activity plays a role in pneumococcal biofilm formation but not in virulence in a mouse model (20, 21, 49). However, we did not detect significant changes in the expression of TCS11 genes in any of our treatment groups. The discrepancy between our results for CSE-TIGR4 and those previous publications may be attributed to differences in pneumococcal strains, exposure time, and/or cigarette brand. Here, we exposed TIGR4 (serotype 4) for 2 h to CSE generated by burning Marlboro cigarettes. In contrast, Manna et al. exposed *S. pneumoniae* strain EF3030 (serotype 19F) for 45 min to CSE from research-grade cigarettes, whereas Cockeran et al. exposed strain 172 (serotype 23F) to 160 μ g/ml cigarette smoke condensate (20, 21). Since biofilm formation was enhanced in both CSE-TIGR4 and EVE^{+NIC}-TIGR4, we can also argue that CSE and EVE^{+NIC} can enhance TIGR4 biofilms in a TCS11-independent manner.

Our differential gene expression analyses reveal that several genes encoding pneumococcal virulence effectors are upregulated in CSE-TIGR4 and EVE^{+NIC}-TIGR4. Notably, virulence gene expression in TIGR4 is unaffected by exposure to EVE^{-NIC}. This led us to evaluate the effects of exposure to EV with or without nicotine or CSE on TIGR4 pathogenesis using *in vitro* assays and an *in vivo* mouse model of acute pneumonia. We observed a modest but consistent augmentation of biofilm formation by EVE^{+NIC}-TIGR4 but not by EVE^{-NIC}-TIGR4, suggesting a role for nicotine in this phenomenon. The role of nicotine in biofilm augmentation was further confirmed as the pretreatment of TIGR4 with 2 mg/ml nicotine also enhanced TIGR4 biofilm. We also observed increased biofilm formation by CSE-TIGR4, as previously reported (19, 20). Multiple studies have established that biofilm-bound pneumococci exhibit downregulation of the virulence genes *ply*, *pavA*, *pspA* (pneumococcal surface protein, SP_0117), and *licD2* (SP_1273, opaque phenotype), as well as the upregulation of competence genes such as the transcriptional regulator gene *mgrA* (50, 51). We observed downregulation of *ply* and *pavA* in CSE-TIGR4 and EVE^{+NIC}-TIGR4, but *licD2* was downregulated only in EVE^{+NIC}-TIGR4. Interestingly, expression of *pspA* and *comD* was affected, and *mgrA* was downregulated.

Compared to those in TS-TIGR4, significant changes in other virulence characteristics, such as epithelial adherence, hydrophobicity, and H₂O₂ sensitivity, were not

observed in CSE-TIGR4, EVE^{+NIC} -TIGR4, and EVE^{-NIC} -TIGR4. Most importantly, at 24 h postinfection in a mouse model of acute pneumonia, we did not observe differences in the CFU recovered from the nasal cavity or the lungs. Similarly, we did not observe differences in the cytokine expression levels among animals infected with CSE-TIGR4, EVE^{+NIC} -TIGR4, EVE^{-NIC} -TIGR4, and the TS-TIGR4 control.

Using a variety of genomic and microbiological tools, such as comparative analysis of transcriptome profiles, *in vitro* assays, and a mouse model of acute pneumonia, a number of studies have established that CS exposure augments the virulence of Gram-positive methicillin-resistant *S. aureus* (MRSA) (16–18). In contrast to these results, our differential gene expression analyses of CS-exposed *S. pneumoniae* show induction of pathways required predominantly for stress response, detoxification, and survival, with only minor effects on the expression of a small number of virulence genes. Consistent with previous studies, we also observed CS-mediated induction of pneumococcal biofilm formation, suppression of pneumolysin production, and altered expression of genes predominantly involved in pneumococcal survival and stress response (19–21). Acute (2-h-long) exposure to EVE^{+NIC} augments pneumococcal biofilm formation, whereas exposure to EVE^{-NIC} does not. Moreover, compared to CSE exposure, EVE^{+NIC} exposure caused more widespread alterations in TIGR4 transcriptome-wide gene expression, although a majority of differentially expressed genes in EVE^{+NIC} -TIGR4 were those involved in pneumococcal survival and stress response. In a mouse model of acute pneumonia, compared to TS-TIGR4, neither EVE^{+NIC} -TIGR4 nor CSE-TIGR4 showed any differences in pathogenesis. Most notably, in contrast to the case for CSE and EVE^{+NIC} , the chemicals in EVE^{-NIC} appear to have a mild effect on the TIGR4 transcriptome (resulting in a modest upregulation of expression of 14 genes). EVE^{-NIC} appears to have no effect on TIGR4 virulence in a mouse model.

In interpreting the physiological relevance of our observations, we must emphasize that they provide only snapshot focused on 2-h-long exposure of pneumococcus to EV from strawberry-flavored e-liquid with or without 3 mg/ml nicotine. To avoid exposing the murine host to EV chemicals, after preexposure to EVE or CSE, pneumococci were washed in sterile PBS before inoculation in the mouse model. This protocol allowed us to specifically track EV-mediated alterations solely in bacterial physiology, without its confounding effects on the host. However, this also constitutes an important limitation of our study in that it does not address the real-life scenario wherein e-cigarette vapor would affect the complex interplay between bacterial factors and host immune defenses in the nasopharyngeal milieu. In the future, it will be of tremendous public health importance to define the effects of chronic exposure to many flavors of e-liquid with various concentrations of nicotine on the host physiology and bacterial virulence. Given the rapidly increasing popularity of vaping, a better understanding of the effects of exposure to EV chemicals on the host and the colonizing microbiome is urgent.

MATERIALS AND METHODS

Bacterial strain, cell lines, and reagents. *Streptococcus pneumoniae* strain TIGR4 was cultured in tryptic soy (TS) broth with 150 U/ml catalase at 37°C in a candle extinction jar (39). The immortalized human upper airway epithelial cell line A549 (ATCC CCL-185) was maintained in minimal essential medium (MEM) (Gibco) supplemented with 10% fetal bovine serum. All the reagents were purchased from Fisher Scientific unless otherwise specified.

Mice. All animal studies were approved by the Institutional Animal Care and Use Committee at University of Louisiana at Lafayette. Mice were purchased from Charles River Laboratories, Inc., and were housed in the animal facility in the Department of Biology at the University of Louisiana at Lafayette. Mice were maintained at 20 to 23°C under a 12-h light/12-h dark cycle and 45 to 65% humidity. Standard laboratory food and water were provided *ad libitum*.

Preparation of CSE and EVE. Cigarette smoke extract (CSE) was prepared by bubbling smoke from 3 Marlboro cigarettes in 20 ml TS broth as described previously (16). Strawberry flavored e-liquid formulations (APII by Bomb Sauce, Atlanta, GA) with 70% vegetable glycerin and 30% propylene glycol, either with 3 mg/ml nicotine or nicotine free, were purchased from a local vape store. Fresh e-cigarette vapor extract (EVE) from e-liquid that contained nicotine (EVE^{+NIC}) or was nicotine free (EVE^{-NIC}) was prepared for each experiment by drawing 20 puffs of vapor into a 20-ml syringe containing 10 ml TS

broth. Each puff was mixed well with the medium before drawing the next puff. Both CSE and EVE were filter sterilized with 0.22- μ m syringe filter prior to use for exposing pneumococci *in vitro*.

Pneumococcal exposure to CSE and EVE. A TIGR4 culture was grown in TS broth to log phase (optical density at 600 nm [OD₆₀₀] = 0.6) and centrifuged at 13,000 rpm for 5 min. Bacterial pellets were resuspended in 3 ml of 100% EVE^{+NIC}, 100% EVE^{-NIC}, 25% CSE, or fresh TS broth and maintained for 2 h at 37°C in a candle extinction jar. Prior to their use in experiments, pretreated TIGR4 was washed twice in sterile PBS or TS broth. The various pretreatment paradigms are referred to as CSE-TIGR4, EVE^{+NIC}-TIGR4, EVE^{-NIC}-TIGR4, and TS-TIGR4 (control).

RNA extraction, sequencing, and differential expression analyses. Total RNA was extracted from bacteria grown in CSE, EVE^{+NIC}, EVE^{-NIC}, or medium alone using the RiboPure bacterial RNA extraction kit (Invitrogen), treated with DNase according to the manufacturer's instructions, and rRNA purified using the RiboZero rRNA depletion kit (Illumina). The amount and quality of extracted RNA were verified using Synergy HTX multimode microplate reader (BioTek). Library preparation and RNA-Seq were performed by Genewiz (Plainfield, NJ). Briefly, one barcoded library was prepared for each of the 12 samples (three each of CSE-TIGR4, EVE^{+NIC}-TIGR4, EVE^{-NIC}-TIGR4, and TS-TIGR4) with the NEBNext Ultra RNA Library Prep kit (New England Biolabs) using default protocols. Libraries for all samples were sequenced as 150-bp paired-end reads on a single lane of an Illumina Hi-Seq 4000 instrument (Illumina). Reads were bioinformatically demultiplexed and trimmed to remove adapter sequences and poor-quality bases using Trimmomatic v.0.36 (52). The trimmed reads were then mapped to the TIGR4 reference genome (GenBank accession no. [AE005672.3](#)) using the Bowtie2 aligner v.2.2.6 (53). Read counts for each gene were calculated by using the featureCounts script from the Subread package v.1.5.2 (54). Only unique reads that fell within gene regions were counted. The DESeq2 Bioconductor package (55) was used to normalize read counts using the relative log expression method and identify differentially expressed genes (DEGs) between pairs of treatments (i.e., CSE-TIGR4 versus TS-TIGR4, EVE^{+NIC}-TIGR4 versus CSE-TIGR4, EVE^{-NIC}-TIGR4 versus TS-TIGR4, and EVE^{+NIC}-TIGR4 versus EVE^{-NIC}-TIGR4). Genes with an adjusted *P* value (*P*_{adj}) of ≤ 0.05 (56) and an absolute log₂ fold change (log₂FC) of ≥ 1 were identified as differentially expressed for each comparison. For the comparison of each treatment with the control, we also assessed the overall similarity among biological replicates by calculating the regularized log (rlog)-transformed Euclidean distance for all sample pairs as well as by principal-component analysis.

A custom script was used to extract gene ontology (GO) (57) and KEGG ontology (KO) (58) terms for each gene in the TIGR4 genome from the UniProt (59) and KEGG (58) websites, respectively. GO enrichment analyses were conducted with GOATOOLS (60) to identify significantly overrepresented molecular function, biological process, and cellular component GO terms among the DEGs for each pair of treatments. Similarly, KEGG pathway enrichment analyses were conducted with the Bioconductor KEGGREST R module (61) to identify significantly overrepresented terms among the DEGs for each pair of treatments.

qRT-PCR. RNA from murine lungs was extracted using the RNAqueous-4PCR total RNA isolation kit (Thermo Fisher). TIGR4 RNA was isolated using the RiboPure bacterial RNA extraction kit (Thermo Fisher). DNase-treated RNA was reverse transcribed to cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative reverse transcription-PCR (qRT-PCR) was carried out using Power SYBR green master mix in a StepOne Plus thermal cycler (Applied Biosystems). Relative quantification (RQ) values were calculated using a comparative threshold cycle ($\Delta\Delta C_T$) program (StepOne software version 2.0).

Biofilm assay. TIGR4 biofilm formation was assayed using the crystal violet staining method as described previously (16). Following 2 h of exposure, CSE-TIGR4, EVE^{+NIC}-TIGR4, EVE^{-NIC}-TIGR4, and TS-TIGR4 were washed twice with fresh TS broth and resuspended at a 1:40 dilution in TS broth. Two hundred microliters of bacterial suspension was transferred to 96-well plates and incubated at 37°C for 18 h in the presence of 5% CO₂. The biofilms were washed with 0.9% NaCl, baked at 60°C for 1 h, and stained with crystal violet for 15 min at room temperature. Excess stain was removed by washing the biofilms twice in deionized (DI) water. The plates were dried at room temperature, biofilm-bound stain was extracted in a 70% ethanol–10% methanol mixture, and absorbance was measured at 590 nm.

Adherence assay. A549 cells were grown to confluence in 12-well plates. CSE-TIGR4, EVE^{+NIC}-TIGR4, EVE^{-NIC}-TIGR4, and TS-TIGR4 were washed twice and resuspended in sterile PBS. The inoculum CFU were enumerated by dilution plating on 5% sheep blood agar to ensure that pneumococci were added at a multiplicity of infection (MOI) of 10. In order to facilitate the bacterial contact with A549 cells, plates were centrifuged at 800 $\times g$ for 5 min at room temperature and incubated for 1 h at 37°C in the presence of 5% CO₂. After incubation, bacteria in the supernatant (supernatant CFU [SC]) were enumerated by dilution plating. The wells were washed three times with sterile PBS containing 1 mM Ca²⁺ and 1 mM Mg²⁺ to remove nonadherent bacteria. Next, adherent bacteria (AB) were enumerated by dilution plating, and the percent adherence was calculated as $[AB/(AB + SC)] \times 100$.

Hydrophobicity test. CSE-TIGR4, EVE^{+NIC}-TIGR4, EVE^{-NIC}-TIGR4, and TS-TIGR4 were washed with PBS, and the bacterial CFU in the inoculum (IC) were enumerated. Hexadecane was added to the bacterial suspension, and the mixture was vortexed and incubated at room temperature for 30 min. After incubation, bacterial CFU in the aqueous phase (AC) were enumerated, and the proportion of bacteria in the organic phase was determined as $IC - AC$. The percent hydrophobicity was calculated as $[(IC - AC)/IC] \times 100$.

Mouse model of acute pneumonia. Different cohorts of 6- to 8-week-old C57BL6 mice were anesthetized by intraperitoneal injection of a ketamine-xylazine mixture and intranasally inoculated with 5×10^7 CFU of CSE-TIGR4, EVE^{+NIC}-TIGR4, EVE^{-NIC}-TIGR4, or TS-TIGR4 that was washed and resuspended in 50 μ l of sterile PBS. After 24 h, animals were sacrificed using CO₂ asphyxiation followed by cervical

dislocation. Nasal lavage, nasal septum, bronchoalveolar lavage fluid (BALF), lungs, and spleen were collected for each animal. Bacterial CFU in lavage fluid samples and tissue homogenates were enumerated by dilution plating. Total cells in BALF were microscopically counted using a hemocytometer. Tissue for RNA extraction was stored in RNeasy lysis buffer (Qiagen) at -20°C , while tissue for total protein estimation and cytokine ELISA was flash frozen in liquid nitrogen and stored at -80°C . Lavage fluid samples for ELISA were stored at -80°C .

H₂O₂ sensitivity assay. Bacterial sensitivity toward H₂O₂ was assessed as described previously (62). CSE-TIGR4, EVE^{+NIC}-TIGR4, EVE^{-NIC}-TIGR4, or TS-TIGR4 was washed, resuspended in fresh medium, and mixed with 40 mM H₂O₂ before being incubated at 37°C for 30 min. CFU were enumerated after incubation by serial dilution and plated on blood agar. The results were expressed as percent survival of CFU relative to the control.

Total protein estimation. Total protein in supernatant from cell lines, BALF, and lung homogenates was estimated by using the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher).

ELISA. Amounts of IFN- γ and IL-6 in BALF were estimated by using the ELISA Ready-SET-Go! kit (Invitrogen) according to the manufacturer's protocol.

Statistical analysis for data other than transcriptome sequencing. Data were analyzed using Prism 8.0 (GraphPad). Normally distributed data from two groups were compared using the unpaired *t* test, while comparisons among three or more groups were performed using a one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test. In the case of bacterial enumeration data (CFU/ml) that were normally distributed, the Mann-Whitney U statistic was used to evaluate the difference between two groups and the Kruskal-Wallis analysis was used to evaluate differences among more than two groups ($P < 0.05$).

Data availability. The raw Illumina reads have been deposited in NCBI's BioProject database under accession no. [PRJNA573106](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA573106).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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