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Preclinical Development of Virulence-attenuated *Streptococcus pneumoniae* Strains Able to Enhance Protective Immunity against Pneumococcal Infection

To the Editor:

The existing vaccination strategies for prevention of adult *Streptococcus pneumoniae* lung infections are only partially effective (1) and novel preventive approaches are required. Recent data have shown that adults develop immunity to *S. pneumoniae* through repeated episodes of asymptomatic nasopharyngeal colonization (2–6). This naturally acquired immunity includes protective responses to both protein and capsular antigens (2–6) and is boosted by recolonization events (4, 7). These data suggest that deliberate nasopharyngeal administration of live *S. pneumoniae* could prevent serious *S. pneumoniae* infections by strengthening preexisting cross-serotype protective immunity that inhibits nasopharyngeal colonization with virulent strains, increases antigen-specific systemic immunity, and perhaps strengthens alveolar macrophage–mediated innate immunity (2, 3, 6, 7). This strategy would require *S. pneumoniae* strains able to stimulate protective immunity but unable to cause disease in a population with an underlying increased susceptibility to

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Table 1. *In Vivo* Phenotype Analysis of Δ*proABC/piaA* and Δ*fhs/piaA* Mutant Strains in Mouse Infection Models

Infection Conditions and Model	Target Organ and (if Applicable) Group	6B Wild Type		Δ*proABC/piaA*		Δ*fhs/piaA*		PBS Control Mice	
		Log_{10} cfu/ml	*n*	Log_{10} cfu/ml	*n*	Log_{10} cfu/ml	*n*	Log_{10} cfu/ml	*n*
Virulence models									
Pneumonia	Lungs	5.26 (0.55)	5	3.78 (1.50)*	5	4.54 (0.35)	5	N/A	—
	Blood	3.30 (0.77)	5	0 (0)†	5	0 (0)†	5	N/A	—
Colonization	Nasal wash	4.13 (0.41)	10	3.99 (0.05)	5	3.55 (0.46)	5	N/A	—
Colonization-then-challenge models									
6B pneumonia‡	Lungs	3.70 (1.86)	15	2.51 (2.03)	16	2.40 (3.24)†	11	3.66 (2.68)	16
	Blood	0 (0)§	15	0 (0)§	16	0 (0)§	11	2.09 (3.89)	16
	BALF	2.00 (2.18)†	15	2.00 (2.38)	16	0 (1.09)*	11	2.83 (1.20)	16
6B pneumonia in μMT mice	Lungs, wild-type control mice	4.54 (0.86)	6	4.10 (0.50)	6	3.11 (1.46)	6	N/A	—
	Lungs, μMT mice	4.08 (0.32)	3	4.18 (0.88)	6	4.28 (0.39)	6	N/A	—
	Blood, wild-type control mice	0 (0)	6	0 (0)	6	0 (0)	6	N/A	—
	Blood, μMT mice	3.54 (0.77)†	3	0 (2.48)	6	3.23 (0.43)*	6	N/A	—
6B pneumonia in $CD4^+$ cell–depleted mice	Lungs, untreated control mice	3.27 (2.91)	6	3.59 (2.89)	6	3.24 (2.73)	6	N/A	—
	Lungs, $CD4^+$ cell–depleted mice	3.54 (2.19)	5	1.33 (2.88)	6	3.47 (1.40)	6	N/A	—
	Blood, untreated control mice	0 (0)	6	0 (0)	6	0 (0)	6	N/A	—
	Blood, $CD4^+$ cell–depleted mice	0 (0)	5	0 (0)	6	0 (0)	6	N/A	—
6B colonization‡	Nasal wash	2.71 (1.46)†	10	1.54 (1.81)§	10	2.54 (0.94)*	10	3.88 (0.16)	10
TIGR4 colonization	Nasal wash, untreated control mice	1.00 (1.78)*	5	1.98 (2.99)†	6	1.75 (1.66)†	6	3.84 (0.47)	6
	Nasal wash, $CD4^+$ cell–depleted mice	2.52 (0.92)	6	2.90 (1.03)	6	3.38 (0.40)	6	3.36 (0.73)	6

Definition of abbreviations: BALF = BAL fluid; N/A = not applicable; PBS = phosphate-buffered saline; *S. pneumoniae* = *Streptococcus pneumoniae*.
For the pneumonia model, colony-forming units were obtained 28 hours after intranasal inoculation with 1×10^7 *S. pneumoniae* CFU in 50 μl PBS under deep isoflurane anesthesia, and for the colonization model, 7 days after colonization with 1×10^7 cfu in 10 μl PBS under light isoflurane anesthesia (4, 6, 8). For colonization-then-challenge experiments, mice underwent two episodes of colonization (Days 0 and 14) with 1×10^7 cfu in 10 μl PBS under light isoflurane anesthesia of wild-type BHN418 6B, Δ*proABC/piaA*, or Δ*fhs/piaA S. pneumoniae* strains or sham colonization with PBS before challenge between Days 30 and 42. For these, the data presented are pooled from two experiments except the colonization-with-Δ*proABC/piaA*-and-then-challenge experiment, which includes pooled data from three experiments. The $\mu MT^{-/-}$ mice were in the C57B/6J background (kind gift from Claudia Mauri, University College London); all other experiments used CD1 mice aged 4–8 weeks. $CD4^+$ cells were depleted by intraperitoneal injection of 250 μg anti-CD4 mAb (GK 1.5; BioxCell) 48 and 24 hours before *S. pneumoniae* challenge (6); flow cytometry confirmed >99% depletion of splenic $CD4^+$ cells (data not shown). Data are presented as the median of log_{10} cfu/ml (interquartile range) recovered from target organs after infection with wild-type, Δ*proABC/piaA*, Δ*fhs/piaA*, or PBS control mice. *P* values were obtained using Kruskal-Wallis tests with Dunn's *post hoc* test comparing groups with the wild-type 6B strain (for virulence models) or the PBS sham-colonized group (for colonization-then-challenge data). The Mann-Whitney test was used for experiments with $\mu MT^{-/-}$ mice and $CD4^+$ cell–depleted mice, comparing wild-type/untreated mouse data with $\mu MT^{-/-}$/$CD4^+$ cell–depleted mouse data, respectively, for each target organ and *S. pneumoniae* strain combination.
*$P < 0.01$.
†$P < 0.05$.
‡Combined data from two repeated experiments.
§$P < 0.001$.

S. pneumoniae. Here we describe the development and preclinical characterization of two live attenuated *S. pneumoniae* strains with these characteristics that are suitable for future use in human trials. Animal procedures were approved by the local ethical review process and conducted in accordance with UK national guidelines under project license PPL70/6510.

Fourteen mutant strains of the BHN418 6B *S. pneumoniae* strain containing deletions of known or potential virulence determinants (selected using data from published virulence screen and transcriptomic studies) were screened in mouse infection models for their virulence phenotypes and ability to induce protective immunity after nasopharyngeal colonization. Eight mutations reduced virulence without affecting colonization (data not shown). Of these, deletion of *fhs* or *proABC* (both with poorly understood roles during *S. pneumoniae* infections) caused particularly strong impairments of virulence. These genes were selected along with *piaA* (which encodes an iron transporter required for systemic virulence) as targets to make double mutant strains for investigation as candidate strains for prevention of *S. pneumoniae* infections. Double mutations were made to increase the degree of virulence attenuation and minimize the risk of revertant strains developing when used in human studies. Target genes were replaced from start to stop codon with the kanamycin or spectinomycin antibiotic resistance cassettes using established transformation techniques (8) to create the Δ*fhs/piaA* (BHN418 *fhs*::*aad9*; *piaA*::*aphIII*; spc^R kan^R) and Δ*proABC/piaA* (BHN418 *proABC*::*aad9*; *piaA*::*aphIII*; spc^R kan^R) strains. Whole-genome sequencing (MicrobesNG, Birmingham

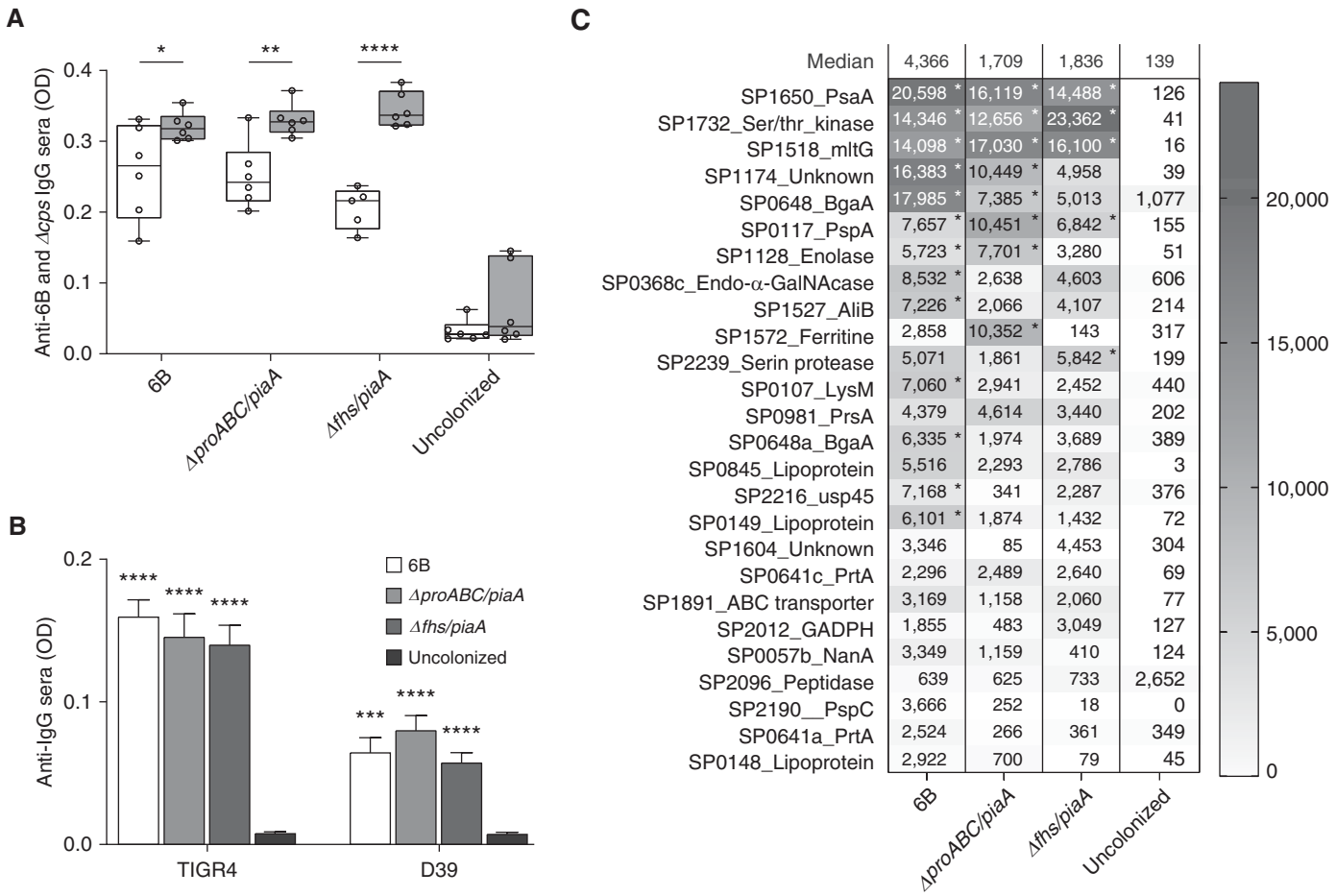

Figure 1. Serological responses in serum from mice colonized with wild-type and the Δ*proABC/piaA* or Δ*fhs/piaA Streptococcus pneumoniae* strains. (*A* and *B*) Whole-cell IgG ELISAs were performed as previously described (4, 6) using mouse sera recovered 28 days after colonization with the wild-type BHN418 6B or double mutant strains compared with uncolonized control mice. (*A*) Whole-cell ELISAs to the homologous wild-type BHN418 6B (white) or unencapsulated Δ*cps* BHN418 6B mutant (gray) strains (serum concentration 1 in 50). (*B*) Whole-cell ELISAs to the heterologous TIGR4 or D39 strains (serum concentration 1 in 50). Data in *A* are presented as box-and-whisker plots (whiskers represent the full range of the data) and in *B* as means (with error bars representing SDs); asterisks represent statistical significance between wild-type and unencapsulated strains in *A* or uncolonized control mice in *B* (Kruskal-Wallis test with Dunn's correction for multiple comparisons; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$). (*C*) Data on IgG binding to an *S. pneumoniae* protein antigen array probed with sera from mice colonized twice with the 6B strain, Δ*proABC/piaA*, or Δ*fhs/piaA* strains. The protein array contains 289 *S. pneumoniae* proteins selected for their known antigenicity in humans and high degree of conservation among *S. pneumoniae* strains (5). The array was constructed using cell-free *in vitro* transcription/translation expression and printing onto nitrocellulose-coated glass AVID slides (Grace Bio-Labs, Inc.), then probed with 1:25 mouse serum, and images were acquired and analyzed using an ArrayCAM Imaging System from Grace Bio-Labs (5). (*C*) Heat map of mean IgG binding levels to the top 26 proteins recognized by IgG in colonized mouse sera ($n = 6$ mice). Results with an asterisk are statistically significantly different from the sham-colonized group (Kruskal-Wallis test to identify significant differences between groups, $P < 0.05$ uncorrected for multiple comparisons). OD = optical density.

University) identified 11 and 2 nonsynonymous SNPs in the Δ*fhs/piaA* and Δ*proABC/piaA* strains, respectively, but no other major unexpected mutations compared with the parental 6B strain (data not shown).

The Δ*fhs/piaA* and Δ*proABC/piaA* strains were strongly reduced in systemic virulence in a mouse model of pneumonia yet in a colonization model maintained nasopharyngeal colony-forming unit densities similar to the wild-type strain (Table 1). After two episodes of colonization with the double mutant strains, mice developed significant serum IgG responses to the homologous BHN418 6B strain and two heterologous *S. pneumoniae* strains (TIGR4 and D39) (Figures 1A and 1B). Serum IgG responses from mice colonized with wild-type, Δ*proABC/piaA*, or Δ*fhs/piaA* strains were increased against the unencapsulated compared with encapsulated 6B strain (Figure 1A), and when assessed using flow cytometry, recognized whole wild-type 6B, TIGR4, and D39 *S. pneumoniae* bacteria (data not shown). Together, these data suggest that colonization with the virulence-attenuated strains induced significant antibody responses mainly against noncapsular antigens. Compatible with these data, no significant anticapsular responses were detected in serum from colonized mice using a Meso Scale Discovery multimeric bead assay (6) (data not shown). Instead, immunoblots confirmed that serum

IgG from colonized mice recognized multiple protein bands in lysates of *S. pneumoniae* BHN418 6B, TIGR4, and D39 strains (data not shown).

The *S. pneumoniae* protein antigens recognized by serum IgG from colonized mice were identified by probing a protein array containing the majority of conserved *S. pneumoniae* proteins recognized by naturally acquired IgG found in human sera (5). Significant IgG responses were detected to 26 proteins (Figure 1C), with considerable overlap in the antigens recognized between mice colonized with the mutant and wild-type strains. These included well-recognized immunodominant *S. pneumoniae* antigens (e.g., PsaA, PspA, and SktP) as well as conserved proteins with few data on their utility as protective antigens (e.g., MltG, Bga, and PhtE). Importantly, subsequent pneumonia challenge in mice previously colonized with the double mutant strains was not associated with enhanced pulmonary or systemic cytokine responses (measured by Meso Scale Discovery), or major changes in recruited inflammatory cell subsets (assessed by flow cytometry of lung preparations) compared with sham (with phosphate-buffered saline) or *S. pneumoniae* wild-type 6B-colonized control mice (data not shown).

When challenged using the *S. pneumoniae* BHN418 6B strain pneumonia model, mice previously colonized with the wild-type BHN418 6B, Δ*proABC/piaA*, or Δ*fhs/piaA* strains were totally protected against bacteremia (Table 1). In addition, mice colonized with the wild-type or Δ*fhs/piaA* strains had reduced lung colony-forming units (Table 1). Repeat colonization and 6B pneumonia rechallenge experiments in B cell–deficient μMT mice or mice depleted of $CD4^+$ cells before challenge demonstrated an important role for antibody rather than $CD4^+$ cells for colonization-induced protection against septicemia (Table 1). Previous colonization with the Δ*proABC/piaA* or Δ*fhs/piaA* strains also protected against recolonization of the nasopharynx with the wild-type homologous BHN418 6B strain or the heterologous TIGR4 strain, reducing nasal wash colony-forming units by >1.5 $\log_{10}$ 7 days after recolonization challenge (Table 1). If replicated in human studies, this reduction in nasopharyngeal colony-forming units is likely to impair successful nasopharyngeal colonization by *S. pneumoniae* and thereby reduce the incidence of subsequent invasive infections. Compatible with published data showing that protection against recolonization is mediated by $CD4^+$ effector cells targeting protein antigens (6, 9, 10), $CD4^+$ cell depletion abrogated the protective effect of prior colonization against recolonization (Table 1).

To summarize, we propose that administering to the nasopharynx mutant attenuated *S. pneumoniae* strains could be a novel strategy to overcome some of the limitations of the existing vaccines. Here, we have described the design and preclinical evaluation of the Δ*fhs/piaA* or Δ*proABC/piaA* BHN418 6B mutant strains and demonstrated they are good candidate strains for testing this strategy in humans. These mutant strains can now be investigated for their protective efficacy against *S. pneumoniae* colonization in a trial using an established controlled human *S. pneumoniae* infection challenge model (3, 7). ■

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Aerosol Transmission of SARS-CoV-2: Inhalation as well as Exhalation Matters for COVID-19

To the Editor:

We read with great interest the article by Echternach and colleagues (1) on the topic of aerosol dispersion during singing and speaking as a potential coronavirus disease (COVID-19) transmission pathway. In the article, as has been the case more broadly regarding this mode of transmission, attention has focused on factors that influence the emission of virus (i.e., aerosol production by the infected individual) when singing or speaking. However, the ventilatory pattern of individuals exposed to the aerosolized virus is also an important factor, as this is likely to be a key modulator of the "dose" of virus-containing aerosol inhaled. As such, the inclusion of such parameters in discussion regarding aerosol transmission is important when considering why certain contexts such as choirs, restaurants, and bars, where speaking, singing, and shouting are common, have been linked to infection clusters (2). Such an appreciation may reframe the discussion to include "superreceptiveness" as a component of "superspreader" events.

Ventilatory parameters vary greatly depending on both the type and intensity of activity and should feature more prominently when considering aerosol transmission. We recently investigated the physiological demands of "Singing for Lung Health" in healthy volunteers (3) and found that when participating in the singing component of the protocol, $\dot{V}_E$ increased from resting volumes of 11 (9–13) L/min (median, interquartile range [IQR]) to 22.42 L/min (IQR, 16.83–30.54 L/min), and the median volume per breath increased from 0.69 L (IQR, 0.63–0.77 L) to 2.11 L (IQR, 1.92–2.70 L). Other researchers, comparing talking with quiet breathing, found increases in parameters including $\dot{V}_E$, V_T, and breathing frequency (4, 5).

Both increased $\dot{V}_E$ and increased V_T are likely to be relevant to aerosol transmission. First, the more aerosolized viral particles inhaled the larger the inoculum, which will impact the chance of developing a disease, and may also influence disease severity (6). Second, greater inhalation will increase the alveolar area exposed to virus-containing aerosols, which may have implications for the viral processing and the immune response (7).

Considering patterns of inhalation as well as exhalation should enable a more complete appreciation of context-specific viral transmission dynamics. This is particularly relevant to contexts in which $\dot{V}_E$ is increased because of physical activity (gyms, supermarkets, etc.) but also where groups of people are vocalizing, such as choral singing, restaurants, bars, and sports crowds. Appreciating these factors does not change the fundamental focus of mitigation measures of hygiene, face coverings, physical distance, and avoiding contexts with poor ventilation. However, given these considerations, particularly with new more infectious severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants in circulation, there may now be a stronger argument for face coverings that reduce the risk of inhaling aerosols rather than just reducing their emission, especially in contexts in which people are vocalizing or exercising or other risk factors are present. ■

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