Preclinical Development of Virulence Attenuated *Streptococcus pneumoniae* Strains Able to Enhance Protective Immunity Against Pneumococcal Infection

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To the Editors,

The existing vaccination strategies for prevention of adult *Streptococcus pneumoniae* lung infections are only partially effective [1] and novel preventative approaches are required. Recent data have shown that adults develop immunity to *S. pneumoniae* through repeated episodes of asymptomatic nasopharyngeal colonisation [2-6]. This naturally acquired immunity includes protective responses to both protein and capsular antigens [2-6], and is boosted by re-colonisation events [4, 7]. These data suggest deliberate nasopharyngeal administration of live *S. pneumoniae* could prevent serious *S. pneumoniae* infections by strengthening pre-existing cross-serotype protective immunity that inhibits nasopharyngeal colonisation with virulent strains, increases antigen-specific systemic immunity, and perhaps stengthens 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 *S. pneumoniae*. Here we describe the development and pre-clinical characterisation of two live attenuated *S. pneumoniae* strains with these characteristics that are suitable for future use in human trials.

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 colonisation. Eight mutations reduced virulence without affecting colonisation (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 minimise 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 *Δfhs/piaA* (BHN418 *fhs*::*aad9*; *piaA*::*aphIII*; spc^R kan^R) and *ΔproABC/piaA* (BHN418 *proABC*::*aad9*; *piaA*::*aphIII*; spc^R kan^R) strains. Whole genome sequencing (MicrobesNG, Birmingham University) identified 11 and 2 non-synonmous SNPs in the *Δfhs/piaA* and *ΔproABC/piaA* strains respectively, but no other major unexpected mutations compared to the parental 6B strain (**data not shown**).

The *Δfhs/piaA* and *ΔproABC/piaA* strains were strongly reduced in systemic virulence in a mouse model of pneumonia, yet in a colonisation model maintained nasopharyngeal CFU densities similar to the wild type strain (**Table 1**). After two episodes of colonisation 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) (**Figure 1A and B**). Serum IgG responses from mice colonised with wild type, *ΔproABC/piaA* or *Δfhs/piaA* strains were increased against the unencapsulated compared to encapsulated 6B strain (**Figure 1A)**, and when assessed using flow cytometry recognised whole wild type 6B, TIGR4 and D39 *S. pneumoniae* bacteria (**data not shown**). Together, these data suggest colonisation with the virulence-attenuated strains induced significant antibody responses mainly against non-capsular antigens. Compatible with these data, no significant anti-capsular responses were detected in serum from colonised mice using a Meso Scale Discovery (MSD) multimeric bead assay [6] (**data not shown**). Instead, immunoblots confirmed serum IgG from colonised

mice recognised multiple protein bands in *S. pneumoniae* BHN418 6B, TIGR4 and D39 strains lysates (**data not shown**).

The *S. pneumoniae* protein antigens recognised by serum IgG from colonised mice were identified by probing a protein array containing the majority of conserved *S. pneumoniae* proteins recognised 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 recognised between mice colonised with the mutant and wild type strains. These included well-recognised immunodominant *S. pneumoniae* antigens (eg PsaA, PspA, SktP) as well as conserved proteins with few data on their utility as protective antigens (eg MltG, Bga, and PhtE). Importantly, subsequent pneumonia challenge in mice previously colonised with the double mutant strains was not associated with enhanced pulmonary or systemic cytokine responses (measured by MSD), or major changes in recruited inflammatory cell subsets (assessed by flow cytometry of lung preparations) compared to sham (with PBS) or *S. pneumoniae* wild type 6B colonised controls (**data not shown**).

When challenged using the *S. pneumoniae* BHN418 6B strain pneumonia model, mice previously colonised with the wild type BHN418 6B, *ΔproABC/piaA*, or *Δfhs/piaA* strains were totally protected against bacteraemia (**Table 1**). In addition, mice colonised with the wild type or *Δfhs/piaA* strains had reduced lung CFU (**Table 1**). Repeat colonisation and 6B pneumonia rechallenge experiments in B cell deficient μMT mice or mice depleted of CD4+ cells pre-challenge demonstrated an important role for antibody rather than CD4+ cells for colonisation-induced protection against septicaemia (**Table 1**). Previous colonisation with the *ΔproABC/piaA*, or *Δfhs/piaA* strains also protected against recolonisation of the nasopharynx with the wild type homologous BHN418 6B strain or the heterologous TIGR4 strain, reducing

nasal wash CFU by >1.5 $\log_{10}$ 7 days after re-colonisation challenge (**Table 1**). If replicated in human studies this reduction in nasopharyngeal CFU is likely to impair successful nasopharyngeal colonisation by *S. pneumoniae* and thereby reduce the incidence of subsequent invasive infections. Compatible with published data showing protection against recolonisation is mediated by CD4+ effector cells targeting protein antigens [6, 9, 10], CD4+ cell depletion abrogated the protective effect of prior colonisation against recolonisation (**Table 1**).

To summarise, we propose 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 *Δfhs/piaA* or *ΔproABC/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* colonisation in a trial using an established controlled human *S. pneumoniae* infection challenge model [3, 7].

Acknowledgements

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Figure 1. Serological responses in serum from mice colonised with wild type and the *ΔproABC/piaA* or *Δfhs/piaA S. pneumoniae* strains. (A) and (B) Whole cell IgG ELISAs were performed as previously described [4, 6] using mouse sera recovered 28 days post colonisation with the wild type BHN418 6B or double mutant strains compared with uncolonised controls. (A) Whole cell ELISAs to the homologous wild type BHN418 6B (white) or unencapsulated *Δcps* BHN418 6B mutant (grey) strains (serum concentration 1 in 50). (B) Whole cell ELISAs to the heterologous TIGR4 or D39 strains (serum concentration 1 in 50). Error bars represent standard deviation and asterisks represent statistical significance between wild type and unencapsulated strains (panel A) or uncolonised controls (panel B) (Kruskal–Wallis test with Dunn's correction for multiple comparisons; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$). (C) IgG binding data to a *S. pneumoniae* protein antigen array probed with sera from mice colonised twice with the 6B strain, *ΔproABC/piaA*, or *Δfhs/piaA* strains. The protein array contains 289 *S. pneumoniae* proteins selected for their known antigenicity in humans and high degree of conservation amongst *S. pneumoniae* strains [5]. The array was constructed using cell free *in vitro* transcription/translation (IVTT) expression and printing onto nitrocellulose coated glass AVID slides (Grace Bio-Labs, Inc., Bend, OR), then probed with 1:25 mouse serum, and images acquired and analysed using an ArrayCAM® Imaging System from Grace Bio-Labs [5]. (C) Heat map of mean IgG binding levels to the top 26 proteins recognised by IgG in colonised mouse sera (n = 6 mice). Results with an asterisk are statistically significantly different to the sham-colonised group (Kruskall-Wallis test to identify significant differences between groups, $p < 0.05$ uncorrected for multiple comparisons).

Table 1.

Infection conditions and model	Target organ and (if applicable) group	6B wild type Log$_{10}$ CFU / ml	n	ΔproABC/piaA Log$_{10}$ CFU / ml	n	Δfhs/piaA Log$_{10}$ CFU / ml	n	PBS controls 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	
Colonisation	**Nasal wash**	4.13(0.41)	10	3.99(0.05)	5	3.55(0.46)	5	n/a	
COLONISATION 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 μMT mice	**Lungs wild type controls**	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 controls	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 CD4+ depleted	**Lungs untreated controls**	3.27(2.91)	6	3.59(2.89)	6	3.24(2.73)	6	n/a	
	Lungs CD4+ depleted	3.54(2.19)	5	1.33(2.88)	6	3.47(1.40)	6	n/a	
	Blood untreated controls	0(0)	6	0(0)	6	0(0)	6	n/a	
	Blood CD4+ depleted	0(0)	5	0(0)	6	0(0)	6	n/a	
6B colonisation†	**Nasal wash**	**2.71(1.46)***	10	**1.54(1.81)*****	10	**2.54(0.94)****	10	3.88(0.16)	10
TIGR4 colonisation	**Nasal wash untreated controls**	**1.00(1.78)****	5	**1.98(2.99)***	6	**1.75(1.66)***	6	3.84(0.47)	6
	Nasal wash CD4+ depleted	2.52(0.92)	6	2.90(1.03)	6	3.38(0.40)	6	3.36(0.73)	6

†combined data from two repeated experiments

Table 1: *In vivo* phenotype analysis Δ*proABC/piaA* and Δ*fhs/piaA* mutant strains in mouse infection models. For the pneumonia model CFU were obtained 28 hours post-intranasal inoculation with 1×10^7 *S. pneumoniae* CFU in 50 µl PBS under deep isoflurane anaesthesia, and for the colonisation model 7 days post colonisation with 1×10^7 CFU in 10 µl PBS under light isoflurane anaesthesia [4, 6, 8]. For colonisation then challenge experiments mice underwent two episodes of colonisation (day 0 and 14) with 1×10^7 CFU in 10 µl PBS under light isoflurane anaesthesia of wild type BHN418 6B, Δ*proABC/piaA* or Δ*fhs/piaA S. pneumoniae* strains or sham-colonisation with PBS before challenge between days 30 to 42. For these, the data presented are pooled from two experiments except colonisation with Δ*proABC/piaA* and then challenge experiment, which includes pooled data from three experiments. The µMT$^{-/-}$ mice were in the C57B/6J background (kind gift Claudia Mauri, UCL); all other experiments used CD1 mice aged 4 to 8 weeks. CD4+ cells were depleted by intraperitoneal injection of 250 µg anti-CD4 mAb (GK 1.5, BioxCell) 48 and 24hrs prior to *S. pneumoniae* challenge [6]; flow cytometry confirmed >99% depletion of splenic CD4+ cells (data not shown). Data are presented as median of $\log_{10}$ CFU/ml (IQR) recovered from target organs after infection with wild type, Δ*proABC/piaA*, Δ*fhs/piaA* or PBS controls. *P* values (*<0.05, **<0.01, ***<0.001) were obtained using Kruskal-Wallis tests with Dunn's post hoc test comparing groups to the wild type 6B strain (for virulence models), the PBS sham-colonised (for colonisation then challenge data). The Mann-Whitney test was used for µMT$^{-/-}$ mice and CD4+ depleted mice experiments, comparing wild type / untreated mice data to µMT$^{-/-}$ / CD4+ depleted mice data respectively for each target organ and *S. pneumoniae* strain combination. Animal procedures were approved by the local ethical review process and conducted in accordance with UK national guidelines under project license PPL70/6510.
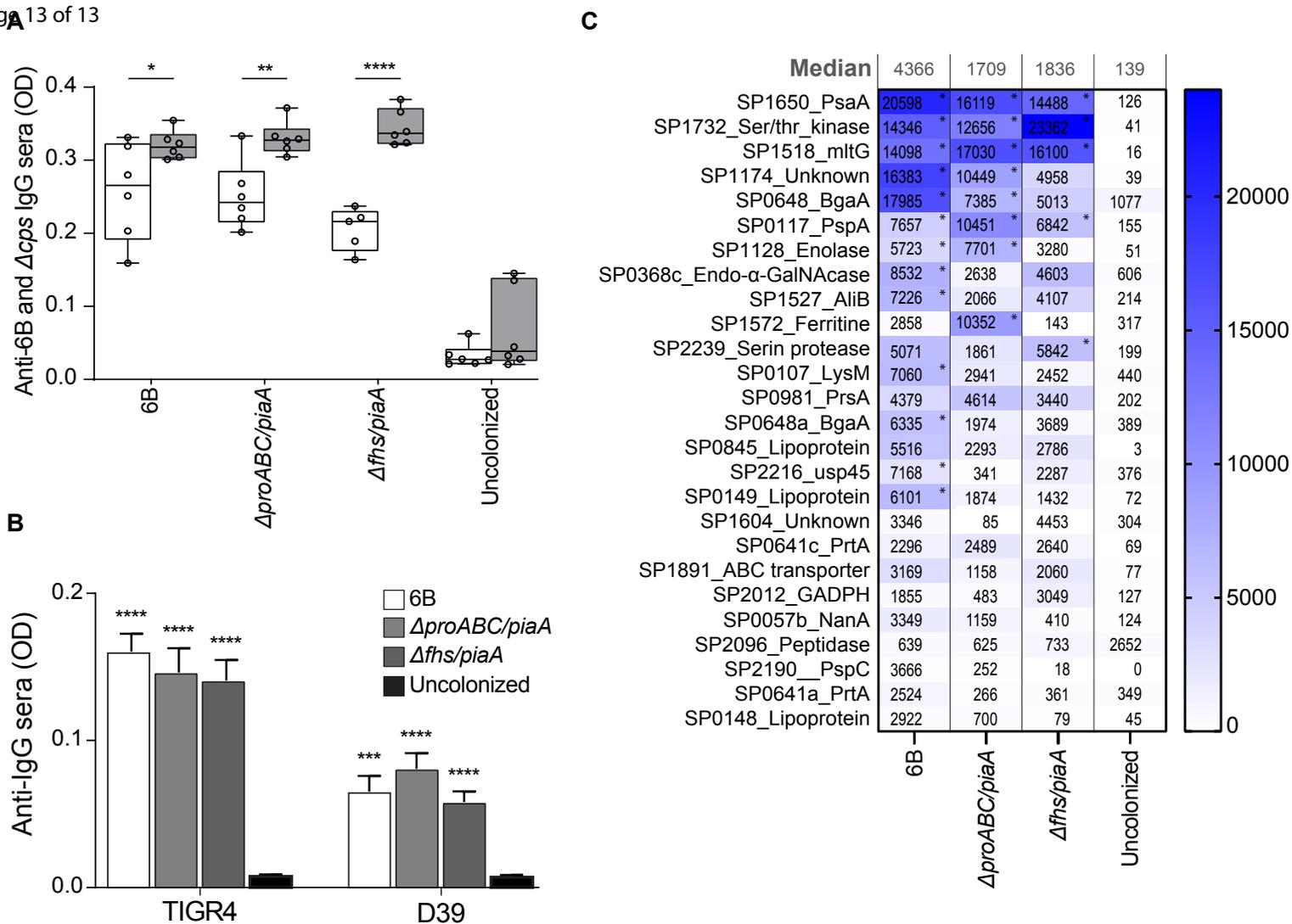
A

B

C

Median	4366		1709		1836		139
SP1650_PsaA	20598	*	16119	*	14488	*	126
SP1732_Ser/thr_kinase	14346	*	12656	*	23362	*	41
SP1518_mltG	14098	*	17030	*	16100	*	16
SP1174_Unknown	16383	*	10449	*	4958		39
SP0648_BgaA	17985	*	7385		5013		1077
SP0117_PspA	7657	*	10451	*	6842	*	155
SP1128_Enolase	5723	*	7701	*	3280		51
SP0368c_Endo-α-GalNAcase	8532	*	2638		4603		606
SP1527_AliB	7226	*	2066		4107		214
SP1572_Ferritine	2858		10352	*	143		317
SP2239_Serin protease	5071		1861		5842	*	199
SP0107_LysM	7060	*	2941		2452		440
SP0981_PrsA	4379		4614		3440		202
SP0648a_BgaA	6335	*	1974		3689		389
SP0845_Lipoprotein	5516		2293		2786		3
SP2216_usp45	7168	*	341		2287		376
SP0149_Lipoprotein	6101	*	1874		1432		72
SP1604_Unknown	3346		85		4453		304
SP0641c_PrtA	2296		2489		2640		69
SP1891_ABC transporter	3169		1158		2060		77
SP2012_GADPH	1855		483		3049		127
SP0057b_NanA	3349		1159		410		124
SP2096_Peptidase	639		625		733		2652
SP2190__PspC	3666		252		18		0
SP0641a_PrtA	2524		266		361		349
SP0148_Lipoprotein	2922		700		79		45

6B ΔproABC/piaA Δfhs/piaA Uncolonized

Figure 1