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RESEARCH ARTICLE

Streptococcus pneumoniae DNA Initiates Type I Interferon Signaling in the Respiratory Tract

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ABSTRACT The mucosal epithelium is the initial target for respiratory pathogens of all types. While type I interferon (IFN) signaling is traditionally associated with antiviral immunity, we demonstrate that the extracellular bacterial pathogen *Streptococcus pneumoniae* activates the type I IFN cascade in airway epithelial and dendritic cells. This response is dependent upon the pore-forming toxin pneumolysin. Pneumococcal DNA activates IFN- β expression through a DAI/STING/TBK1/IRF3 cascade. *Tlr4*^{-/-}, *Myd88*^{-/-}, *Trif*^{-/-}, and *Nod2*^{-/-} mutant mice had no impairment of type I IFN signaling. Induction of type I IFN signaling contributes to the eradication of pneumococcal carriage, as IFN- α/β receptor null mice had significantly increased nasal colonization with *S. pneumoniae* compared with that of wild-type mice. These studies suggest that the type I IFN cascade is a central component of the mucosal response to airway bacterial pathogens and is responsive to bacterial pathogen-associated molecular patterns that are capable of accessing intracellular receptors.

IMPORTANCE The bacterium *Streptococcus pneumoniae* is a leading cause of bacterial pneumonia, leading to upwards of one million deaths a year worldwide and significant economic burden. Although it is known that antibody is critical for efficient phagocytosis, it is not known how this pathogen is sensed by the mucosal epithelium. We demonstrate that this extracellular pathogen activates mucosal signaling typically activated by viral pathogens via the pneumolysin pore to activate intracellular receptors and the type I interferon (IFN) cascade. Mice lacking the receptor to type I IFNs have a reduced ability to clear *S. pneumoniae*, suggesting that the type I IFN cascade is central to the mucosal clearance of this important pathogen.

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The airway epithelium is a primary site for the recognition of both viral and bacterial pathogens and initiates the host defense against respiratory pathogens. Epithelial cells produce chemokines and cytokines, such as interleukin-8 (IL-8), IL-6, IL-1 β , granulocyte-macrophage colony-stimulating factor (GM-CSF), and G-CSF, that function to recruit and activate phagocytic cells to eradicate organisms and infected cells (1). These mucosal epithelial cells are also a major source of the type I interferons (alpha interferon [IFN- α] and IFN- β) that are critical for viral clearance and coordinating immune regulation (2). The role of type I IFNs in pulmonary defenses is exemplified by the increased susceptibility of mice lacking the IFN- α/β receptor to influenza virus infection and secondary bacterial pneumonia (3). Bacterial induction of type I IFNs has been examined primarily within the context of infection due to intracellular pathogens and limited to pathways expressed in macrophages and dendritic cells (DCs) (4). These studies indicate that the release of bacterial pathogen-associated molecular patterns (PAMPs) within the phagolysosome, from the phagolysosome into the cytosol (5), or from bacteria within the cytosol also induces type I IFN responses, a situation which closely mimics viral infection (6). However, there is little data to indicate how nonphagocytic cells could produce type I IFNs

in response to extracellular bacteria, such as the organisms which most commonly cause pneumonia.

The upper airway is the initial site of colonization by *S. pneumoniae*. Aspiration of these organisms into the lower airways induces a florid proinflammatory response ascribed to the stimulation of Toll-like receptors (TLRs) on the surface of airway and immune cells and activation of NF- κ B-dependent signaling. *S. pneumoniae* produces a cholesterol-dependent, pore-forming toxin, pneumolysin (Ply), which has inherent proinflammatory activity (7) and is critical in pathogenesis (8, 9). As autolysis and the release of cell fragments accompany pneumococcal replication, the pneumolysin pore provides a conduit for these PAMPs to gain access to the mucosal airway cells (10). The presence of bacterial components, especially DNA, within the cytosol is a potent stimulus for the type I IFN cascade (11) that can be recognized by a number of recently described receptors, including RIG-1-like helicases, DAI (DNA-dependent activator of IFN regulatory factors), and several others (6, 11–15). In the experiments described herein, we demonstrate that pneumolysin and DNA are involved in the activation of type I IFN signaling, a response which contributes to pneumococcal clearance from the airway.

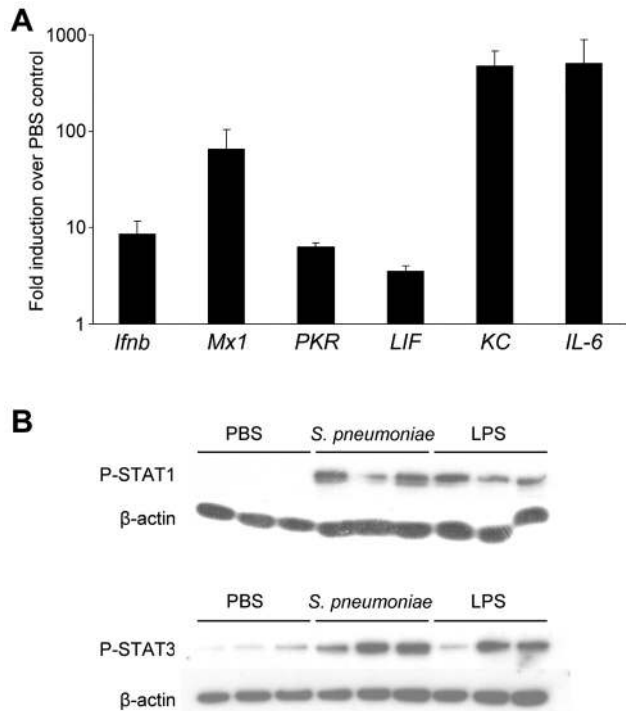


FIG 1 *S. pneumoniae* activates the type I IFN response *in vivo*. Mice were infected with 2×10^7 CFU of *S. pneumoniae* and analyzed 24 h later. (A) qRT-PCR of type I IFN and inflammatory cytokine genes from RNA extracted from infected mouse lungs. Graphs display means with standard deviations ($n = 3$). (B) Immunoblots of transcription factors P-STAT1 and P-STAT3 from mouse lungs. β -Actin was used as a loading control. Each lane represents an individual mouse. Data are representative of two experiments. LPS from *E. coli* (50 μ g per mouse) was used as a positive control.

RESULTS

***S. pneumoniae* induces expression of type I IFN genes in the murine lung.** To test the hypothesis that *S. pneumoniae* activates type I IFN signaling, C57BL/6J mice were infected intranasally with 2×10^7 CFU of *S. pneumoniae* D39 for 24 h and induction of *Ifnb* and other cytokine-encoding genes in lung tissue was analyzed using quantitative reverse transcription (qRT)-PCR (Fig. 1A). A 10-fold increase in *Ifnb* was observed in addition to induction of the IFN- β -inducible genes *Mx1*, *PKR*, and *LIF* (16, 17). The chemokine *KC* (as a positive control) and the cytokine *IL-6* (also a type I gene product) were up-regulated by almost 1,000-fold. To document that these type I IFN-dependent genes were induced through the expected JAK/STAT signaling cascade, phosphorylation of the STAT1 (P-STAT1) and STAT3 (P-STAT3) transcription factors in the infected mouse lung tissues was demonstrated and compared to a lipopolysaccharide (LPS) control (Fig. 1B).

Pneumolysin contributes to induction of type I IFN signaling *in vivo* and *in vitro*. *S. pneumoniae* pneumolysin is reported to interact with TLR4 (7, 18), which interacts with TRIF (TIR domain-containing adapter inducing IFN- β) to participate in the induction of type I IFN signaling. Recent data (19) have suggested that the immune response to pneumolysin does not involve TLR4. Pneumolysin also functions as a pore that allows bacterial PAMPs access to the epithelial cytosol and pathogen recognition receptors

(10). We assessed *Ifnb* production in mice 4 h following intranasal infection with 10^7 CFU of wild-type (WT) *S. pneumoniae* D39 or a *ply* null mutant. There was 40-fold induction of *Ifnb* by the WT *S. pneumoniae* strain under these conditions but only a 3-fold induction in mice infected with the *ply* mutant ($P = 0.029$) (Fig. 2A). At this 4-h time point, *LIF* and *Mx1* were expressed and this expression was pneumolysin dependent. Significantly reduced levels of *KC* and *IL-6* were also associated with exposure to the *ply* mutant ($P < 0.001$).

Despite equal numbers of WT and mutant bacteria in the lung at this 4-h time point (Fig. 2B), there were significant differences in the immune cell populations. Pneumolysin expression was required for the recruitment of neutrophils, as the *ply* mutant and the phosphate-buffered saline (PBS) control attracted similar proportions of neutrophils into the lung (Fig. 2C). Infection with the *ply* null mutant resulted in the retention of a significantly ($P = 0.0002$) larger percentage of CD11c⁺ cells (analogous to PBS mice), representing macrophages and DCs, than did infection with WT *S. pneumoniae* (Fig. 2D), which is consistent with the idea that Ply is a major immunostimulant (7, 20, 21).

To assess epithelial cell involvement in the signaling of the type I IFN response, we tested the induction of *Ifnb* in murine airway epithelial cells in primary culture. Murine airway epithelial cells stimulated with *S. pneumoniae* had 10-fold induction of *Ifnb* at 4 h compared with the 2-fold induction stimulated by the *ply* null mutant (Fig. 2E). *Ifnb* induction in epithelial cells was not inhibited by cytochalasin D, indicating that endocytosis was unlikely to be involved in this signaling (Fig. 2F).

Pneumolysin-dependent pore formation is involved in induction of type I IFN signaling. The role of pore formation in the stimulation of *Ifnb* was examined using a Ply W433F mutant, a toxoid (PdB)-expressing strain in the *S. pneumoniae* P1121 background that has 0.1% of the WT hemolytic activity but is still capable of binding and oligomerizing into cellular membranes (22, 23). STAT1 phosphorylation in murine lungs exposed to the *S. pneumoniae* toxoid-expressing strain was substantially less than that induced by the P1121 parent strain (Fig. 2G). We used fluorescent (Alexa Fluor 488 [AF488]-labeled) Ply to follow the distribution of the toxin in polarized airway monolayers, which was limited to the apical surfaces, consistent with a role as a pore (Fig. 2H). Activation of *Ifnb* was not detected when purified recombinant Ply was applied to the cells (Fig. 2I), as a control for signaling that p38 phosphorylation was still detected (see Fig. S1 in the supplemental material). However, when Ply was applied in the presence of a *ply* pneumococcal cell lysate, a significant increase ($P = 0.045$) in *Ifnb* induction was observed, but not with a toxoid version of the protein (Fig. 2J and Fig. S2 in the supplemental material shows that the toxoid has significantly reduce hemolytic activity). These results suggest that pneumolysin pore activity is involved in the delivery of a ligand that activates type I IFN signaling.

Induction of *Ifnb* by *S. pneumoniae* is TLR4, MyD88, trif, and Nod2 independent. We next examined which signaling pathways are involved in mediating pneumococcal activation of IFN- β . As it has been suggested that Ply functions as a TLR4 agonist (7, 18, 24), we tested the participation of TLR4 and its adaptor TRIF in mediating pneumococcal type I IFN signaling (Fig. 3). C57BL/6J WT, *Tlr4*^{-/-}, and *Trif*^{-/-} mice were intranasally inoculated with either PBS or 10^7 CFU of *S. pneumoniae*, and induction of *Ifnb* and STAT1 phosphorylation was examined 24 h later.

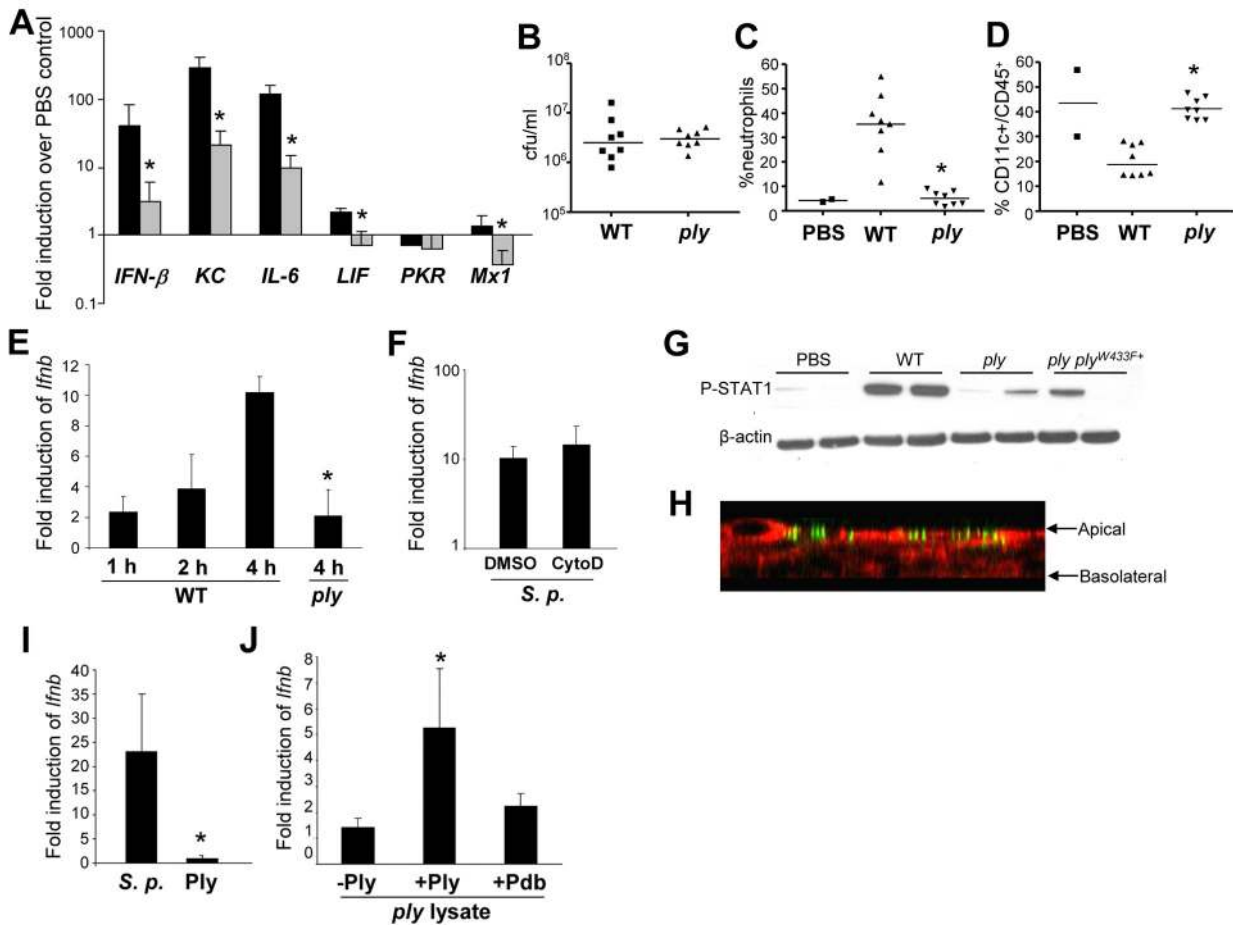


FIG 2 Pneumolysin is involved in the induction of type I IFN. C57BL/6J mice were infected with 2×10^7 CFU of *S. pneumoniae* (*S. p.*) intranasally and studied at 4 h (A to D). (A) WT-infected lung data are shown in black, and *ply* mutant-infected lung data are shaded grey. *, $P < 0.05$ (Student's *t* test; $n = 8$). Lung homogenates were analyzed for bacterial counts (B), neutrophils (percentage of Ly6G Ly6C⁺ CD45⁺ cells) (C), and CD11c⁺ cells (D) by FACS. Lines represent median values. *, $P < 0.05$ (WT versus *ply* mutant; Mann-Whitney test; $n = 8$, except for the PBS controls). (E) Murine nasal epithelial cells in primary culture were stimulated for the times indicated with WT or *ply* mutant *S. pneumoniae* D39 in the presence or absence of cytochalasin D (CytoD) (F). DMSO, dimethyl sulfoxide. *, $P < 0.05$ (Student's *t* test; $n = 3$). (G) Mice were intranasally administered *S. pneumoniae* strains (P1121 background) expressing WT pneumolysin, no pneumolysin, or a nonhemolytic toxoid variant and examined 4 h later. Lung homogenates were immunoblotted for P-STAT1. Each lane represents an individual mouse. (H) AF488-labeled pneumolysin is visualized in murine nasal epithelial cells at 1 h poststimulation. Murine epithelial cells in primary culture following stimulation with *S. pneumoniae* D39 or purified pneumolysin (Ply) (I) or with *ply* lysates with and without purified pneumolysin or Pdb (J). *, $P < 0.05$ compared to unstimulated cells (Student's *t* test; $n = 3$). RNA was extracted and analyzed for levels of IFN- β by qRT-PCR. All graphs display means with standard deviations.

Not only did all of the mice express *Ifnb*, but the *Tlr4*^{-/-} mice had increased levels of P-STAT1 (Fig. 3A and B) and *Ifnb* production (3-fold higher than those of the WT; Fig. 3B), which were not due to increased bacterial numbers in the lung (data not shown), as has been reported by others (25). *Trif*^{-/-} mice were also able to phosphorylate STAT1, indicating that endosomal TLR3 and TLR4, which use Trif/Tram adaptors, are not involved in the response to *S. pneumoniae*.

Type I IFN signaling through the other endosomal TLRs (TLR7, TLR8, and TLR9) that utilize the adapter MyD88 (7) was also excluded. In response to *S. pneumoniae*, *MyD88*^{-/-} null mice had increased activation of STAT1 and *Ifnb* induction compared to that of WT mice (Fig. 3A and B), consistent with increased levels of infection (26).

We next screened for involvement of the cytosolic receptor Nod2 (nucleotide-binding oligomerization domain 2), which

senses peptidoglycan (27) and can be transported via the pneumolysin pore (10, 28). Although a link between Nod2 and type I IFN production has been observed (29–31), *Nod2*^{-/-} mice still responded to *S. pneumoniae* (Fig. 3A and B).

Pneumococcal DNA activates IFN- β expression. During active growth, pneumococci spontaneously lyse, releasing cellular components, including DNA. *S. pneumoniae* produces an autolysin (LytA, an amidase) required for autolysis and release of cell wall components (9). Incubation of epithelial cells with a *lytA* strain of *S. pneumoniae* did not activate type I IFN signaling (Fig. 4B), indicating that the inducing ligand is liberated from lysed cells. DNA is a potent activator of type I IFN signaling, and Ply could permit bacterial DNA to enter the cytosol, where it would interact with one or more of the several different types of DNA sensors that induce type I IFN signaling (11). Pneumococcal lysates incubated with DNase were found to stimulate signifi-

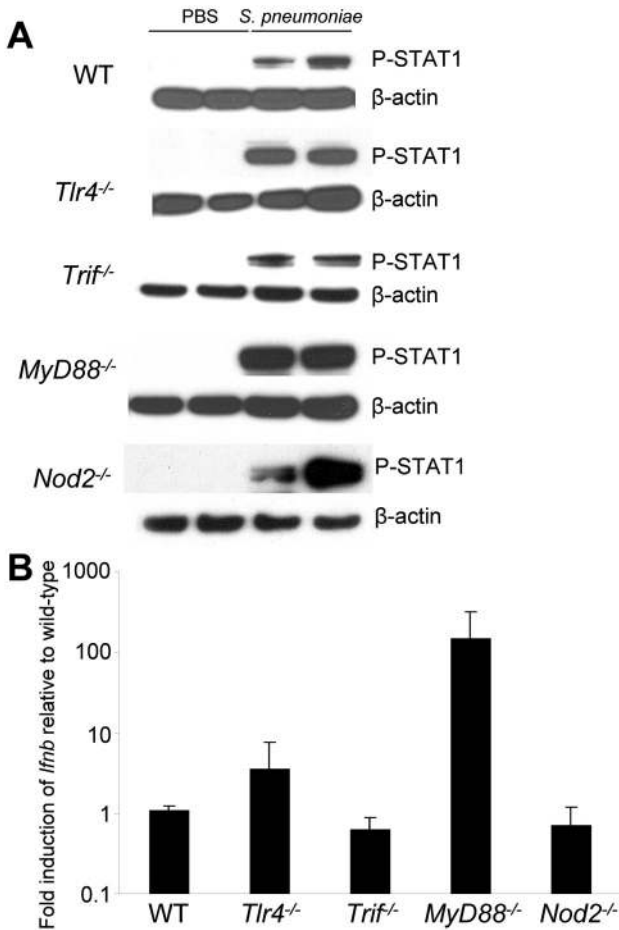


FIG 3 Induction of IFN- β production by *S. pneumoniae* in mice with mutations in TLR4, Nod2, and innate adapters. WT and *Tlr4*^{-/-}, *Trif*^{-/-}, *MyD88*^{-/-}, and *Nod2*^{-/-} mutant C57BL/6J mice were infected intranasally with 10⁷ CFU of *S. pneumoniae* and examined 24 h later. (A) Lung homogenates were assayed for STAT1 phosphorylation. Each lane represents an individual mouse. Shown are representative experiments. (B) Lung tissue was analyzed for induction of IFN- β by qRT-PCR ($n = 4$, except for *Nod2*^{-/-} mutant mice [$n = 6$]).

cantly less ($P = 0.046$) *Ifnb* than control lysates (Fig. 4A), further implicating pneumococcal DNA as the ligand. To further impli-

cate pneumolysin in enabling DNA entry into the epithelial cells, we demonstrated increased amounts of pneumococcal DNA in murine epithelial cells incubated with WT *S. pneumoniae* compared to the *ply* mutant (Fig. 4C). However, reconstitution of the system *in vitro*, i.e., adding purified Ply and *S. pneumoniae* DNA to murine airway epithelial cells in primary culture, did not result in *Ifnb* induction.

DAI participates in sensing of pneumococcal DNA. There are numerous receptors that respond to foreign DNA but relatively few that do not involve TLRs. DNA receptors that stimulate IFN- β expression are available within both endosomes and the cytosol (11). DAI is a cytosolic double-stranded DNA sensor that responds to DNA from several sources (32), although it has been shown to be dispensable for DNA detection in cells isolated from DAI null mice (33). In response to *S. pneumoniae* in the lung, there was a significant (15-fold, $P = 0.002$) increase in DAI transcription, consistent with its known induction by IFN- β (32) and suggestive of involvement in pneumococcal signaling (Fig. 5A).

We took advantage of macrophage cell lines and dendritic cells in primary culture to evaluate the participation of the expected components of the DAI cascade in response to pneumococcal DNA. To confirm that pneumococcal DNA was a ligand in immune cells, we incubated pneumococcal lysate treated with DNase or RNase before stimulating dendritic cells (Fig. 5B). We observed a significant (90%, $P = 0.007$) decrease in *Ifnb* induction in the DNase-treated lysate. No loss of induction was observed in the RNase-treated sample. The requirement for DAI in recognizing pneumococcal DNA was confirmed in DAI-deficient macrophages (Fig. 5C). A significant (50%, $P = 0.048$) decrease in *Ifnb* induction was observed in DAI-deficient cells compared to that in WT controls. This was not specific to just pneumococcal genomic DNA, as DNA from *Pseudomonas aeruginosa* and *Staphylococcus aureus* also stimulated reduced IFN- β signaling in the DAI null background (bone marrow-derived DCs) (see Fig. S3 in the supplemental material; Fig. S4 with controls). We also investigated the role of the recently identified factor STING (stimulator of IFN genes) (14), which is essential for the recognition of non-CpG intracellular DNA species (Fig. 5C). Transfection of bone marrow-derived macrophages with pneumococcal DNA resulted in a complete absence of *Ifnb* induction in the *STING*^{-/-} cells ($P < 0.0001$).

To confirm that the expected distal components of the DNA-sensing cascade were involved, we transfected pneumococcal

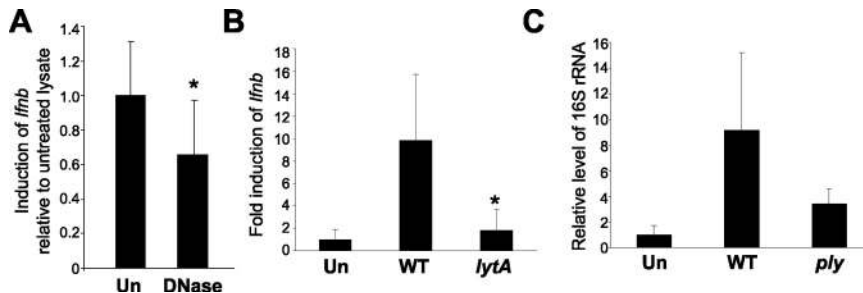


FIG 4 *S. pneumoniae* DNA activates type I IFN production. (A) Murine nasal epithelial cells were stimulated for 4 h with pneumococcal lysate that had been left untreated or treated with DNase. (B) Murine nasal epithelial cells were stimulated for 4 h with the WT or *lytA* mutant strain ($n = 3$). IFN- β induction was assessed by qRT-PCR and is displayed as fold induction over that in PBS-only controls. (C) Murine nasal epithelial cells were stimulated for 4 h with the WT and *ply* mutant strains ($n = 3$). DNA was extracted from the epithelial cells, and levels of *S. pneumoniae* 16S rRNA were measured by qRT-PCR. Un, untreated lysate. *, $P < 0.05$ compared to WT or untreated samples (Student's *t* test). All results shown are representative of at least two independent experiments.

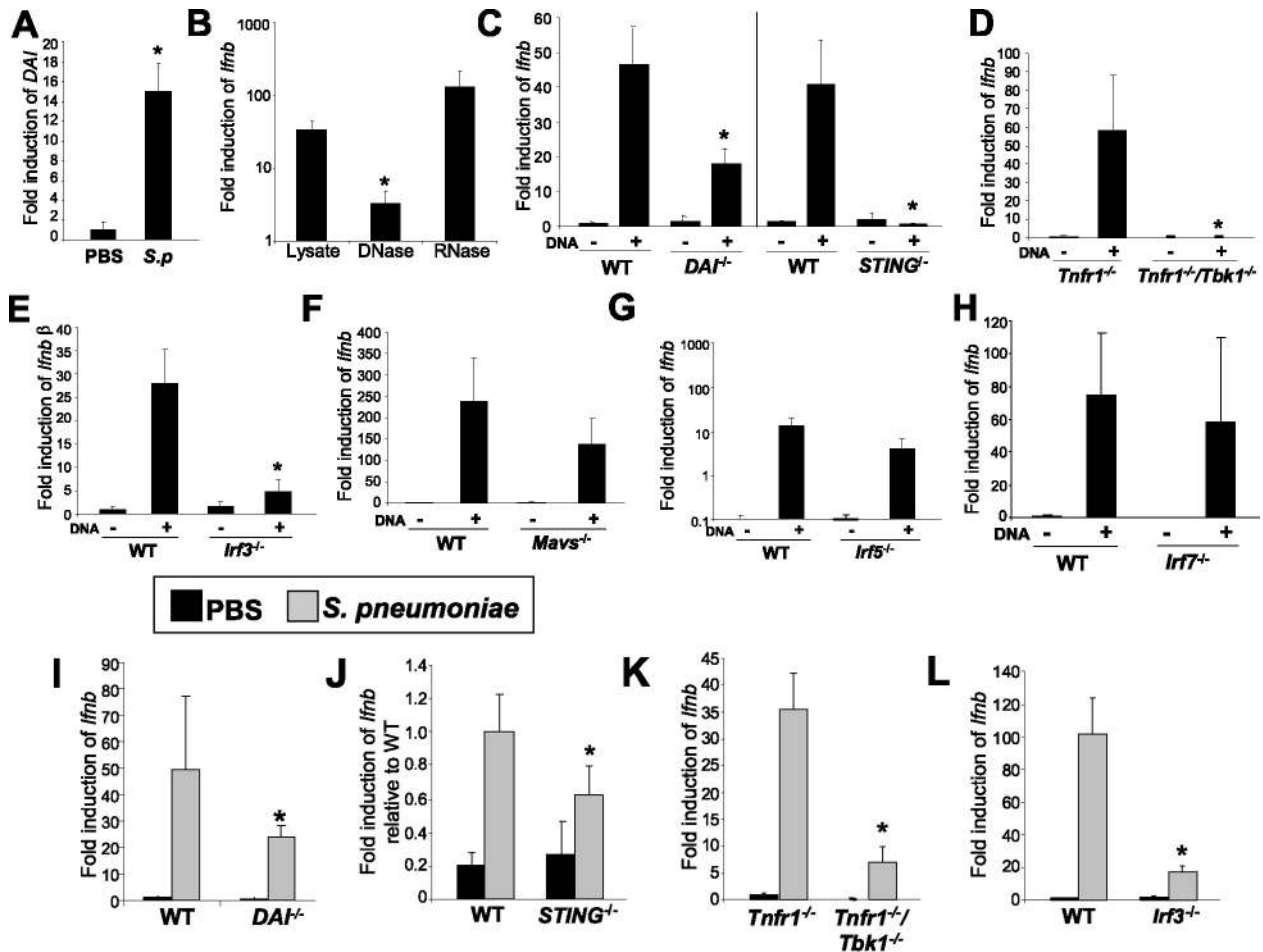


FIG 5 Pneumococcal DNA is sensed by the cytosolic DAI sensor. (A) Mice were infected with 10^7 CFU of *S. pneumoniae* D39 (*S.p*), and lung tissue was analyzed for induction of *DAI* 24 h later ($n = 4$). (B) Bone marrow-derived cells were stimulated with enzymatically treated *S. pneumoniae* lysates and then analyzed for *Ifnb* production by qRT-PCR ($n = 3$). (C) Bone marrow-derived cells from *DAI*^{-/-} ($n = 6$) and *STING*^{-/-} ($n = 6$) mice were transfected with pneumococcal DNA, and *Ifnb* induction was analyzed by qRT-PCR. *Ifnb* induction of pneumococcal DNA-stimulated knockout bone marrow-derived cells and their respective controls downstream of DAI (D and E) and other type I signaling components (F to H) ($n = 3$). Bone marrow-derived DCs and macrophages from *DAI*^{-/-} (I) and *STING*^{-/-} (J) mice ($n = 6$) and *TBK1*^{-/-} (K) and *IRF3*^{-/-} (L) mice ($n = 3$) were stimulated with *S. pneumoniae* for 2 h, and *Ifnb* levels were quantitated by qRT-PCR. Graphs display means plus standard deviations and are representative of two experiments (excluding *STING* data), and data are expressed as fold induction compared to that of the control cell line stimulated with PBS alone. *, $P < 0.05$ compared to unstimulated or WT samples (Student's *t* test).

DNA into macrophage cell lines cells lacking TANK-binding kinase 1 (TBK1) (in a TNFR1 null background since TBK1 null mice are embryonic lethal [34, 35]) (Fig. 5D) and IFN regulatory factor 3 (IRF3) (Fig. 5E) transcription factors, both of which were required for *Ifnb* induction, results consistent with the involvement of DAI in the sensing of cytosolic DNA (15).

Participation of RIG-1, which stimulates helicase activity in a complex with MAVS, resulting in IFN- β expression through TBK1 and IRF3 (12, 13), was excluded (Fig. 5F), as was signaling through IRF5 (Fig. 5G) and IRF7 (Fig. 5H).

The significance of DNA sensing in the context of live organisms was also addressed. *S. pneumoniae*-induced *Ifnb* transcription in bone marrow-derived cells from *DAI*^{-/-} mice was $>50\%$ decreased ($P = 0.048$) and that in cells isolated from *STING*^{-/-} mice was $>40\%$ decreased ($P = 0.0081$) compared with that in controls (Fig. 5I and J). Macrophage lines deficient in TBK1 and IRF3 displayed an even greater reduction of *Ifnb* induction (Fig. 5K and L), confirming the participation of the expected components of DAI-mediated IFN- β signaling.

Type I IFN signaling contributes to *S. pneumoniae* clearance.

The physiological role of type I IFNs in the pathogenesis of pneumococcal infection was then explored by comparing the colonization of WT mice with that of *Ifnar*^{-/-} mice. At 7 days postinoculation of 129 SvEv mice infected intranasally with 10^7 CFU of *S. pneumoniae*, *Ifnar* null mice had significantly higher bacterial counts (7-fold increase, $P = 0.0102$) in nasal lavage fluid than WT controls (Fig. 6A). There were increased numbers of monocytes in the lungs of infected *Ifnar*^{-/-} mice (Fig. 6B; 14% versus 9%, $P = 0.0051$) and dendritic cell populations (Fig. 6C; 4% versus 1.6%, $P = 0.0079$) compared with the WT controls, consistent with the known effects of type I IFN signaling on the mobilization of immune cells to the lung (36). Neutrophil recruitment was equivalent between WT and *Ifnar*^{-/-} mice (Fig. 6D). We did observe a difference in neutrophils in Fig. 2C, but this was in cells taken at an early time point from an intranasal infection, and pneumolysin can activate cytokines other than type I IFNs (37, 38). We did not observe a difference in bacterial numbers in the lung in this model (data not shown), consistent with other studies (39). Thus, despite

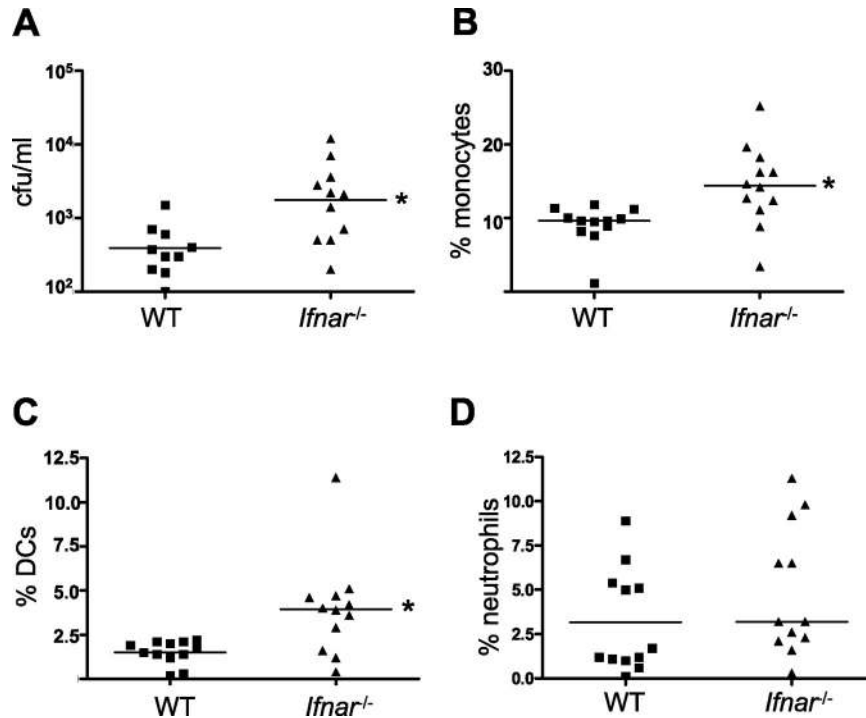


FIG 6 Type I IFN production contributes to pneumococcal clearance. WT and *Ifnar*^{-/-} mutant mice (129/SvEV background for both) were infected with 10⁷ CFU of *S. pneumoniae* intranasally and examined 7 days later. Shown are bacterial counts recovered from nasal lavage fluid samples (A) and percentages of monocytic cells (percentage of CD11b⁺ CD45⁺ cells) (B), DCs (percentage of CD11b⁺ CD11c⁺ CD45⁺ cells) (C), and neutrophils (percentage of Ly6G⁺ Ly6C⁺ CD45⁺ cells) (D) from lung homogenates analyzed by FACS. Each data point represents an individual mouse ($n = 12$). Data are combined from two independent experiments. Lines represent median values. *, $P < 0.05$ compared to WT mice (Mann-Whitney test).

the ability to recruit phagocytic cells, in the absence of type I IFN signaling, there was increased colonization with *S. pneumoniae*.

DISCUSSION

In this report, we demonstrate the participation of airway epithelial cells in the initial recognition of an important bacterial pathogen, *S. pneumoniae*, in the respiratory tract. This immune response is mediated by activation of the epithelial type I IFN cascade by the intracellular accumulation of pneumococcal DNA. As this pathway is readily activated by viral nucleic acids that accumulate in airway epithelial cells, it is not unexpected that bacterial nucleic acids could have a similar effect. The upper respiratory tract is continually exposed to commensal flora, but the bacterial components, including DNA released from lysed organisms, do not appear to activate mucosal signaling. *S. pneumoniae*, a pathogen, is distinct from commensal flora by virtue of its expression of pneumolysin, the pore-forming toxin that enables ligands to gain access to the epithelial cytosol and initiate type I IFN signaling. As the induction of proinflammatory responses in the lung is so potentially deleterious to respiration, it seems logical that mucosal signaling is activated only by PAMPs that are sensed intracellularly, by organisms that have breached the barrier function of the airway mucosa. Our findings suggest that type I IFN signaling may be a general host response to perceived cellular invasion and does not discriminate among types of pathogens, whether viral or bacterial, but instead responds to the nature of the specific PAMPs within the cell.

In a model of nasopharyngeal colonization, the usual route of pneumococcal infection, the type I IFN response analogous to that

induced by influenza virus contributed to bacterial clearance. Production of pneumolysin seems to be critical in triggering immune recognition of *S. pneumoniae* as a pathogen. The role of type I IFN signaling in the host response to pneumococcal infection has been previously reported by Weigent et al. (40), who demonstrated increased susceptibility to infection in mice treated with anti-IFN- α/β antibodies. Mancuso et al. (41) similarly investigated the importance of type I IFN signaling in *S. pneumoniae* sepsis and meningitis models. The significance of nasopharyngeal colonization in the initiation of host defenses against *S. pneumoniae* was recently highlighted in a microarray study that identified the induction of a number of type I IFN-related genes in murine nasal lymphoid tissue early in the course of a 6-week colonization (42). Less clear is how necessary type I IFN induction is in the context of active pneumococcal pneumonia, as models of pneumonia using direct tracheal inoculation of the organisms fail to demonstrate a notable phenotype (39). However, as upper airway colonization precedes the development of pneumonia, this is a critical stage in the pathogenesis of systemic pneumococcal infection.

It is increasingly apparent that the mucosal epithelium of the lung provides much more than a physical barrier to infection but actively participates in sensing and initiating immune signaling in response to inhaled pathogens, both viral and bacterial. This signaling includes not only the proinflammatory cascades set off by the apically exposed TLRs but also intracellular sensing systems. For at least two important pulmonary pathogens, *S. aureus* and *S. pneumoniae*, shed components that are either endocytosed (*S. aureus* protein A [36]) or taken up via pore formation (pneumolysin) trigger IFN- β production in both epithelial and immune

cells. The ability of these PAMPs from Gram-positive extracellular bacteria to access intracellular receptors suggests a central role for the type I IFN cascade in mucosal defenses against respiratory pathogens of all types.

The expression of pneumolysin is critical in the activation of type I IFN signaling by *S. pneumoniae*. Pneumolysin has been shown previously to facilitate the introduction of peptidoglycan into host cells, as well as induce host signaling from osmotic changes (28, 43). DNA from lysed commensal flora in the upper respiratory tract could potentially be immunostimulatory, if the organisms were able to deliver it into mucosal cells. The data suggest that pneumolysin facilitates entry of DNA via its pore formation. We observed the requirement for pneumococcal lysis and release of pneumococcal DNA as an autolysin null strain was unable to induce type I IFN signaling. We could obviate this requirement artificially by transfection of DNA or exposing naturally phagocytic DCs to cell lysates. This suggests that the ability to access cytosolic receptors may also contribute significantly to virulence. However, the addition of purified pneumolysin and DNA did not activate signaling (data not shown). The inability to artificially recapitulate the system *in vitro* indicates a missing component, alternative ligand, or stoichiometric effects necessary to activate signaling.

Several discrete receptors in both the cytosol and endosomes of the cells that make up the respiratory mucosa participate in type I IFN signaling. Our data suggest that DAI, whose role in immune signaling has been debated (15, 33), may be important in the response to pneumococcal DNA and other bacterial DNA. There was a significant induction of DAI expression by *S. pneumoniae* and a significant requirement for DAI to induce *Ifnb* in response to pneumococcal DNA. In addition, the contribution of other potential DNA sensors and signaling components (MAVS, RIG-I, and TLRs) was specifically excluded (although there was a trend to reduced induction in the MAVS null background, suggesting a limited role for RNA polymerase III [12, 13]). The expected downstream components of this pathway, including the recently identified STING (stimulator of IFN genes), TBK1, and IRF3 proteins, were also shown to be involved in the induction of *Ifnb* in the contexts of both DNA and the whole organism.

There is a growing literature detailing how bacterial pathogens induce the type I IFN cascade in macrophages (6, 30, 44–47). This report provides a second example of how an extracellular pathogen can activate type I IFN signaling, an intracellular response (36). We now show that epithelial cells similarly participate in the induction of type I IFN signaling. Exactly how the epithelial cells, which are not actively phagocytic, are stimulated to express IFN- β in response to other types of airway pathogens remains to be established. However, based upon our observations with the pneumococcus, it seems likely that a general induction of type I IFN signaling in the lung, including the airway mucosal cells, is an important consequence of both bacterial and viral infections of the lung.

MATERIALS AND METHODS

Bacterial strains. *S. pneumoniae* strains D39 (48), D39 *ply* (49), 1725 (P1121, a derivative of P833, a human carriage isolate [50]), 1726 (P1121 *ply* [10]), 1727 (P1121 *ply ply*^{W433F+}, a PdB toxoid-expressing strain [28]), and D39 *lytA* (51) were grown on Trypticase soy agar (supplemented with erythromycin for D39 *ply*) or broth supplemented with 200 U/ml catalase (Worthington). Plate cultures were grown at 37°C in

the presence of carbon dioxide (5%). *Escherichia coli* strain BL21AI (Invitrogen), *P. aeruginosa* PAO1, and *S. aureus* USA300 were grown at 37°C on LB. All chemicals were purchased from Sigma unless otherwise stated.

RNA analysis. RNA was isolated using the RNAqueous-4PCR kit (Ambion) with DNase treatment. cDNA was made using the iScript Synthesis Kit (Bio-Rad). qRT-PCR was performed using Power SYBR Green PCR Master Mix in a StepOne Plus thermal cycler (Applied Biosystems). Samples were normalized to β -actin or glyceraldehyde 3-phosphate dehydrogenase levels (sense, 5' ACCACAGTCCATGCCATCAC 3'; antisense, 5' TCCACCACCCTGTTGCTGTA 3'). 16S rRNA primers for *S. pneumoniae* were 5' GCCTACATGAAGTCGGAATCG 3' (sense) and 5' TACAAGGCCCGGAACGT 3' (antisense). Primers for mouse actin, *Ifnb*, *Mx1*, *PKR*, *LIF*, *KC*, *IL-6*, and *DAI* have been described elsewhere (32, 36, 52).

Protein purification and labeling. *E. coli* BL21AI carrying pET29a/pneumolysin or pneumolysin W433F (37, 53) was grown to mid-exponential phase and induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and 0.2% arabinose for 3 h at 37°C. Cells were lysed using Bugbuster (Novagen) and sonication in the presence of the HALT protease cocktail (Pierce). His-tagged protein was purified from clarified lysates with nickel-charged agarose resin using Poly-Prep gravity flow columns (Bio-Rad). Eluted protein was also passed through an endotoxin removal column (Detoxi-Gel columns; Thermo Scientific). When required, protein was concentrated to 2 mg/ml using Amicon centrifugation columns and labeled with AF488 according to the manufacturer's instructions (AF488 labeling kit; Molecular Probes-Invitrogen). Hemolytic activity of proteins was confirmed (see Fig. S2 in the supplemental material).

Confocal microscopy. AF488-labeled pneumolysin (25 μ g/ml) was incubated with murine nasal epithelial cells in primary culture for 1 h. Cells were washed three times in PBS and fixed in 4% paraformaldehyde before being blocked in 5% normal goat serum with 0.3% Triton X-100 to permeabilize cells. Phalloidin conjugated to rhodamine was used to stain actin. Imaging was performed on a Zeiss LSM 510 META scanning confocal microscope and analyzed using LSM Image Browser software (version 4.2).

Cell culture. Mouse nasal epithelial cells grown polarized from septa were isolated from the indicated strains of adult mice and grown as described elsewhere (54). Cells were stimulated with 5×10^8 CFU/ml of bacteria. Purified pneumolysin was applied to cells at a final concentration of 2 μ g/ml for 4 h. Experiments were performed with cytochalasin D at 20 μ M and included a 30-min preincubation of inhibitor before the organism was applied. Pneumococcal lysate experiments were performed using 5 mg/ml of protein. *S. pneumoniae* lysates (1 mg/ml) were treated with 100 U/ml DNase (Ambion) or 25 μ g/ml RNase (digestion verified by gel electrophoresis) for 2 h at 37°C before heat inactivation at 70°C for 10 min. DCs and macrophages were generated from bone marrow isolated from femurs and tibias (from DAI and STING null mice). DCs were cultured for 7 days in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), streptomycin, penicillin, and 20 ng/ml of GM-CSF (PeproTech). Macrophages were grown in Dulbecco's modified Eagle's medium with 10% FBS, ciprofloxacin, and 20% L929 supernatant. Immortalized macrophages (from TBK1, IRF3, IRF5, IRF7, and MAVS null mice) were prepared as described previously (55) and stimulated with 4×10^7 CFU/ml *S. pneumoniae*. Cell transfections were carried out using bacterial genomic DNA at a final concentration of 2 μ g/ml. DNA was isolated using the DNeasy kit (Qiagen), and transfections were performed for 6 h using FuGENE according to the manufacturer's instructions (Roche).

Mouse studies. Lung infections with *S. pneumoniae* were performed using 6-week-old C57BL/6J mice. *Nod2*^{-/-} mice were obtained from Jackson Laboratories (originally from Regeneron Pharmaceuticals). Mice were anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine and intranasally inoculated with 2×10^7 to 5×10^7 CFU of the organism. LPS from *E. coli* (50 μ g per mouse) was used as a control. Colonization studies were performed with *Ifnar*^{-/-} and WT 129/SvEV mice. Six- to 7-week-

old mice were inoculated with *S. pneumoniae* D39 without anesthesia. For fluorescence-activated cell sorter (FACS) analysis, red blood cells were lysed from lung homogenates. Cells were suspended in PBS with 2% FBS and stained with combinations of phycoerythrin-labeled anti-CD45 (Caltag Laboratories), fluorescein isothiocyanate-labeled anti-Ly6G/Ly6C (clone RB6-8C5; BD Biosciences-Pharmingen), PerCP-Cy5.5-labeled anti-CD11b (M1/70; eBioscience), and allophycocyanin-labeled anti-CD11c (N418; eBioscience) antibodies in the presence of 10% normal mouse serum and Fc block (2.4G2; BD Biosciences). Negative controls were stained with relevant isotype-matched antibodies. Cells were fixed with 1% paraformaldehyde and analyzed on a FACScalibur (Becton Dickinson) using CellQuest software (version 3.3; BD). Cells were gated on their side and forward scatter and CD45 expression. Data were analyzed using WinMDI (version 2.8; Joseph Trotter). All mouse infections were performed under the guidelines of the Institutional Animal Care and Use Committee of Columbia University.

Western blotting. Phosphorylation of STATs was detected using P-STAT1 (Y701; Abcam) and P-STAT3 (Y705; Cell Signaling Technology) and normalized to β -actin (Sigma), while phosphorylation of p38 was detected using P-p38 (T180/Y182; Cell Signaling Technology) and normalized to total p38 (Cell Signaling Technology). All immunoblot assays were followed by secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology Inc.). Samples from at least two individual mice were typically analyzed on two separate occasions. Protein separation, transfer, and immunoblotting were performed as previously described (36).

Statistics. Significance of data that followed a normal distribution was determined using a two-tailed Student *t* test, and for data that did not follow a normal distribution, a nonparametric Mann-Whitney test was used. Statistics were performed with GraphPad Prism software.

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D.P. performed the experiments, F.J.M. assisted with mice and FACS experiments, G.S. performed confocal microscopy, B.S.H. and J.L.A. provided technical support, A.J.R., K.A.F., and C.S. provided reagents, A.J.R. and A.P. supervised the research, and D.P. and A.P. conceived the experiments and wrote the manuscript.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbo.asm.org/lookup/suppl/doi:10.1128/mBio.00016-11/-DCSupplemental>.

Figure S1, TIF file, 0.073 MB.

Figure S2, TIF file, 0.042 MB.

Figure S3, TIF file, 0.046 MB.

Figure S4, TIF file, 0.092 MB.

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