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# Ethanol-Induced Alcohol Dehydrogenase E (AdhE) Potentiates Pneumolysin in *Streptococcus pneumoniae*

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**Alcohol impairs the host immune system, rendering the host more vulnerable to infection. Therefore, alcoholics are at increased risk of acquiring serious bacterial infections caused by *Streptococcus pneumoniae*, including pneumonia. Nevertheless, how alcohol affects pneumococcal virulence remains unclear. Here, we showed that the *S. pneumoniae* type 2 D39 strain is ethanol tolerant and that alcohol upregulates alcohol dehydrogenase E (AdhE) and potentiates pneumolysin (Ply). Hemolytic activity, colonization, and virulence of *S. pneumoniae*, as well as host cell myeloperoxidase activity, proinflammatory cytokine secretion, and inflammation, were significantly attenuated in *adhE* mutant bacteria (Δ*adhE* strain) compared to D39 wild-type bacteria. Therefore, AdhE might act as a pneumococcal virulence factor. Moreover, in the presence of ethanol, *S. pneumoniae* AdhE produced acetaldehyde and NADH, which subsequently led Rex (redox-sensing transcriptional repressor) to dissociate from the *adhE* promoter. An increase in AdhE level under the ethanol condition conferred an increase in Ply and $H_2O_2$ levels. Consistently, *S. pneumoniae* D39 caused higher cytotoxicity to RAW 264.7 cells than the Δ*adhE* strain under the ethanol stress condition, and ethanol-fed mice (alcoholic mice) were more susceptible to infection with the D39 wild-type bacteria than with the Δ*adhE* strain. Taken together, these data indicate that AdhE increases Ply under the ethanol stress condition, thus potentiating pneumococcal virulence.**

Alcoholism is one of the most important risk factors for pulmonary infections involving *Streptococcus pneumoniae* (pneumococcus) or *Klebsiella pneumoniae* (1–4). Alcohol impairs alveolar macrophages, which are the first cellular line of defense against pathogens in the lower airways (5). Impaired alveolar macrophages produce fewer proinflammatory cytokines and chemokines in response to pulmonary infection, resulting in an elevated bacterial burden and increased mortality (6, 7). Alcoholics are particularly susceptible to pulmonary infection by pneumococcus (8, 9); current or former alcohol abuse patients have an increased risk of pneumococcal infection (10). Pneumococcus spread in the bloodstream of ethanol (EtOH)-fed rats more rapidly than it did in control rats (11). However, the role of bacterial gene(s) induced by alcohol in virulence remains unclear.

Alcohol dehydrogenase (Adh; EC 1.1.1.1), which is produced by many different types of organisms ranging from bacteria to mammals, is involved in ethanol oxidation. Adh facilitates the conversion of alcohol to aldehyde with the reduction of $NAD^+$ to NADH (12).

Previously, we showed that among six Adh genes in the *S. pneumoniae* type 2 D39 strain, including SPD_0265, SPD_1126, SPD_1636, SPD_1834, SPD_1865, and SPD_1985, only SPD_1834 (104-kDa protein), which encodes a bifunctional two-domain protein (named AdhE) containing Adh and acetaldehyde-coenzyme A (CoA) dehydrogenase domains in the C terminus and the N terminus, respectively, was strongly induced after ethanol shock (13). Ethanol-induced production of AdhE constituted 23% of all induced proteins (13).

Most of the studies on Adh in bacteria are focused on metabolism (14, 15) and tolerance, including nitrosative stress (16) and ethanol tolerance (17, 18). There have been a few studies on the contribution of Adh to bacterial virulence. Although the Gram-positive *Listeria monocytogenes* alcohol acetaldehyde dehydrogenase (LAP) is secreted and binds to the host-cell receptor Hsp60 to promote adherence (19, 20), *S. pneumoniae* type 2 AdhE was not secreted and did not influence pneumococcal adherence (data not shown). Therefore, the role of Adh in bacterial virulence, in particular, the mechanism through which AdhE contributes to pneumococcal virulence under ethanol-tolerant conditions, needs further clarification.

Here, we demonstrated that *S. pneumoniae* D39 could tolerate ethanol stress, which subsequently increased pneumolysin (Ply) via upregulation of AdhE. Mice were more susceptible to infection with D39 wild-type (WT) bacteria than with *adhE* mutant bacteria. Moreover, ethanol-fed mice (alcoholic mice) were more susceptible to infection with D39 WT bacteria than normal control mice infected with D39 WT and *adhE* mutant bacteria, suggesting that AdhE can potentiate *S. pneumoniae* type 2 virulence under the ethanol stress condition.

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**TABLE 1** Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristic(s) | Antibiotic resistance[a] | Reference or source |
|---|---|---|---|
| Strains | | | |
| *S. pneumoniae* strains | | | |
| D39 | Encapsulated, type 2 | | 22 |
| Δ*adhE* strain | D39 Δ*adhE*::*ermB* | Ery$^r$ | This study |
| Δ*rex* strain | D39 Δ*rex*::*ermB* | Ery$^r$ | This study |
| Δ*spxB* strain | D39 Δ*spxB*::*ermB* | Ery$^r$ | This study |
| pMV158-*adhE* strain | D39 Δ*adhE*::*ermB* containing pMV158-*adhE* | Ery$^r$, Tet$^r$ | This study |
| WU2 | Encapsulated, type 3 | | 23 |
| WU2 Δ*adhE* | WU2 Δ*adhE*::*ermB* | Ery$^r$ | This study |
| *E. coli* BL21(DE3) | F$^-$ *ompT hsdS*$_B$($r_B^-$ $m_B^-$) *gal dcm* (DE3) | | Novagen |
| Plasmids | | | |
| pET30a(+) | 5,422 bp | Kan$^r$ | Novagen |
| pET32b(+) | 5,549 bp | Ery$^r$ | Novagen |
| pPSH01 | His-tagged AdhE in pET30a(+) | Kan$^r$ | This study |
| pKEH01 | His-tagged Rex in pET32b(+) | Amp$^r$ | This study |
| pMV158 | 5,300 bp; streptococcal plasmid | Tet$^r$ | 24 |

[a] Ery, erythromycin; Tet, tetracycline; Kan, kanamycin; Amp, ampicillin.

## MATERIALS AND METHODS

**Ethics statement.** Male CD-1 (ICR) mice, 4 to 6 weeks old, were obtained from OrientBio, Inc. (Seongnam, Kyonggi, Republic of Korea), and housed in a specific-pathogen-free barrier facility (12-h light/dark cycle, 22 ± 2°C, 50% ± 10% relative humidity) at the School of Pharmacy at Sungkyunkwan University (Suwon, Republic of Korea). The animals received water and sterilized food *ad libitum*. All animal experiments were approved by the Animal Care and Use Committee of the SungKyunKwan University (Suwon, Republic of Korea). Infection procedures followed protocol PH-530518-06, in accordance with the animal care guidelines of the Korean Academy of Medical Sciences, and all efforts were made to minimize animal suffering.

***In vivo* survival and colonization.** For the *in vivo* survival test, mice were infected with *S. pneumoniae* via the intranasal route (i.n.). Mouse survival was recorded eight times during the first 4 days and four times until the end of this experiment (14 days) (21).

For the *in vivo* colonization test, mice were infected with $1.5 \times 10^7$ CFU of *S. pneumoniae* D39 or the Δ*adhE* strain in phosphate-buffered saline (PBS) i.n. Mice were anesthetized, and nasopharynx, lung wash, lung, and blood were collected and subjected to viable cell counting on Todd-Hewitt–yeast (THY) agar supplemented with 5% sheep blood after appropriate serial dilution using PBS. Blood agar contained 10 μg/ml gentamicin only (for D39) or both 10 μg/ml gentamicin and 2.5 μg/ml erythromycin (for the Δ*adhE* strain) (21).

**Bacterial strains, culture, transformation, and cell culture conditions.** The bacterial strains (*Escherichia coli* and *S. pneumoniae*) and plasmids used in this study are shown in Table 1. Encapsulated *S. pneumoniae* D39 (serotype 2; NCTC7466) (22) and WU2 (serotype 3) (23) strains and their isogenic Δ*adhE* (serotype 2 and 3), Δ*rex* (serotype 2), and Δ*spxB* (serotype 2) mutants were used. The mutants were created using an *ermB* cassette inserted opposite to the target gene orientation to disrupt its expression (24). To create an insertion-deletion mutation in the *S. pneumoniae adhE* and *rex* genes, an 860-bp *ermB* cassette was amplified with primers prs3 and prs4 (24) and used to disrupt *adhE* and *rex* expression. Two arms were flanked by *S. pneumoniae* D39 genomic DNA, and the triple PCR product was integrated into *S. pneumoniae* D39 by homologous recombination. Transformants were selected by 2.5 μg/ml erythromycin and confirmed by PCR and Western blotting as well as by DNA sequencing.

The complemented strain that contains a recombinant pMV158 plasmid with the functional *adhE* gene in the isogenic Δ*adhE* mutant background was used. The full *adhE* gene, including its promoter, was amplified with primers (available upon request), which incorporated HindIII and EcoRI restriction enzyme sites from the D39 genome. Tetracycline-resistant transformants containing the recombinant plasmid were screened (1 μg/ml) and confirmed by PCR and Western blotting as well as by DNA sequencing. All pneumococci were cultured in Todd-Hewitt broth containing 0.5% (wt/vol) yeast extract (THY broth).

Murine macrophage RAW 264.7 cells were obtained from the American Type Culture Collection. Cells were cultured at 37°C in 95% air–5% $CO_2$. RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/liter glucose, 10% fetal bovine serum (Gibco BRL), 100 units/ml penicillin G, and 100 μg/ml streptomycin.

**Cloning and purification of recombinant AdhE and Rex.** The *adhE* (GenBank accession number ABJ54182.1) and *rex* (GenBank accession number ABJ53704.1) genes in *S. pneumoniae* D39 were amplified by PCR using D39 genomic DNA as a template with AdhE and Rex primers (available upon request) that incorporated HindIII and EcoRI (AdhE) or BamHI and XhoI (Rex) restriction enzyme sites. The PCR products were digested with the appropriate enzymes (New England BioLabs) and cloned into the corresponding restriction sites in pET30a(+) (AdhE) or pET32b(+) (Rex) (Novagen) plasmids that were used to transform *E. coli* BL21(DE3) to generate the strains pPSH01 and pKEH01 (Table 1), respectively. The nucleotide sequences in the recombinant strains were confirmed by DNA sequencing (Cosmo, Seoul, Republic of Korea). *E. coli* strains were cultured in Luria broth (LB), and kanamycin (50 μg/ml) or ampicillin (100 μg/ml) was used to select recombinant colonies. Plasmid DNA was extracted and purified from *E. coli* with a commercially available kit (Cosmo, Seoul, Republic of Korea). Proteins were then induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 6 h at 37°C and purified using a nickel-nitrilotriacetic acid column (Ni-NTA; Probond, Invitrogen) in default buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl) and imidazole, according to the manufacturer's instructions. The eluted fraction was dialyzed against 50 mM Tris-HCl buffer (pH 7.5).

**Histological analysis of lung tissue.** Whole lungs of control and infected mice ($n = 2$) were fixed in 10% formalin (Sigma). The samples were embedded in paraffin, sectioned (5 μm), and stained with hematoxylin-eosin. Histological changes were evaluated randomly in nonconsecutive fields using an AxioPlan instrument (Zeiss, Germany) at a magnification of ×200. Images were analyzed using AxioVision, version 3.1 (Zeiss, Germany).

**AO-EB stain.** RAW 264.7 cells were infected with *S. pneumoniae* D39 or the Δ*adhE* strain (multiplicity of infection [MOI] of 100) for 2 h. Cells were stained with acridine orange and ethidium bromide (AO-EB) for

apoptosis detection (25). Samples were visualized immediately using an IX70 instrument (Olympus).

**MPO assay.** Myeloperoxidase (MPO) was quantified to evaluate neutrophil infiltration in lung tissue (26). Mice were asphyxiated, and lungs were collected and homogenized in 50 mM sodium phosphate buffer (pH 6.5). The crude homogenate was added to hexadecyltrimethylammonium bromide (Sigma) to a final concentration of 0.5% before samples were frozen at −70°C. Frozen samples were thawed, sonicated, and centrifuged (20,000 × *g* for 30 min at 4°C). The MPO assay was performed as described previously (26). A 10-μl sample was added to a 96-well plate, and subsequently 100 μl of 3,3′,5′,5-tetramethylbenzidine liquid substrate was added. The colorimetric assay was recorded at $A_{650}$ using a microplate reader (Molecular Device, USA).

**Hemolytic assay.** Hemolytic activity was determined, as previously described (27), using sheep red blood cells (Hanil Komed, Seongnam, Republic of Korea). *S. pneumoniae* was incubated to log phase ($A_{550}$ of 0.3) prior to harvest, and the pellet was resuspended in PBS. Sodium deoxycholate (Sigma) was added to the suspension to a final concentration of 0.1% for lysis. A 50-μl sample was added to each well of a 96-well plate with 50 μl of 1.5% sheep red blood cell solution. The hemolytic titer was recorded as equal to the dilution at which 50% of red blood cells were lysed at $A_{540}$.

**Acetaldehyde assay.** To measure acetaldehyde concentration, the *S. pneumoniae* D39 and Δ*adhE* strains were cultured until they reached an $A_{550}$ of 0.3. Acetaldehyde level was measured with an aldehyde quantification kit (Abcam, England), according to the manufacturer's instructions. THY broth was used as a blank control.

**ADH activity.** AdhE activity was determined, as described previously (12), based on NADH release. Fresh, recombinant AdhE (300 nM) was added to a solution that contained 33 mM sodium phosphate buffer, pH 7.5, 1 mM $NAD^+$, and 5 mM ethanol. NADH release was measured at $A_{340}$.

**Alcohol administration and determination of *in vivo* ethanol level.** Mice were fed alcohol directly in their drinking water. The alcohol or water and standard chow intake were monitored every day. The alcohol group drank 10% (vol/vol) alcohol for 2 days, 15% (vol/vol) alcohol for 5 days, and 18% (vol/vol) alcohol until the end of the study (28). Ethanol level *in vivo* was determined from 100 μl of mouse serum, nasopharynx (nasal tissue) homogenates, or lung wash using alcohol dehydrogenase, $NAD^+$ (Sigma), and sodium phosphate buffer, pH 8.0, to check the release of NADH, measured at $A_{340}$ for 5 min. Ethanol (Merck) was used to construct a standard curve of NADH release.

**Antisera and Western blotting.** To prepare antisera, 5-week-old male CD1 mice were immunized via the intraperitoneal route (i.p.) with 10 μg of purified AdhE or Rex protein mixed with 100 μg of aluminum adjuvant (Sigma) at 14-day intervals. Sera were collected (after mice were asphyxiated) 1 week after the third immunization.

To collect whole-cell lysates, *S. pneumoniae* was grown in THY broth and lysed in lysis buffer (50 mM Tris-Cl, pH 8.0, 1 mM dithiothreitol [DTT], 0.1% Triton X-100) (24). To collect released protein, the supernatant of *S. pneumoniae* culture was precipitated with 10% trichloroacetic acid (Sigma) (23). Protein samples were separated by 7.5 to 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) prior to transfer onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Membranes were then blocked by 5% (wt/vol) skim milk (Merck) and probed with the appropriate primary antibody (AdhE, Rex, Ply, SpxB, and PsaA). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Promega) was used as the secondary antibody.

**RNA isolation and qRT-PCR.** Bacterial RNA was isolated by the hot-phenol method (29), and eukaryotic RNA was isolated using TRIzol reagent (Invitrogen), as instructed by the manufacturer. All RNA samples were treated with DNase I (TaKaRa). Briefly, a 1-μg RNA sample was subjected to reverse transcription into cDNA using random primers (TaKaRa). Quantitative reverse transcription-PCR (qRT-PCR) was performed according to the manufacturer's instructions (Applied Biosystems), as described previously (24).

**ELISA.** Groups of mice ($n = 5$) were infected with *S. pneumoniae* D39 or the Δ*adhE* strain ($1.5 \times 10^7$ CFU/mouse) i.n. for 4, 8, 12, 24, or 48 h. After mice were asphyxiated, lungs were collected and homogenized, and cytokine (tumor necrosis factor alpha [TNF-α], interleukin-1β [IL-1β], and IL-6) levels were measured by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (BD Biosciences).

Recombinant AdhE protein was time and dose dependently added to RAW 264.7 cell culture. Supernatant was collected and used to check cytokine levels by ELISA, as instructed by the manufacturer (BD Biosciences). Lipopolysaccharide (LPS), heat-inactivated AdhE, and VncR were used as controls.

**Electrophoretic mobility shift assay (EMSA).** To determine the binding ability of Rex to the *adhE* promoter, a band shift assay was carried out with minor modifications (30). Purified recombinant Rex or 65°C-denatured Rex was coincubated with 4 nM purified *adhE* promoter DNA in reaction buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 3% [wt/vol] Ficoll 400, 0.5 mM DTT, 150 mM NaCl) for 15 min at 25°C. The reaction mixture was then analyzed in a 7.5% native acrylamide/bisacrylamide gel at 4°C. The shift bands were visualized under UV light after 45-min of Gel Red staining (Biotium, CA, USA).

To analyze the influence of $NAD^+$ and NADH on the binding affinity of Rex to the *adhE* promoter, another similar experiment was performed but in the presence of $NAD^+$ (Sigma) or NADH (Sigma). To prevent DNA-nicotinamide coprecipitation, HEPES buffer was used instead of Tris-HCl buffer, as above (30).

**$NAD^+$-NADH pool isolation and NAD cycling assay.** To extract $NAD^+$ and NADH, the *S. pneumoniae* D39 and Δ*adhE* strains were cultured to the log phase and incubated with 100 mM ethanol for 30 min. The dinucleotides were extracted as described previously, with some modifications (31). The cell pellet was immediately frozen in liquid nitrogen before being suspended in a final volume of 250 μl of either 0.2 M HCl (for $NAD^+$ extraction) or 0.2 M NaOH (for NADH extraction). $NAD^+$-NADH levels were determined with a cycling assay (31) measured at $A_{570}$.

**Hydrogen peroxide production assay.** To measure the concentration of released $H_2O_2$, 5 ml of *S. pneumoniae* bacteria was cultured in a 15-ml glass tube at 37°C with 100-rpm agitation to supply oxygen. A total of 900 μl of bacterial culture supernatant was mixed with 100 μl of reaction solution (0.3% [wt/vol], 2,2′-azinobis [ABTS; 3-ethylbenzthiazolinesulfonic acid]; Sigma), and 0.0002% (wt/vol) horseradish peroxidase (HRP) (Sigma) in 0.1 M sodium phosphate buffer, pH 7.0. Before the $A_{540}$ was measured, the mixture was incubated at 25°C for 3 min. The $H_2O_2$ concentration was determined with an $H_2O_2$ (Sigma) standard curve (32).

**Cytotoxicity assay.** RAW 264.7 cells were infected with *S. pneumoniae* D39 or the Δ*adhE* strain (MOI of 100) for 2 h. Cytotoxicity was determined using 3-(4,5-dimethylthiazole-2-yl)-2,5-dephenyl-tetrazolium bromide) (MTT), as described previously (33).

**ChIP assay.** *S. pneumoniae* D39 or the Δ*adhE* strain at log phase was treated with 100 mM ethanol for 30 min, and the bacterial pellet was subjected to a chromatin immunoprecipitation assay (ChIP), as described previously (34). The bacterial pellets were fixed in 1% formalin for 15 min, and fixation was stopped by the addition of 125 mM glycine, pH 2.5. Bacterial cell lysate was immunoprecipitated overnight at 4°C with anti-Rex and protein A-agarose beads (Santa Cruz) with gentle agitation. The beads were rinsed with lysis buffer and Tris-EDTA (TE) buffer before elution with 50 μl of TE buffer containing 1% SDS. Samples were then treated with 100 μg of proteinase K. DNA from treated samples was precipitated by the cold-ethanol method before PCR with *adhE* promoter primers (WT; 300 bp) (available upon request). Input was determined using 16S RNA primers.

**Statistical analysis.** Most graphs were created and statistical analyses performed using SigmaPlot, version 11.0, software (Systat Software). *In vivo* survival (see Fig. 3C and 6E) and qRT-PCR (see Fig. 3A) analyses were performed using GraphPad Prism, version 5.02. Statistical analysis was

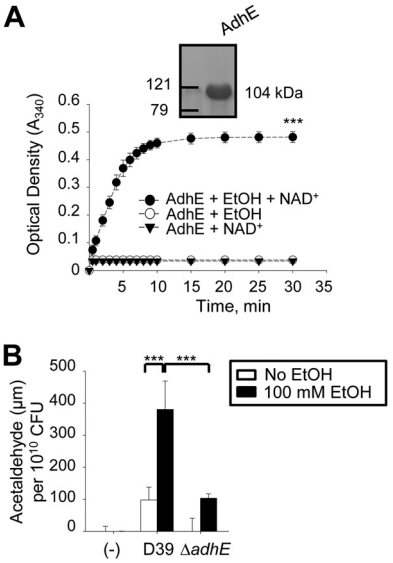


**FIG 1** Pneumococcal AdhE produces NADH and acetaldehyde. (A) ADH activity of recombinant AdhE was determined at $A_{340}$ using 1 mM $NAD^+$, 5 mM ethanol, and 0.3 mM AdhE. (B) *S. pneumoniae* D39 or the Δ*adhE* strain was cultured until an $A_{550}$ of 0.3 was reached, and then AdhE was induced by supplementation with 100 mM ethanol for 30 min. Bacterial cultures were used to detect acetaldehyde. Data are representative of three independent experiments. Significant differences were analyzed by one-way ANOVA (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). (−), blank.

calculated using one-way analysis of variance (ANOVA) (Holm-Sidak method), two-way ANOVA (Bonferroni method), a Mann-Whitney U test (nonparametric), or a log rank (Mantel-Cox) test, as appropriate. *P* values of ≤0.05 (denoted on the figures by *), $P \leq 0.01$ (**), and $P \leq 0.001$ (***) were considered significant. Data presented are the means and standard deviations (SD) of results of three to four independent experiments.

## RESULTS

**EtOH-induced *S. pneumoniae* type 2 AdhE promotes reducing conditions.** Although AdhE in *S. pneumoniae* D39 (type 2) was identified by sequence homology (13) and is highly conserved among streptococci (92 to 99% identity with *Streptococcus mitis*, *Streptococcus australis*, *Streptococcus. sanguinis*, *Streptococcus pyogenes*, and *Streptococcus agalactiae*) and *S. pneumoniae* strains (99% identity with types 1, 3, 4, 6B, 19A, 19F, and 14), its catalytic activity has not been identified. To confirm whether *S. pneumoniae* AdhE can catalyze ethanol and $NAD^+$ to form acetaldehyde and NADH, recombinant *S. pneumoniae* AdhE (Fig. 1A) was expressed in *E. coli*, and the activity was determined. The results demonstrate that *S. pneumoniae* AdhE produced more NADH ($P < 0.001$) than the control (Fig. 1A), indicating that AdhE has ADH activity.

To determine the role of AdhE in *S. pneumoniae* D39, an Δ*adhE* mutant was constructed with the triple-PCR method by inserting an *ermB* cassette to disrupt *adhE* gene expression. In the presence of ethanol, the acetaldehyde level in *S. pneumoniae* D39 WT was 4-fold higher ($P < 0.001$) than in the untreated sample (Fig. 1B). Ethanol treatment consistently increased the NADH/$NAD^+$ ratio in *S. pneumoniae* by approximately 19-fold (Table 2). These results indicate that ethanol-induced AdhE produces both NADH and acetaldehyde.

*S. pneumoniae* growth might be affected by culture medium (THY broth in normal cultures or DMEM during host cell infection) or by ethanol. To investigate this possibility, *S. pneumoniae* growth in THY broth or DMEM or in the presence of ethanol was performed; however, these media do not significantly affect the growth of the WT or *adhE* mutant (data not shown). To confirm that ethanol induced AdhE expression, *S. pneumoniae* D39 WT was treated with ethanol. The results showed that ethanol induced AdhE (50 mM) time dependently (Fig. 2A and B). In addition, a complemented strain of the Δ*adhE* mutant, in which *adhE* expression is reconstituted by introducing a pMV158 vector harboring the *adhE* gene, had increased levels of AdhE after ethanol treatment (Fig. 2C). To verify AdhE induction at the mRNA level, mRNA expression was measured by RT-PCR. Consistently, ethanol treatment induced *adhE* mRNA production (Fig. 2E), demonstrating that ethanol induced *adhE* production at both the mRNA and protein levels.

Since NADH is known to generate superoxide anions, the immediate precursor of $H_2O_2$ (35), and since ethanol-induced AdhE increases NADH levels (Table 2), $H_2O_2$ levels were determined after ethanol treatment. After ethanol treatment, the $H_2O_2$ level in treated D39 WT bacteria increased 2-fold compared to the level in nontreated D39 WT bacteria. The complemented strain had a significantly higher $H_2O_2$ level than the Δ*adhE* mutant (Fig. 2D). In contrast, the $H_2O_2$ level in the Δ*adhE* mutant increased only 1.2-fold compared to the control. The $H_2O_2$ level of a pyruvate oxidase deletion (Δ*spxB*) mutant was nearly abrogated (Fig. 2D) because SpxB is the major protein that produces $H_2O_2$ (36), and the SpxB level did not vary between the D39 WT and Δ*adhE* mutant strains (Fig. 3A); thus, the Δ*spxB* mutant was used as a negative control. These data suggest that ethanol-induced AdhE increases $H_2O_2$ production.

**Attenuation of virulence by *adhE* mutation.** To determine whether *adhE* expression affects expression of *S. pneumoniae* virulence factors, pneumococcal lysates at the log phase were subjected to Western blotting. Among several pneumococcal virulence factors at an $A_{550}$ of 0.3, only intracellular pneumolysin (Ply) levels were decreased (1.7-fold) in the Δ*adhE* mutant compared to those in D39 WT bacteria. No significant differences in choline binding protein A (CbpA), pneumococcal surface adhesin A (PsaA), or SpxB were found (Fig. 3A). The difference in $H_2O_2$ production between the *S. pneumoniae* D39 WT strain and the Δ*adhE* mutant (Fig. 2D) was relevant to NADH levels (Table 2) but not because of SpxB levels (Fig. 3A). These data demonstrate that AdhE affects the Ply level but not CbpA, PsaA, and SpxB.

Moreover, the intracellular Ply level in the Δ*adhE* mutant at an $A_{550}$ of 0.6 significantly decreased (1.58-fold) compared to that in the D39 WT strain (data not shown). Additionally, the Ply level released from the Δ*adhE* mutant to the culture supernatant was 1.59-fold lower than that from the D39 WT strain (data not

**TABLE 2** Increase of NADH after ethanol exposure

| Strain | Ethanol treatment | Concn[a] | | Concn ratio (NADH/$NAD^+$) |
|---|---|---|---|---|
| | | $NAD^+$ | NADH | |
| D39 | Untreated | 5.203 | 0.025 | 0.00480 |
| | Treated | 3.740 | 0.339* | 0.09064 |
| Δ*adhE* strain | Untreated | 3.464 | 0.098 | 0.02829 |
| | Treated | 4.149 | 0.22 | 0.05302 |

[a] Micromoles per ml of culture ($7 \times 10^8$ CFU/ml). *, $P < 0.01$.

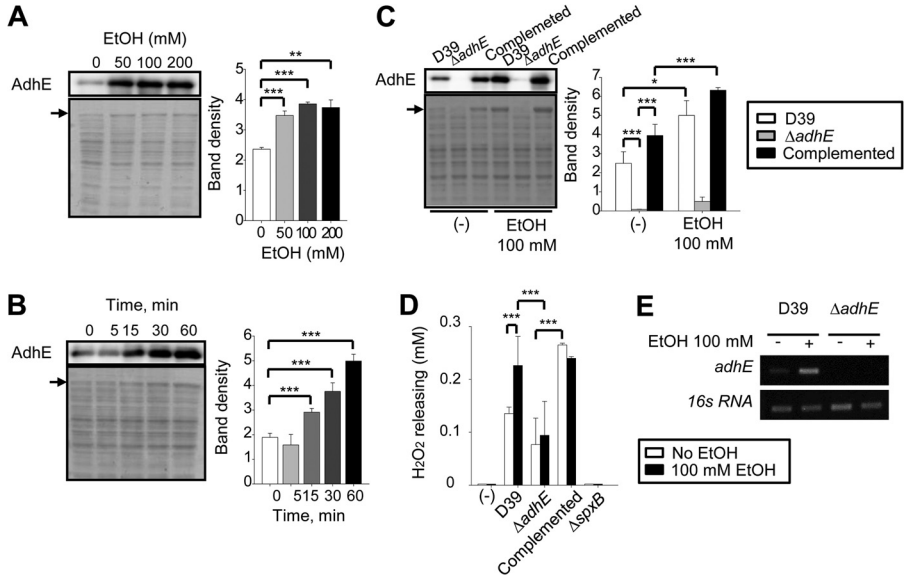


**FIG 2** Ethanol-induced AdhE increases $H_2O_2$ production. (A and B) *S. pneumoniae* D39 was cultured until an $A_{550}$ of 0.3 was reached, and then ethanol was added. Cell lysates were used for Western blotting. AdhE induction was dose (A) and time (B) dependent. (C, D, and E) *S. pneumoniae* strains were cultured until an $A_{550}$ of 0.3 was reached, and then AdhE was induced with 100 mM ethanol for 30 min. The cell lysate was used for Western blotting (C). Bacterial cultures were used to determine $H_2O_2$ concentration (D) or to isolate RNA and determine mRNA levels by RT-PCR (E). Data are representative of three independent experiments. Significant differences were analyzed by one-way ANOVA (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

shown). To determine whether the released protein level was associated with autolysis, *S. pneumoniae* was cultured in THY broth with an autolysis inhibitor, choline chloride (23). Consistently, when the pneumococci were cultured in the presence of 2% choline chloride, the level of Ply released from the Δ*adhE* mutant to the culture supernatant significantly decreased compared to that from the D39 WT strain (data not shown). These results indicate that the Ply level is increased by AdhE, but this occurs autolysis independently.

In addition, the effect of AdhE on hemolytic activity, primarily caused by Ply, was determined. The D39 WT and complemented strains had significantly higher hemolytic activities than the Δ*adhE* mutant ($P < 0.05$) (Fig. 3B), suggesting that AdhE increases hemolysis. This result confirms that AdhE increases the Ply level of *S. pneumoniae*.

To further corroborate that the *adhE* mutation attenuated virulence *in vivo*, 4-week-old mice were infected i.n. with *S. pneumoniae* D39 and its isogenic Δ*adhE* mutant at a high dose ($1.5 \times 10^7$ CFU/mouse) and low dose ($1.5 \times 10^6$ CFU/mouse). Mice infected with the D39 WT or complemented strains showed higher mortality than those infected with the Δ*adhE* mutant (Fig. 3C). In addition, high-dose infection showed a significantly higher mortality than low-dose infection (Fig. 3C). Thus, high-dose infection was used for further studies.

To further confirm the role of AdhE on virulence, another *S. pneumoniae* strain, type 3 WU2, and its isogenic WU2 Δ*adhE* mutant were used to infect mice via the i.n. route at both a low dose ($1.5 \times 10^6$ CFU/mouse) and high dose ($4.5 \times 10^7$ CFU/mouse) (37). No mice died after infection with the WU2 Δ*adhE* mutant at either infection dose although mice infected with the high dose were more morbid than those infected with the low dose. Our results showed that WU2 infection at the high dose and low dose caused 46.7% ($P = 0.0023$) (Fig. 3D) and 13.3% (data not shown) mortality, respectively. The survival results with these mice demonstrate that the *S. pneumoniae* Δ*adhE* mutation attenuated virulence in serotypes 2 and 3 and that high-dose infection produced a more significant difference in mortality between WT and *adhE* mutant infections than low-dose infection.

Furthermore, significantly fewer viable cells colonized the lung, nasopharynx, nasal fluid, and blood time dependently after i.n. infection ($n = 7$/group) with the Δ*adhE* mutant than after infection with the D39 WT strain (Fig. 3E). Taken together, these results demonstrate that the Δ*adhE* mutation attenuated *S. pneumoniae* virulence *in vitro* and *in vivo*.

**AdhE increases cytokine production, inflammation, and cell death.** Although the Δ*adhE* mutant affected virulence *in vitro* and *in vivo* (Fig. 3), its adherence to various cell types, including macrophage RAW 264.7, lung A549, and nasopharynx Detroit 562 cells, was not significantly different from that of the D39 WT strain (data not shown). Thus, we investigated how AdhE modulates host gene expression by using a systems biology approach. RAW 264.7 cells were infected with *S. pneumoniae*, and host gene expression was analyzed by microarray and systems biology analyses. NF-κBIZ, Cxcl, and TNF-α expression after infection with D39 WT were significantly higher than after infection with the Δ*adhE* strain (data not shown). To confirm this result, RAW 264.7 cells were infected with *S. pneumoniae*, and mRNA levels were measured by qRT-PCR. mRNA levels of the ATF-3, NF-κB, Cxcl2, TNF-α, and IL-6 genes were significantly higher after infection with D39 WT than after infection with the control or Δ*adhE* mutant. Cxcl2 and TNF-α levels were increased 5- and 3-fold, respectively, by infection with D39 WT (Fig. 4A). To corroborate the effect of AdhE on cytokine production, mice ($n = 5$/group) were infected i.n. with $1.5 \times 10^7$ CFU of *S. pneumoniae*, and cytokine levels were measured. Infection with the D39 WT strain significantly increased cytokine (TNF-α, IL-1β, and IL-6) levels time

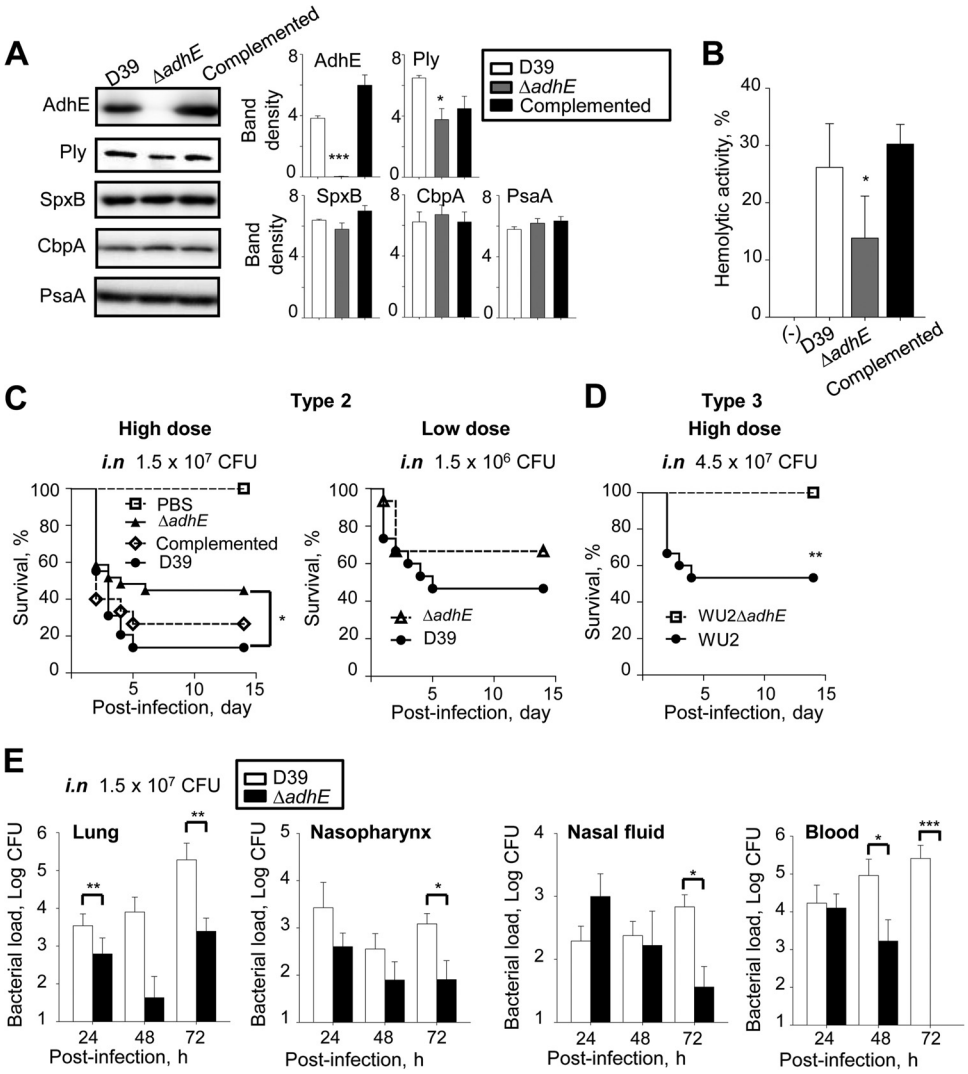


**FIG 3** AdhE potentiates virulence. (A) *S. pneumoniae* D39, the Δ*adhE* mutant, and complemented strains were cultured until an $A_{550}$ of 0.3 was reached. Cell lysates were subjected to Western blotting. (B) The hemolytic activity of the *S. pneumoniae* D39, Δ*adhE*, and complemented strains was determined with a 1.5% (vol/vol) red blood cell solution followed by measurement of the $A_{540}$. Data are representative of three independent experiments. (C) Mice ($n = 10$ to 15) were infected with a high ($1.5 \times 10^7$ CFU/mouse) or low ($1.5 \times 10^6$ CFU/mouse) dose of pneumococcal strain serotype 2 intranasally (i.n.), and survival time was recorded until 14 days postinfection. (D) Mice ($n = 10$ to 15) were infected with a high dose ($4.5 \times 10^7$ CFU/mouse) of pneumococcal strain serotype 3 i.n., and survival time was determined. (E) Mice ($n = 7$) were infected i.n. with $1.5 \times 10^7$ CFU of the pneumococcal strains of serotype 2, and viable cells were counted. Significant differences were analyzed using one-way ANOVA (A and B), a log rank test (C and D), or a Mann-Whitney rank sum test (D) (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

dependently compared to infection with the mutant (Fig. 4B). Moreover, to identify whether AdhE could affect cytokine secretion indirectly or directly, a recombinant AdhE was added to RAW 264.7 cell culture, and the levels of TNF-α, IL-6, and IFN-γ were determined. Results showed that recombinant AdhE induced TNF-α time dependently (Fig. 4C) but did not induce IL-6 and IFN-γ (data not shown); however, the effect of recombinant AdhE on TNF-α induction was not as potent as that of recombinant Ply (Fig. 4C). Taken together, these results suggest that AdhE could directly affect TNF-α production but not as potently as Ply. Therefore, AdhE-induced Ply production might be a major origin of inflammatory response enhancement.

To confirm these results *in vitro*, RAW 264.7 cells were infected with the Δ*adhE* mutant and stained with acridine orange and ethidium bromide (AO-EB). Infection with the Δ*adhE* mutant induced less apoptosis than infection with the WT (Fig. 4D), indicating that cell death induced by AdhE was potentiated.

To corroborate inflammation after i.n. infection ($n = 2$/group), mouse lungs were stained with hematoxylin and eosin, and histopathological changes were examined. Mouse lungs infected with the Δ*adhE* mutant had less inflammation than lungs infected with the WT (Fig. 4E), suggesting that AdhE induced lung inflammation in mice.

Furthermore, myeloperoxidase (MPO) activity, which is a hallmark of neutrophil infiltration (38), was determined. MPO levels in the lungs of mice ($n = 7$/group) infected with D39 WT was significantly higher than those in mice infected with the Δ*adhE* mutant (Fig. 4F).

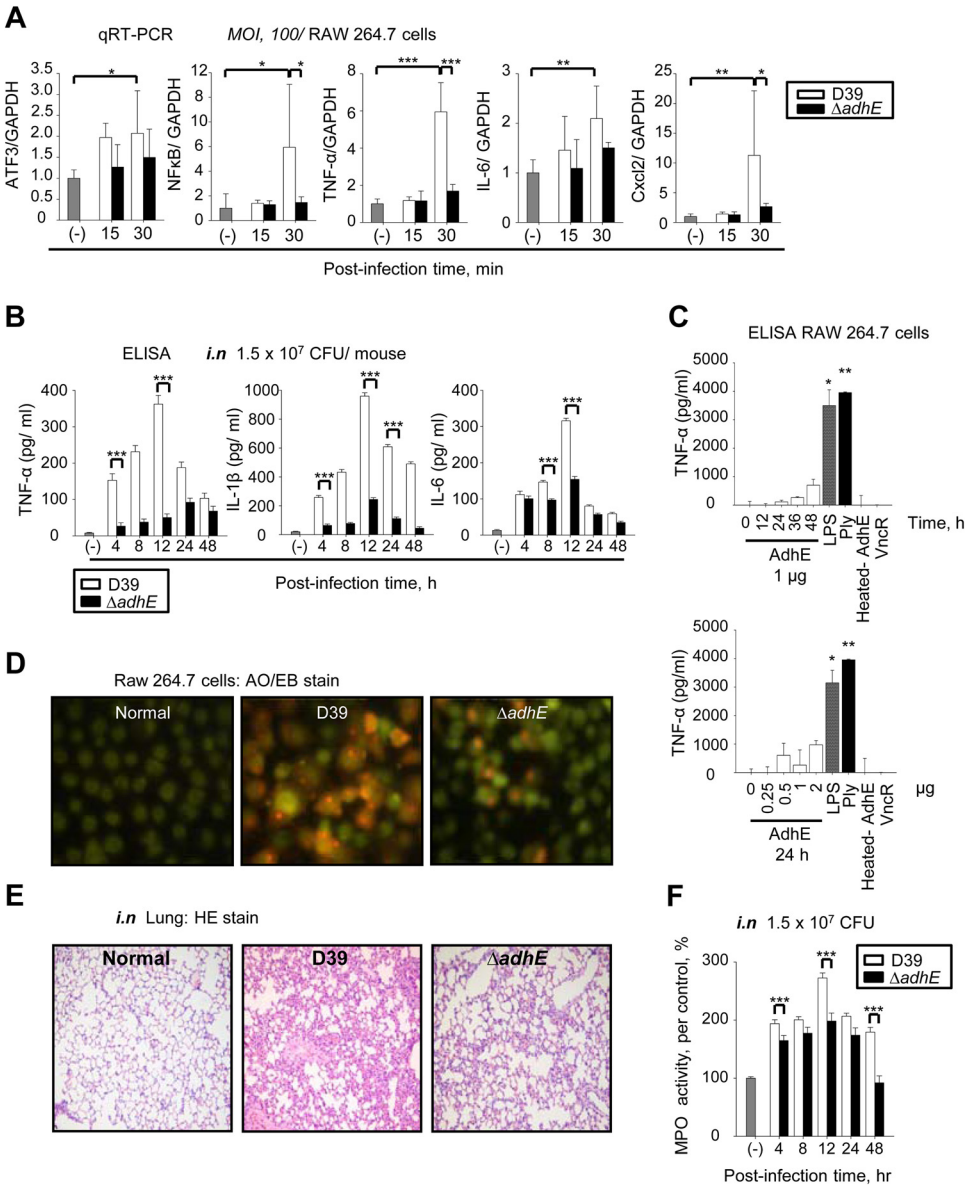


**FIG 4** AdhE increases cytokine production, neutrophil infiltration, inflammation, and cell death. (A) RAW 264.7 cells were infected with *S. pneumoniae* D39 or the Δ*adhE* mutant (MOI 100). Total RNA was isolated and used for qRT-PCR. Data are representative of three independent experiments. (B) Mice ($n = 5$) were infected i.n. with $1.5 \times 10^7$ CFU of the pneumococcal strains, and lung homogenates were used to determine cytokine levels. (C) TNF-α was induced by recombinant AdhE time dependently in RAW 264.7 cells. Recombinant AdhE was added into RAW 264.7 cell culture, and the TNF-α level in the culture supernatant was determined. LPS, heat-inactivated AdhE, and VncR were used as controls. (D) RAW 264.7 cells were infected with the pneumococcal strains (MOI of 100) for 2 h and stained with acridine orange-ethidium bromide (AO-EB) to determine cell death. (E) Mice ($n = 2$) were infected i.n. with $1.5 \times 10^7$ CFU of the pneumococcal strains, and lung histology was examined 24 h after infection via hematoxylin and eosin (HE) staining (magnification, ×200). (F) Mice ($n = 3$) were infected i.n. with $1.5 \times 10^7$ CFU of the pneumococcal strains, and lung homogenates were used for an MPO assay. Significant differences were analyzed by two-way ANOVA (A) or one-way ANOVA (B, C, and F) (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

These results demonstrate that AdhE enhances inflammatory cytokine production, lung inflammation, virulence, and cell death and might be considered a potential virulence factor.

**Rex binds to the *adhE* promoter and represses *adhE* expression in the absence of NADH.** In *Staphylococcus aureus*, Rex represses *adhE* expression by binding to the *adh* promoter, and this binding is competitively modulated by NADH and $NAD^+$. In the presence of NADH, Rex dissociates from the *adhE* promoter; thus, the NADH/$NAD^+$ ratio could potentially signal AdhE induction (30). *S. pneumoniae* has a conserved putative Rex binding motif in the *adhE* promoter (data not shown), and after ethanol treatment, Rex was induced in the D39 WT strain (Fig. 5A). Therefore, Rex could affect *adhE* expression. We examined the relationship between AdhE and Rex after ethanol treatment. AdhE expression in the Δ*rex* mutant at both the mRNA and protein levels was significantly increased compared to the levels in the control (Fig. 5B), demonstrating that Rex downregulates *adhE* expression.

To investigate whether Rex binds to the *adhE* promoter, re-

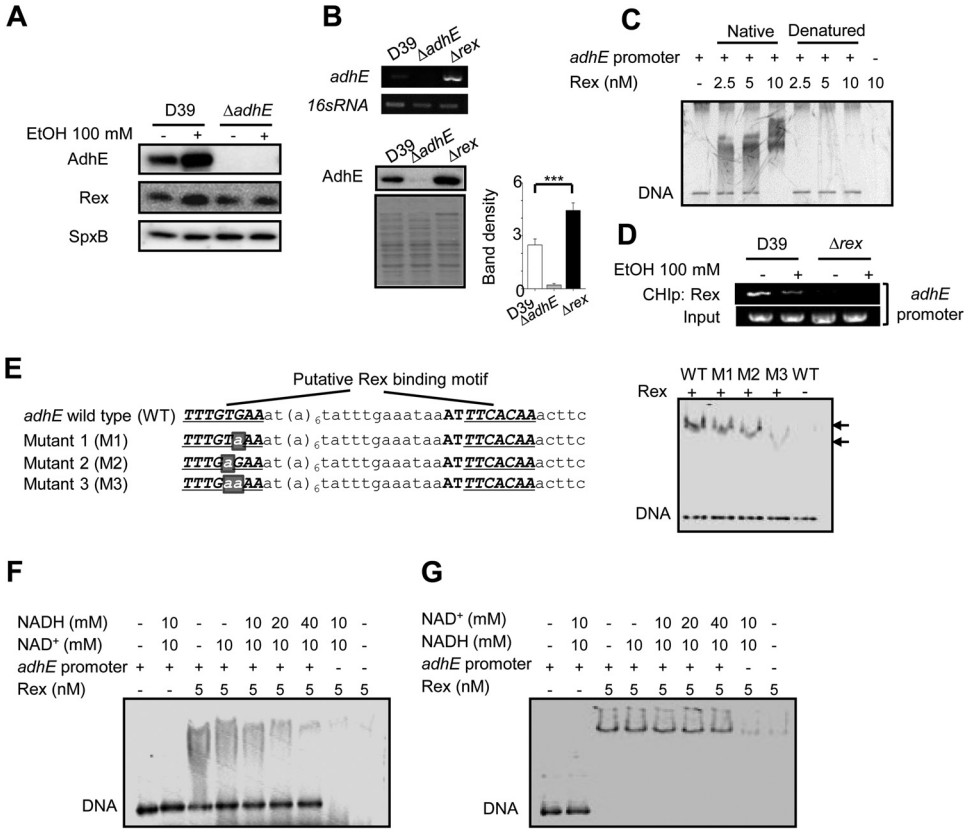


**FIG 5** Rex binds to the *adhE* promoter and represses *adhE* expression, but NADH reverses this repression. (A) Increase of Rex level in ethanol shock depends on AdhE. *S. pneumoniae* D39 and the Δ*adhE* strain were incubated ($A_{550}$ of 0.3) and induced with 100 mM ethanol for 30 min. Cell lysates were collected and subjected to Western blotting. (B) Pneumococcal strains were cultured until an $A_{550}$ of 0.3 was reached. Total RNA was isolated for RT-PCR, or cell lysates were used for Western blotting. (C) Native recombinant Rex binds to the *adhE* promoter. The *adhE* promoter (final concentration, 4 nM) was incubated with 2.5, 5, or 10 nM native or heat-denatured Rex for 10 min, and EMSA was performed. (D) *S. pneumoniae* D39 and Δ*rex* strains were cultured until an $A_{550}$ of 0.3 was reached, and ethanol was added for 30 min. The cell lysates were subjected to a ChIP assay. The Δ*rex* mutant was used as a negative control. (E) A point mutation was introduced in the Rex binding motifs of the *adhE* promoter, and the mutated AdhE promoter (4 nM) was incubated with 20 nM Rex for 10 min. Rex binding was examined by EMSA. (F and G) To determine Rex binding to the *adhE* promoter, the *adhE* promoter was incubated with various concentrations of NADH and $NAD^+$ (final concentration, 4 nM), and EMSA was performed. Data are representative of two (A, C, D, E, F, and G) or three (B) independent experiments. Significant differences were analyzed using one-way ANOVA (C) (***, $P \leq 0.001$).

combinant Rex was purified from *E. coli* (data not shown), and an electromobility shift assay (EMSA) was performed. Purified Rex bound to the *adhE* promoter dose dependently, but heat-denatured Rex did not (Fig. 5C). To examine the effect of ethanol on Rex binding to the *adhE* promoter, a chromatin immunoprecipitation (ChIP) assay was performed. Antibodies raised against Rex were used to precipitate the Rex-*adhE* promoter complex from D39 WT lysate. Rex-bound DNA fragments were purified and amplified by PCR. Rex binding to the *adhE* promoter was decreased after ethanol treatment (Fig. 5D), demonstrating that ethanol increases AdhE expression by diminishing Rex binding to the *adhE* promoter.

To corroborate the Rex binding motif in the *adhE* promoter, point mutations were introduced into the putative Rex binding site (Fig. 5E), and EMSA was performed. A single point mutation decreased Rex binding to the *adhE* promoter, and a double mutation nearly abolished Rex binding. These results suggest that Rex binds to the *adhE* promoter and represses *adhE* expression (Fig. 5E).

Rex plays an important role in redox balance by sensing the pyridine nucleotide NAD(H) (39). Moreover, NADH inhibits Rex binding to DNA in *S. aureus* (30). Therefore, in the presence of ethanol, higher NADH levels (Fig. 1A and Table 2) could cause a dissociation of Rex from the *adhE* promoter. To test this hypothesis, EMSA was performed *in vitro*. When the reaction solution was supplemented with NADH, Rex binding to the *adhE* promoter was decreased (Fig. 5F). Supplementing the reaction mixture with $NAD^+$, however, did not significantly affect Rex binding to the *adhE* promoter (Fig. 5G).

These results indicate that Rex binding might depend on the relative $NAD^+$ and NADH concentrations and that Rex could repress *adhE* expression by binding to the *adhE* promoter. Under ethanol shock conditions, however, increased NADH decreased Rex binding to the *adhE* promoter, resulting in AdhE induction. These data demonstrate that Rex is a redox-dependent repressor for *adhE* expression.

**Alcohol potentiates *S. pneumoniae* type 2 virulence by upregulating AdhE.** To determine the effect of ethanol on *S. pneumoniae* virulence *in vitro*, the cytotoxicity of *S. pneumoniae* on RAW 264.7 cells was measured. After ethanol treatment, D39 WT cytotoxicity increased significantly compared to that of the non-ethanol-treated control ($P < 0.01$) (Fig. 6A). Cytotoxicity of the

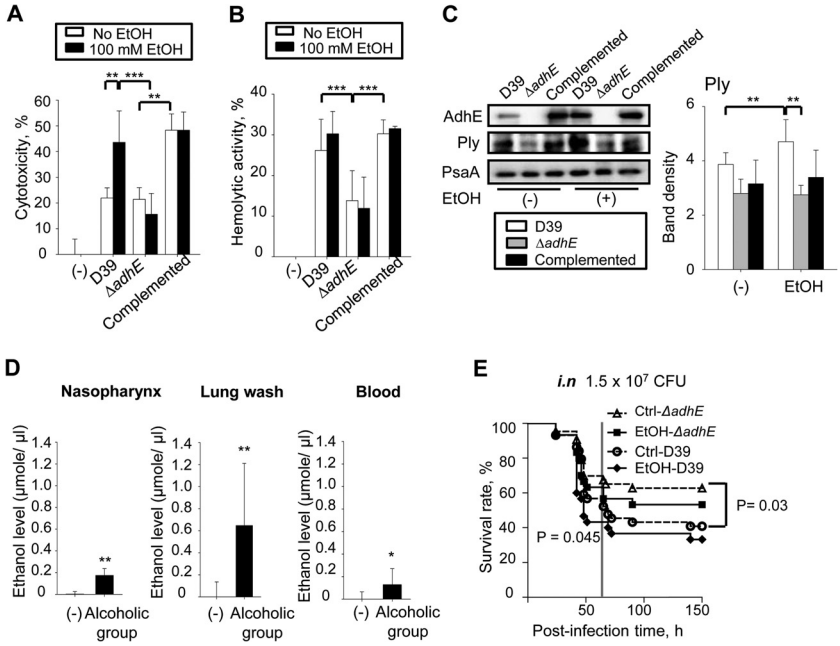


**FIG 6** Pneumococcal virulence increased after exposure to ethanol due to AdhE induction. (A) Ethanol-treated cells were more susceptible to infection with D39 than with the Δ*adhE* mutant. Ethanol-treated RAW 264.7 cells were infected with pneumococcal strains (MOI of 100) for 2 h, and cytotoxicity was determined by MTT assay. (B) Hemolytic activity of the pneumococcal strains after ethanol treatment for 30 min was determined. (C) The cell lysates from the experiment shown in panel B were collected and subjected to Western blotting. (D) Mice ($n = 5$) were fed ethanol for 10 days, and the ethanol levels of the nasopharynx, lung wash, and blood were determined. (E) Mice ($n = 20$) were fed ethanol for 10 days and infected i.n. with $1.5 \times 10^7$ CFU of *S. pneumoniae* D39 or the Δ*adhE* strain. Survival times were determined. Data are representative of three independent experiments (A to C). Significant differences were analyzed by one-way ANOVA (A to C), a Mann-Whitney rank sum test (D), or a log rank test (E) (*, $P \leq 0.05$).

Δ*adhE* mutant did not change after ethanol treatment (Fig. 6A). The complemented strain had a level of cytotoxicity similar to that of the WT after ethanol treatment (Fig. 6A), possibly due to constitutive high expression of AdhE. In addition, the D39 WT and complemented strains had higher hemolytic activity than the Δ*adhE* mutant ($P < 0.001$), and ethanol treatment slightly increased the hemolytic activity of the WT and complemented strains (Fig. 6B). Moreover, ethanol treatment increased the Ply level in the D39 WT strain compared to that in the non-ethanol-treated control ($P < 0.01$) but not in the Δ*adhE* mutant (Fig. 6C).

When ethanol was administered to mice according to the ethanol feeding protocol (28), alcoholic mice did not exhibit any abnormal behavior. Moreover, mice fed up to 20% ethanol for 5 weeks were still considered healthy enough to tolerate experiments (40, 41) prior to infection with *S. pneumoniae* (41) or *Mycobacterium tuberculosis* (40). Therefore, this ethanol feeding procedure is suitable for experiments evaluating the effects of alcohol. To further identify the effect of AdhE on virulence *in vivo*, mice were fed ethanol for 10 days (alcoholic mice), and ethanol levels in the nasopharynx (nasal tissue), lung wash, and blood were measured by an alcohol dehydrogenase assay. The ethanol levels of alcoholic mice were significantly higher than those in the water-fed control group, and the highest ethanol concentration was detected in lung wash (Fig. 6D).

When alcoholic mice were infected i.n. with $1.5 \times 10^7$ CFU of *S. pneumoniae* and mortality rate was examined, alcoholic mice infected with D39 WT became morbid earlier than the other mice and were more susceptible to D39 WT than the control group (Fig. 6E) ($P = 0.045$). Alcoholic mice infected with the Δ*adhE* mutant, however, did not show any significant difference in susceptibility compared with nonalcoholic mice infected with the Δ*adhE* strain (Fig. 6E). Moreover, the control group infected with D39 showed 59% mortality, whereas the group infected with the Δ*adhE* mutant exhibited approximately 34% mortality ($P = 0.03$). These results indicate that AdhE potentiates *S. pneumoniae* virulence under an ethanol stress condition.

## DISCUSSION

Alcohol abuse is a serious health problem in the United States and in most developed countries, especially among young people (3, 10). Approximately 20 million Americans meet the diagnostic criteria for alcoholism, and 20% to 40% of patients admitted to hospitals present with disease exacerbated by alcohol abuse (3). The most widely recognized infectious disease associated with ethanol abuse is bacterial pneumonia (3). Thus, a virulence factor that contributes to pathogenicity during infection might be a candidate target for pneumococcal treatment in alcoholics.

Bacterial metabolism is maintained by balancing the redox state of all cellular components (42). Increased oxidative stress can break this balance and damage essential cellular components. Therefore, bacteria have evolved specialized sensors to monitor redox signals and adapt to altered redox environments. One bacterial redox sensor, Rex, senses the NADH/$NAD^+$ redox state and modulates respiratory gene expression as a redox-regulated repressor (39). Most Gram-positive bacteria, including several important human and animal pathogens, such as *S. aureus*, *L. monocytogenes*, and *S. pneumoniae*, encode Rex homologues (39). The *adhE*-binding activity of Rex is modulated specifically by the ratio of NADH to $NAD^+$. Under aerobic conditions, the $NAD^+$ concentration is higher than the NADH concentration, and Rex has a

high affinity for the *adhE* promoter. Under anaerobic conditions, Rex binds to NADH and loses its affinity for the *adhE* promoter, leading to the transcription of genes involved in respiration and metabolism (39), such as fermentative enzymes comprising AdhE (30, 43, 44). In this study, we identified AdhE as a virulence factor that might be regulated by Rex under an alcoholic condition.

After ethanol ingestion, the *in vitro* antipneumococcal activity of surfactants and pneumococcal clearance were inhibited (10). Moreover, in ethanol-fed rats, more *S. pneumoniae* type 3 bacteria spread into the bloodstream (11). Although alcoholics are highly susceptible to *S. pneumoniae* infection (11), how alcohol contributes to pneumococcal virulence has not been resolved. Among six alcohol dehydrogenases in *S. pneumoniae* type 2 D39, only SPD_1834 (AdhE) was strongly induced by ethanol (15). AdhE has 98% identity with *Streptococcus gordonii* AdhE (AcdH), which uses only ethanol as its substrate (45). Moreover, a BLAST search revealed that SPD_0265 has 93% identity with *S. gordonii* AdhA, which preferentially catalyzes 1-propanol (45). SPD_1126 (GenBank) seems to function as a threonine dehydrogenase in amino acid transport and metabolism. SPD_1636 has 85% identity with *S. gordonii* AdhB, which preferentially catalyzes 1-butanol (45). On the other hand, SPD_1865 (GenBank) seems to function in propanoate metabolism and as a formaldehyde dehydrogenase-like ADH. Furthermore, SPD_1895 (GenBank) has 100% identity with the NADH-dependent methanol dehydrogenase in *S. pneumoniae* 70585. Therefore, in the presence of ethanol, AdhE could preferentially play a major role in catalysis of ethanol.

Here, we demonstrated that ethanol shock increases acetaldehyde and NADH concentrations via AdhE (Fig. 1 and Table 2). A high NADH level restores a reduced condition, which triggers superoxide or $H_2O_2$ generation (46). Thus, under ethanol shock, acetaldehyde and $H_2O_2$ accumulation could cause a toxic response in host cells during pneumococcal infection (Fig. 1B, 4D and E, and 6A and E). In particular, acetaldehyde, which inhibits cell proliferation (47), is a carcinogen in rats (48) and humans (49) and inhibits DNA repair enzymes (50). $H_2O_2$ is a common form of reactive oxygen species (ROS) that damages cell membranes via lipid oxidation (51). $H_2O_2$ in *S. pneumoniae* is a toxin for alveolar epithelial cells (52). *S. pneumoniae* tolerates up to 10 mM $H_2O_2$ (53); therefore, *S. pneumoniae* can survive in $H_2O_2$ concentrations that damage host cells during infection. The ethanol stress condition also increases Ply levels (both intracellular and released) (Fig. 3A and 6C and data not shown). Because Ply is a pore-forming cytolysin that has hemolytic activity, Ply and $H_2O_2$ in combination can impair human ciliated epithelium cells additively (54). An increase in Ply or $H_2O_2$ alone or in combination with ethanol shock could contribute to *S. pneumoniae* pathogenesis involving respiratory tract infection, thus enhancing pneumococcal virulence.

Colonization of the lungs is a key stage in a local pneumococcal infection of the nasopharynx as it converts into a systemic infection (55). This conversion is linked to the activation of alveolar macrophages and, subsequently, heavy neutrophil recruitment (56). A pneumococcal virulence factor, Ply, has been shown to activate nitric oxide (NO) production in neutrophils (57). Moreover, *ply* and *spxB* mRNAs were abundant in the nasopharynx following intranasal challenge with the D39 WT strain (58), suggesting that Ply and SpxB are important for the oxidative stress response. Alcoholics are susceptible to infectious disease, particularly bacterial pneumonia (59). Pneumonia is an inflammation of the alveoli that causes them to fill with excess fluid, and the resulting inhibition of $O_2$ and $CO_2$ exchange can allow pneumococci to proliferate. Therefore, the mechanism causing bacterial pneumonia should be elucidated. Since AdhE was expressed under reducing conditions, it can enhance pneumococcal survival and virulence. Importantly, Ply expression and $H_2O_2$ levels increased in WT bacteria after ethanol exposure. Thus, AdhE might contribute to the ability of pneumococci to colonize the upper respiratory tract and subsequently infect the lower respiratory tract.

*S. pneumoniae* type 2 AdhE increases inflammatory cytokines *in vivo* and *in vitro* (Fig. 4). Recombinant AdhE could increase TNF-α secretion but is not as potent as Ply (Fig. 4C). In addition, AdhE was not released into the culture supernatant (data not shown). Therefore, AdhE increases the Ply level, resulting in an increase of cytokine production. How AdhE stimulates TNF-α production should be studied further.

In conclusion, *S. pneumoniae* type 2 AdhE enhances hemolytic activity, neutrophil infiltration, inflammatory cytokine production, and inflammation (Fig. 3 and 4). Ethanol-induced AdhE produces NADH (Fig. 1 and Table 2), which disrupts the Rex-*adhE* promoter association (Fig. 5) and results in increased acetaldehyde (Fig. 1B) and $H_2O_2$ levels (Fig. 2D) and increased virulence (Fig. 6). Therefore, AdhE contributes to enhanced pneumococcal virulence under alcoholic conditions. These results suggest that *S. pneumoniae* D39 is tolerant to ethanol stress, which subsequently increases virulence through AdhE. Thus, AdhE might be a useful target in the treatment of pneumococcal disease in alcoholics.

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