Role in virulence and protective efficacy in pigs of *Salmonella enterica* serovar Typhimurium secreted components identified by signature-tagged mutagenesis

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Salmonella enterica serovar Typhimurium (*S. Typhimurium*) is a zoonotic enteric pathogen of worldwide importance and pigs are a significant reservoir of human infection. Signature-tagged transposon mutagenesis (STM) was used to identify genes required by *S. Typhimurium* to colonize porcine intestines. A library of 1045 signature-tagged mutants of *S. Typhimurium* ST4/74 Nal^R was screened following oral inoculation of pigs in duplicate. A total of 119 attenuating mutations were identified in 95 different genes, many of which encode known or putative secreted or surface-anchored molecules. A large number of attenuating mutations were located within *Salmonella* pathogenicity islands (SPI)-1 and -2, confirming important roles for type III secretion systems (T3SS)-1 and -2 in intestinal colonization of pigs. Roles for genes encoded in other pathogenicity islands and islets, including the SPI-6-encoded Saf atypical fimbriae, were also identified. Given the role of secreted factors and the protection conferred against other pathogens by vaccination with extracellular and type III secreted proteins, the efficacy of a secreted protein vaccine from wild-type *S. Typhimurium* following intramuscular vaccination of pigs was evaluated. Serum IgG responses against type III secreted proteins were induced following vaccination and a significant reduction in faecal excretion of *S. Typhimurium* was observed in the acute phase of infection compared to mock-vaccinated animals. Vaccination with secreted proteins from an isogenic *S. Typhimurium* *prgH* mutant produced comparable levels of protection to vaccination with the preparation from the parent strain, indicating that protection was not reliant on T3SS-1 secreted proteins. The data provide valuable information for the control of *Salmonella* in pigs.

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INTRODUCTION

Salmonella enterica is an important zoonotic pathogen with the ability to infect a wide range of animal hosts. Infections can range in severity from subclinical infection, through mild diarrhoea, to severe systemic disease. In food-producing animals such as pigs, calves and chickens *S. enterica* is a major cause of disease. Infections in pigs are predominantly associated with serovars Choleraesuis and Typhimurium. *S. enterica* serovar Choleraesuis (*S. Choleraesuis*) causes a systemic, sometimes fatal, infection while *S. Typhimurium* causes a self-limiting enterocolitis but is rarely associated with mortality. A characteristic of

serovar Typhimurium infection of pigs, however, is its ability to produce persistent and asymptomatic infections. Pigs with persistent infections can harbour and shed *S. Typhimurium* in their faeces for several months post-exposure (Berends *et al.*, 1996). These carriers and shedders can contaminate the food chain and environment via meat and faeces and represent a significant reservoir of human *Salmonella* infection (Hurd *et al.*, 2002). In a year-long randomized UK national abattoir survey, *S. Typhimurium* was detected in the caeca of 11.1 % of pigs presented for slaughter and on 2.1 % of carcass swabs (Davies *et al.*, 2004). Although serovar Typhimurium is the principal serovar isolated from UK pig herds our understanding of the pathogenesis of *S. Typhimurium* infections in swine is limited as relatively few studies have been carried out using pigs as an experimental model.

Recent studies have established that the *Salmonella* pathogenicity island (SPI)-1-encoded type III secretion

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Abbreviations: Amp, ampicillin; Km, kanamycin; Nal, nalidixic acid; STM, signature-tagged mutagenesis; SPI, *Salmonella* pathogenicity island; T3SS, type III secretion system.

system (T3SS-1) plays a pivotal role in intestinal, but not tonsillar, colonization in pigs (Boyen *et al.*, 2006b), as well as in invasion of porcine pulmonary alveolar macrophages (Boyen *et al.*, 2006a). Type I fimbriae have also been implicated in intestinal colonization of swine by serovar Typhimurium (Althouse *et al.*, 2003). Most *in vivo* pig studies have focused on serovar Choleraesuis. Experimental studies using this serovar have indicated a role for the SPI-1 transcriptional regulator *hilA* in enteric infection in pigs and, more recently, screening of random *S. Choleraesuis* mutants identified a number of SPI-1 and SPI-2 genes essential for systemic infection of pigs (Ku *et al.*, 2005; Lichtensteiger & Vimr, 2003).

Working towards the aims of enhancing the health and welfare of farmed pigs and improving food safety, we have used signature-tagged mutagenesis (STM; Hensel *et al.*, 1995) as a rapid high-throughput screen for *S. Typhimurium* factors that may be suitable for inclusion in vaccines. STM permits the simultaneous analysis of up to 95 uniquely tagged transposon insertion mutants for defects in virulence and has been used as a prelude to the testing of vaccines against a variety of bacterial pathogens (Collins *et al.*, 2005; Flashner *et al.*, 2004; Ku *et al.*, 2005). This method has revealed that *Salmonella* uses both conserved and host-specific colonization factors in calves, chickens and mice (Bispham *et al.*, 2001; Hensel *et al.*, 1995; Morgan *et al.*, 2004; Shah *et al.*, 2005; Tsolis *et al.*, 1999). Increasing awareness that *Salmonella* requires different genes in different hosts reinforces the need to extend these studies to a porcine model of infection.

Screening of the same serovar Typhimurium mutant library as recently characterized in calves and chickens (Morgan *et al.*, 2004) identified 95 genes required for intestinal colonization of pigs. The data from this comprehensive survey indicate that secreted and surface-anchored molecules, including fimbriae and type III secreted proteins, play pivotal roles in the carriage of *S. Typhimurium* in pigs. Vaccines based on extracellular factors and type III secreted proteins have shown protection against other bacterial pathogens, including *Yersinia pestis* in mice and enterohaemorrhagic *Escherichia coli* in cattle (Leary *et al.*, 1995; Potter *et al.*, 2004). We therefore exploited insights from STM to evaluate the efficacy of a *S. Typhimurium* secreted protein vaccine and probed the role of T3SS-1-secreted proteins in the protection observed.

METHODS

Bacterial strains and culture conditions. A nalidixic acid resistant mutant of *Salmonella* Typhimurium ST4/74 (a progenitor of SL1344; ST4/74 Nal^R) and a *prgH* : : mini-Tn5Km2 mutant of ST4/74 Nal^R were used in this study (Morgan *et al.*, 2004). Both strains exhibited wild-type growth characteristics. *Escherichia coli* strain TOP10F' (Invitrogen) was used for subcloning of transposon-containing fragments in plasmid pBluescript KS(+) (Short *et al.*, 1988). *S. Typhimurium* and *E. coli* strains were grown in LB broth with appropriate antibiotics. Antibiotics were used at the following concentrations unless otherwise indicated: ampicillin (Amp; 100 µg

ml⁻¹), kanamycin (Km; 50 µg ml⁻¹), nalidixic acid (Nal; 20 µg ml⁻¹). Cultures were grown at 37 °C with aeration in a rotary shaker unless otherwise indicated.

Experimental animals. All animal experiments were conducted according to the requirements of the Animals (Scientific Procedures) Act 1986 (licence 30/1998) and were approved by the local ethical review committee. Large White × Landrace crossbred pigs were obtained from an established *Salmonella*-free commercial farm. Pigs were housed in a medium containment unit and fed twice daily on a diet of antibiotic-free weaner pellets with water freely available. All experimental animals were confirmed to be culture-negative for *Salmonella* by enrichment of rectal swabs overnight in Rappaport and Selenite broth followed by subsequent culture on Brilliant Green agar (Oxoid) for 16 h. Analysis of pre-vaccination serum IgG confirmed negligible levels of sero-reactivity to *Salmonella* in the animals used for vaccination and challenge studies.

In vivo screening of a ST4/74 Nal^R STM mutant library. A library of 1045 signature-tagged mini-Tn5Km2 transposon insertion mutants of ST4/74 Nal^R previously described by Morgan *et al.* (2004) was used for this study. Eleven pools of 95 mutants were grown in LB broth in microtitre trays at 37 °C for 16 h with aeration. Aliquots were removed from each of the 11 pools and used for preparation of 'input pool' genomic DNA by the CTAB (*N*-cetyl-*N,N,N*-trimethylammonium bromide) method (Hensel *et al.*, 1995). For each pool, two 6-week-old pigs were fasted for 12 h prior to infection and orally dosed with ~10¹⁰ c.f.u. per input pool. A section of distal ileal mucosa, excluding Peyer's patches, was aseptically excised at post mortem examination 3 days post-infection and triplicate 1 g samples were homogenized in 9 ml 0.9% saline. Bacteria (in excess of 10 000 colonies) were recovered from homogenized ileal mucosa by plating onto MacConkey agar containing Nal and Km. Bacteria were pooled and used for preparation of 'output pool' genomic DNA. Signature tags from input and output pools were amplified from genomic DNA using primers P2 (5'-TACCTACAACCTCAAGCTT-3') and P4 (5'-TACCCATTCTAACCAAGC-3'). Tags were subsequently labelled with [³²P]dCTP by PCR as described previously (Morgan *et al.*, 2004). [³²P]dCTP labelled signature-tag probes were hybridized to a corresponding pool of signature-tag PCR products amplified from each of 95 pUTmini-Tn5Km2 plasmids using primers P6pair (5'-GATCAGATCTGGCCGCTAGGC-3') and PXho (5'-TTATGAGC-CATATTCAACGGG-3'). Dot blots and hybridizations were performed as described previously (Morgan *et al.*, 2004). Duplicate dot blots were prepared for both input pools and output pools from each of two pigs to reduce artefacts.

For spiked pools, selected attenuated mutants identified in the primary screen, and colonization-proficient mutants with compatible tags, were picked into individual wells of 96-well microtitre plates containing LB broth supplemented with Nal and Km. All spiked pools contained less than 25% known attenuated mutants. Pools were tested in the STM pig infection model as described above.

Identification of colonization-defective mutants. Attenuated mutants were identified as producing a weaker hybridization signal in the output pool of at least one pig compared to the input pool, from duplicate dot blots, when compared by eye by two independent researchers. The site of the transposon insertion in attenuated mutants was then determined. *EcoRI*-, *PstI*- or *EagI*-restricted genomic DNA was ligated into similarly restricted pBluescript KS(+) and transformed into chemically competent *E. coli* TOP10F'. Sequencing of DNA flanking the transposon was performed by Lark Technologies using mini-Tn5Km2 specific primers P6 (5'-CCTAGGCGGCCAGATCTG-3') or P10 (5'-TCCTCTAGAGTCGACCTGC-3'). The location of transposon insertions was identified by BLAST-N alignment using the GenBank, National Center for

Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>) and coliBase (<http://colibase.bham.ac.uk>) databases. Sequence data are available from the authors on request.

In vivo co-infection study. Mixed infections with mutant and wild-type strains were used to confirm attenuation of a *S. Typhimurium* *safA* mutant. Cultures of wild-type ST4/74 Nal^R and a ST4/74 Nal^R *safA* : : mini-Tn5Km2 transductant (described below) were adjusted to the same OD₆₀₀ and mixed in a 1 : 1 ratio prior to oral inoculation of two 6-week-old pigs. Each pig was challenged with a total dose of 10¹⁰ c.f.u. Post mortem examination was performed 5 days post-infection and bacteria were recovered from homogenized ileal mucosa by plating onto Brilliant Green agar containing Nal or Nal plus Km to enumerate the mutant strain. The number of viable wild-type bacteria was calculated by subtracting the count on Nal plus Km medium from that on the Nal plates. The ratio of mutant to wild-type bacteria recovered from ileal mucosa was compared to the ratio of mutant to wild-type bacteria in the original inoculum and the data were statistically analysed by *t*-test.

Transduction of *safA* : : mini-Tn5Km2 between strains of *S. Typhimurium*. A *safA* mutation was introduced into the archived *S. Typhimurium* ST4/74 Nal^R strain by transduction with bacteriophage P22 HT/int. A donor lysate of P22 HT/int was prepared by the addition of phage to an exponential-phase LB culture of the original *safA* : : mini-Tn5Km2 mutant strain at a m.o.i. of 0.03. Cultures were reincubated for 3 h at 37 °C with shaking. Bacteria were removed by centrifugation at 11 000 *g* for 10 min and the supernatant filtered through a 0.45 µm filter. The resulting lysate was added to a broth culture of recipient *S. Typhimurium* ST4/74 Nal^R at a m.o.i. of ~0.8. After static incubation at 37 °C for 30 min, the mixture was plated onto LB agar containing Nal and Km. Transposon insertion sites were confirmed by PCR and Southern blot analysis as described previously (Morgan *et al.*, 2004) using transposon-specific primer P10 (as above) and gene-specific primer *safA*_for (5'-TGTTATTACCAGCCACG-GATT-3'). The transduced mutant was checked for non-agglutination with 5% acriflavine/HCl to confirm normal expression of lipopolysaccharide.

Preparation of secreted proteins. Secreted proteins were prepared from ST4/74 Nal^R and ST4/74 Nal^R *prgH* : : mini-Tn5Km2. Bacteria were grown to OD₆₀₀ 1.0 in Dulbecco's Modified Eagle's Medium (DMEM), cells pelleted by centrifugation (8000 *g*, 15 min, 4 °C) and supernatants collected. Supernatants were filtered using 0.45 µm pore size low-protein-binding filters (Millipore) and proteins were precipitated on ice with 10% (v/v; final concentration) trichloroacetic acid for 1 h. After centrifugation (10 000 *g*, 20 min, 22 °C) pellets were washed in ice-cold acetone and resuspended in PBS. At least four independent extractions for each vaccine preparation were pooled to minimize differences in protein recovery from sample to sample. After dialysis an aliquot from each vaccine preparation was removed and boiled in SDS buffer for 5 min, followed by separation using 10% (w/v) SDS-PAGE to assess the yield, size and stability of proteins. Prior to vaccine formulation each protein preparation was analysed by Cambrex Bio Science to ensure that endotoxin levels were within recommended standards of the European Pharmacopeia (Ph.Eur.), which limits the endotoxin content of a porcine vaccine to 1 × 10⁶ international endotoxin units (IEU) per dose (wild-type vaccine, 9 × 10² IEU per dose; *prgH* vaccine 2 × 10³ IEU per dose).

As type III secreted proteins are a major component of wild-type secreted protein preparations, the protein profile of the *prgH* mutant was compared visually with the wild-type secreted protein profile to normalize the *prgH* preparation based on the relative concentrations of the FliC (flagellin) protein. Adjustments based on FliC levels were also confirmed to result in comparable levels of the secreted SPI-4-encoded ~600 kDa SiiE protein, thus equivalent levels of non-SPI-1 related secreted factors were represented in both vaccine formulations.

Experimental immunization and infection procedures. Vaccines were formulated 1 : 1 with Freund's incomplete adjuvant (Sigma-Aldrich) prior to immunization. On day 0 three groups of four 5-week-old pigs were immunized intramuscularly (i.m.) with either 100 µg wild-type secreted protein vaccine, an equivalent quantity of *prgH* mutant vaccine, based on normalization relative to the FliC and SiiE proteins, or mock vaccine (PBS formulated with Freund's incomplete adjuvant) in a 1 ml volume. Due to concerns regarding health status one pig was removed from the *prgH* vaccine group prior to immunization, reducing the *prgH* vaccine group size to 3 pigs. At day 22 each pig was given an i.m. booster vaccination of either 200 µg wild-type secreted protein vaccine, an equivalent quantity of *prgH* mutant vaccine, or mock vaccine in a 1 ml volume. At day 37 all pigs were challenged by oral-gastric intubation with 5 × 10⁹ c.f.u. ST4/74 Nal^R in a 10 ml volume administered with 10 ml antacid [sterile double-distilled water containing 5% (w/v) Mg(SiO₃)₃, 5% (w/v) NaHCO₃, and 5% (w/v) MgCO₃]. Clinical signs of infection, including pyrexia, general demeanour, fluid and food intake, faecal consistency and faecal content, were monitored twice daily for 6 days post-infection. Faecal excretion of ST4/74 Nal^R was quantified by plating serial dilutions of faecal samples collected *per rectum* onto Brilliant Green agar containing Nal. Any pigs reaching predefined humane end points were humanely killed with an overdose of barbiturate. All other pigs were killed 6 days post-infection. Serum was obtained by bleeding from the anterior vena cava prior to primary vaccination (pre-immune), booster vaccination (pre-boost) and challenge (pre-infection). Blood sampling was also performed at post mortem examination.

ELISA. Maxisorp plates (NUNC, Fisher Scientific) were coated with carbonate buffer (Na₂CO₃ 10 mM; NaHCO₃ 35 mM; pH 9.6) containing 0.5 µg ml⁻¹ wild-type or an equivalent quantity of *prgH* mutant vaccine based on normalization relative to the FliC and SiiE for 16 h at 4 °C. After washing three times with PBS-T [PBS plus 0.05% (v/v) Tween 20] plates were blocked with PBS-T containing 3% (w/v) BSA for 1 h at room temperature. Plates were washed a further three times with PBS-T and incubated for 1 h at room temperature with PBS-T plus 3% (w/v) BSA containing diluted pig sera (1 : 100) collected on either day 0 (pre-immune), day 22 (pre-boost), day 37 (pre-infection) or day 43 (post mortem). Plates were washed as above and incubated with PBS-T plus 3% (w/v) BSA and 1 : 5000 rabbit anti-swine IgG (whole molecule)-AP (Sigma-Aldrich) for 1 h at room temperature. Plates were washed as above and 50 µl *p*-nitrophenyl phosphate substrate added. Colour development was allowed to occur for 30 min and stopped with the addition of 3 M NaOH. The absorbance of each well was read at 405 nm.

Statistical analysis. Statistical analyses of data were performed using the Statistical Analysis System (SAS Institute). Data were transformed exponentially, geometric means determined, and statistical significance of differences was calculated using parametric tests. An *F*-test with data taken as repeated measurements was used to determine whether administration of wild-type or *prgH* mutant vaccine resulted in a significant decrease in shedding of ST4/74 Nal^R when compared with mock vaccination. Single comparisons between control and treatment means were made with a paired Student's two-tailed *t*-test when appropriate. *P* values of 0.05 or less were considered significant. All data are expressed as mean ± SEM.

RESULTS AND DISCUSSION

In vivo screening of an *S. Typhimurium* STM mutant library in pigs

A library of ST4/74 Nal^R signature-tagged mutants was screened for ability to colonize the intestinal tract in a

porcine model of infection. In total 1045 signature-tagged mutants were screened in 11 pools of 95 mutants, with each pool screened in two pigs. Of the 1045 mutants screened, 119 mutants representing 95 genes were defective in colonization on the basis that they gave a weak or absent hybridization signal in the output pool recovered from intestinal mucosa of at least one animal. All mutant phenotypes are reported, since numerous mutants which exhibited varying levels of attenuation in replicate animals were significantly attenuated upon retesting in single infection studies (Morgan *et al.*, 2004). Of the 119 attenuating mutations, the transposon insertion sites of 79 had previously been determined by Morgan *et al.* (2004) and were attenuated in calves, chickens or both species. The transposon insertion sites of 36 of the remaining 40 mutants were determined during the course of this study and all of these mutants were attenuated in the pig model only. Despite the use of three alternative restriction enzymes we were unable to subclone the remaining four mutants due to difficulties associated with cloning large DNA fragments, probably a consequence of excessive distance between restriction sites in the genome relative to those in the transposon. In addition to the 119 mutants attenuated in pigs, a further 108 mutants (with defects in a total of 83 different genes) that were attenuated in calves and/or chicks (Morgan *et al.*, 2004) exhibited no defect in intestinal colonization of pigs (Table 1).

To confirm the attenuated phenotypes demonstrated in the primary screen a selection of transposon mutants representing *Salmonella* pathogenicity islands (SPIs) and fimbrial loci (*prgK*, *ssaQ*, *sugR*, *mgtC*, *pipC*, *safA*, *stbC*, *stbD*) were spiked into fresh pools of 95 among tagged but colonization-proficient mutants and retested in the pig infection model as described. All mutants were consistently attenuated in the second screen (Table 1).

Of the 119 mutants attenuated in pigs, 54 contained transposon insertions within known SPIs, with a striking 49 insertions located in 32 genes of SPIs-1 and -2. SPI-1 encodes T3SS-1 and a number of secreted translocator proteins and facilitates *Salmonella* invasion of host epithelial cells by injection of effector proteins into enterocytes that stimulate rearrangements of the subcortical actin cytoskeleton (Galan & Curtiss, 1989; Galan, 1996; Jones & Falkow, 1994). T3SS-1-secreted effectors also act in concert to influence the induction of enteritis (Drecktrah *et al.*, 2005; Galyov *et al.*, 1997; Zhou & Galan, 2001). SPI-2 encodes T3SS-2 and expression of T3SS-2 and associated effectors facilitates the persistence of intracellular bacteria within membrane-bound *Salmonella*-containing vacuoles (Hensel *et al.*, 1995; Hensel, 2000; Shea *et al.*, 1996).

The isolation of multiple independent attenuating mutations in structural and secreted components of T3SS-1 and T3SS-2 markedly reduces the likelihood that attenuation of SPI-1 or -2 mutants is due to second-site or polar effects. Furthermore, our data support a recent study in which a

SPI-1 *sipB* : : kan^R mutant was found to be significantly impaired in its ability to colonize the intestines of pigs following oral inoculation and to invade porcine intestinal mucosa in ligated ileal loops (Boyen *et al.*, 2006b).

An attenuated mutant with a transposon insertion in *safA* was identified in our screen. The *safABCD* operon is located within *Salmonella enterica* centisome 7 genomic island (SCI), also known as SPI-6 in *Salmonella* Typhi (Folkesson *et al.*, 2002; Parkhill *et al.*, 2001) and encodes *Salmonella*-specific putative atypical fimbriae. Although the *safA* mutant was attenuated in our pig model it was not attenuated in a similar STM study in calves and chickens and the *S. Typhimurium* *saf* operon was not required for virulence in mice (Folkesson *et al.*, 1999; Morgan *et al.*, 2004). This may reflect the ability of bacteria to express different adhesins to exploit different animal hosts. To our knowledge this is the first evidence for a role *in vivo* for this island.

To further validate the STM screen and confirm the role of SafA, two pigs were orally inoculated with a mixed inoculum of the wild-type and a P22 transductant harbouring a *safA* : : mini-Tn5Km2 mutation that was found to be attenuating in one of two pigs in the primary screen. The ratio of *safA* mutant to wild-type strain recovered from ileal mucosa 5 days post-infection [0.84 ± 0.014 (mutant log₁₀ c.f.u. g⁻¹/wild-type log₁₀ c.f.u. g⁻¹ $\pm$ SEM)] was significantly different to the ratio of *safA* mutant to wild-type strain in the initial inoculum (0.92 ± 0 ; $P < 0.001$); however, no statistically significant differences in the magnitude or duration of faecal excretion of the mutant strain relative to the parent were detected (data not shown). The consistent attenuation caused by the *safA* mutation detected at the site of recovery of STM output pools suggests a subtle role for Saf fimbriae in intestinal colonization and confirms that the screen sensitively detects meaningful phenotypes.

Of the other fimbrial mutants present within the mutant bank only *stbC* and *stbD* mutants were attenuated. Despite evidence that type I fimbriae play a role in the colonization of pigs (Althouse *et al.*, 2003), a *fimZ* mutant was not attenuated in our screen. However, it is likely that differences in strain, mutation, inoculation dose, mode of delivery and sampling points between the studies account for the disparity in results. Mutation of *fimZ* reduces expression of type I fimbriae, but simultaneously increases flagella-mediated motility (Clegg & Hughes, 2002), and we consider that it would be unsafe to directly compare our findings to the role established for the type I fimbriae major subunit (FimA) described previously (Althouse *et al.*, 2003).

Three of the mutants attenuated in the STM screen had transposon insertions in *Salmonella* pathogenicity island 3 (SPI-3) genes: *sugR*, *misL* and *mgtC*. SPI-3 is thought to have evolved by a multi-step evolutionary process and its phylogenetic distribution within *S. enterica* is not uniform. Not surprisingly therefore SPI-3 has a mosaic organization

Table 1. Colonization phenotypes of STM mutants screened in a pig intestinal colonization model

Classification	Gene name*	Proven or predicted function	Colonization phenotype†
SPI associated genes			
SPI-1	<i>orgA</i> ‡	Type III secretion	—
	<i>prgK</i> §	Type III secretion	—
	<i>prgH</i>	Type III secretion	—
	<i>hilD</i> ‡	Type III secretion regulator	—
	<i>hilA</i>	Type III secretion regulator	—
	<i>sipA</i> ‡	Secreted protein	—
	<i>sipD</i>	Secreted protein	—
	<i>sipC</i>	Secreted protein	—
	<i>sicA</i> ‡	Type III secretion	—
	<i>spaR</i> ‡	Type III secretion	—
	<i>spaO</i> ‡	Type III secretion	—
	<i>invI</i>	Type III secretion	—
	<i>invC</i>	Type III secretion	—
	<i>invE</i>	Type III secretion	—
	<i>invF</i>	Type III secretion	—
	<i>invH</i>	Type III secretion	—
SPI-2	<i>ssrB</i> ‡	Type III secretion regulator	—
	<i>ssrA</i> ‡	Type III secretion regulator	—
	<i>ssaB</i>	Type III secretion	—
	<i>ssaC</i>	Type III secretion	wt
	<i>ssaD</i> ‡	Type III secretion	—
	<i>sseA</i>	Type III secretion	—
	<i>sseC</i>	Secreted protein	—
	<i>ssaG</i>	Type III secretion	—
	<i>ssaJ</i> ‡	Type III secretion	—
	STM1410	Unknown	—
	<i>ssaK</i>	Type III secretion	—
	<i>ssaL</i>	Type III secretion	—
	<i>ssaM</i> ‡	Type III secretion	—
	<i>ssaV</i>	Type III secretion	—
	<i>ssaQ</i> §	Type III secretion	—
	<i>ssaR</i>	Type III secretion	—
	<i>ssaS</i>	Type III secretion	—
SPI-3	<i>sugR</i> §	ATP-binding protein	—
	<i>rmbA</i>	Putative cytoplasmic protein	wt
	<i>misL</i> ‡	Autotransporter protein	—
	<i>slsA</i> ‡	Putative inner membrane protein	wt
	<i>mgtC</i> §	Mg ²⁺ transport protein	—
	STM3784	Putative phosphotransferase system	wt
SPI-4	<i>siiD</i>	Putative membrane transporter	wt
	<i>siiE</i> ‡§	Putative inner membrane protein	wt/—
	<i>siiF</i> ‡	Putative membrane transporter	wt
SPI-5	<i>pipB</i> ‡	TTSS-2 translocated protein	wt
	<i>pipC</i> (<i>sigE</i>)§	SopB specific chaperone	—
SPI-6	<i>safA</i> §	Fimbrial subunit	—
Non SPI-encoded virulence-associated genes	<i>sifB</i>	TTSS-2 translocated effector protein	wt
	<i>sopE2</i> ‡	TTSS-1 translocated protein	wt
	<i>mig-14</i>	Resistance to anti-microbial peptides	wt
	<i>sseK1</i>	TTSS-2 translocated effector protein	—
	<i>steC</i>	Effector protein	wt
Cell envelope	<i>rfbP</i>	Lipopolysaccharide synthesis	wt
	<i>rfbK</i>	Lipopolysaccharide synthesis	—
	<i>rfbN</i>	Lipopolysaccharide synthesis	—
	<i>rfbU</i>	Lipopolysaccharide synthesis	wt
	<i>rfbH</i>	Lipopolysaccharide synthesis	—

Table 1. cont.

Classification	Gene name*	Proven or predicted function	Colonization phenotype†
Surface structures	<i>rfbI</i> ‡	Lipopolysaccharide synthesis	—
	<i>rfbA</i>	Lipopolysaccharide synthesis	wt
	<i>wcaE</i> ‡	Colanic acid biosynthesis transferase	—
	<i>rfaK</i>	Lipopolysaccharide synthesis	—
	<i>rfaB</i>	Lipopolysaccharide synthesis	—
	<i>rfaG</i>	Lipopolysaccharide synthesis	—
	<i>wecE</i>	Enterobacterial common antigen biosynthesis	—
	<i>stbD</i> §	Fimbrial usher protein	—
	<i>stbC</i> §	Fimbrial usher protein	—
	<i>fimZ</i>	Fimbrial transcriptional regulator	wt
	<i>csgD</i>	<i>csg</i> fimbrial operon regulator	wt
	<i>fljA</i>	FliC repressor	—
	<i>fljB</i>	Flagellin	wt
	<i>sthB</i>	Outer membrane fimbrial usher protein	wt
	<i>srgA</i> (<i>orf8</i>)	Disulfide oxidoreductase, PE fimbriae	wt
Carbon compound degradation	<i>orf7</i>	Putative regulator, PE fimbriae	wt
	<i>pilO</i>	Putative membrane protein	wt
	<i>cai</i> T	Carnitine transport	wt
	<i>celF</i> ‡	Cellobiose-6-phosphate hydrolase	—
	<i>kduD</i>	2-Deoxy-D-gluconate 3-dehydrogenase	wt
	<i>kduI</i>	5-Keto-4-deoxyuronate isomerase	—
	STM3600	Putative sugar kinase	wt
	STM3793	Putative carbohydrate kinase	wt
	<i>rbsK</i>	Ribokinase	wt
	<i>sbcD</i>	ATP-dependent dsDNA exonuclease	—
DNA degradation	<i>hscC</i>	Putative heat-shock protein	—
Protein degradation	<i>pcgL</i>	D-alanine-D-alanine dipeptidase	—
	<i>clpB</i>	Heat-shock protein	—
	<i>degQ</i>	Serine endoprotease	wt
Amino acid degradation	STM2196	Putative L-serine dehydratase	wt
Pyrimidine biosynthesis	<i>pyrD</i>	Dihydro-orotate dehydrogenase	—
Polyamine synthesis	<i>speA</i>	Arginine decarboxylase	wt
Amino acid biosynthesis	<i>ilvA</i>	Threonine deaminase	wt
Protein translation and modification	<i>pphB</i>	Serine/threonine protein phosphatase	wt
	<i>slyD</i>	EKBP-type peptidyl-prolyl <i>cis-trans</i> isomerase	—
DNA replication, restriction/modification recombination and repair	<i>dinP</i>	DNA polymerase IV	wt
Central metabolism	STM0036	Putative arylsulphatase regulator	—
	STM0084	Putative sulphatase	wt
	<i>citC</i>	Citrate lyase synthetase	—
	<i>pflA</i>	Pyruvate formate lyase activating enzyme 1	—
	<i>ppk</i>	Polyphosphate kinase	—
	<i>ppx</i>	Exopolyphosphatase	wt
	STM4433	<i>myo</i> -inositol 2-dehydrogenase	—
	STM0053	Citrate histidine kinase regulator	wt
	STM0328	Putative permease	—
	<i>ompX</i>	Outer-membrane protein	wt
Transport/binding	<i>sfbB</i>	ABC transporter	wt
	<i>fepA</i>	Ferric enterobactin receptor	—
	<i>entF</i>	Enterobactin synthetase	wt
	STM0765	Putative cation transporter	—
	<i>mdfA</i>	Multidrug translocase	—
	<i>potH</i>	Putrescine transporter	wt
	<i>focA</i>	Putative formate transporter	wt
	<i>hpaX</i>	4-hydroxyphenylacetate permease	wt
	<i>ompN</i> ‡	Outer-membrane protein	wt

Table 1. cont.

Classification	Gene name*	Proven or predicted function	Colonization phenotype†
Chemotaxis and motility Regulatory	<i>chaA</i>	Sodium-calcium/proton antiporter	wt
	<i>hisM</i>	Histidine transport system permease	wt
	<i>cadB</i>	Putative cadaverine-lysine antiporter	—
	STM2574	Putative permease	—
	<i>gabP</i>	Gamma-aminobutyrate permease	wt
	<i>emrA</i>	Multidrug resistance secretion protein	wt
	<i>trkA</i>	Potassium transport protein	wt
	<i>yneA</i>	ABC transport protein	wt
	STM1657	Putative chemoreceptor protein	wt
	<i>ybdO</i>	Putative transcriptional regulator	wt
	<i>dpiA</i>	Two-component system response regulator	—
	STM1127II	Putative transcriptional regulator	wt
	<i>celD</i>	Putative <i>cel</i> operon repressor	wt
	<i>hns</i>	DNA binding protein; pleiotropic regulator	—
	<i>kdgR</i>	Probable global regulatory protein	wt
	<i>relA</i>	GTP pyrophosphokinase	wt
	<i>agaR</i>	<i>aga</i> operon transcriptional regulator	wt
	STM3602	Putative transcriptional regulator	—
Phage-associated	<i>yjjQ</i>	Putative transcriptional regulator	wt
	STM1043	Gifsy-2 prophage	wt
	STM2243	Putative phage tail fibre protein	—
	STM2586	Gifsy-1 prophage	wt
Plasmid functions	STM2587	Gifsy-1 prophage	wt
	<i>traMII</i>	Plasmid transfer	wt
Unknown function	Unknown	Homology to pHCM1 replication protein	—
	<i>yaiU</i>	Putative autotransporter protein	—
	<i>ybaJ</i>	Putative cytoplasmic protein	—
	<i>ydjA</i>	Putative nitroreductase	—
	STM1527	Putative inner membrane protein	—
	STM1540	Putative secreted hydrolase	wt
	STM1548	Putative isomerase	wt
	<i>yciF</i>	Putative cytoplasmic protein	—
	<i>pagO</i>	Putative integral membrane protein	—
	<i>pagK</i>	Putative PhoPQ-activated protein	—
	<i>yeel</i>	Putative inner membrane protein	—
	<i>ygcF</i>	Putative organic radical activating enzyme	wt
	STM2503	Putative diguanylate cyclase	—
	STM3031II	Ail/OmpX homologue	wt
	<i>yigF</i>	Putative inner membrane protein	—
Hypothetical proteins	STM0037	Conserved hypothetical	wt
	STM0080	Conserved hypothetical	wt
	STM0082	Conserved hypothetical	wt
	STM0438	Conserved hypothetical	—
	STM0557	Conserved hypothetical	—
	STM0731	Hypothetical	wt
	<i>ybhM</i>	Conserved hypothetical	—
	STM1228	Hypothetical	wt
	STM1630	Conserved hypothetical	wt
	STM1668‡	Hypothetical	—
	STM1672II	Hypothetical	wt
	STM1698	Hypothetical	wt
	<i>ynaF</i>	Conserved hypothetical	—
	STM1864‡	Conserved hypothetical	—
	<i>araH</i>	Conserved hypothetical	wt
	STM2245	Hypothetical	—
	STM2743	Hypothetical	wt

Table 1. cont.

Classification	Gene name*	Proven or predicted function	Colonization phenotype†
	STM2761	Conserved hypothetical	wt
	STM2779‡	Hypothetical	wt
	STM3026	Hypothetical	wt
	STM3030‡	Conserved hypothetical	wt
	<i>yfgZ</i>	Conserved hypothetical	—
	STM3278	Hypothetical	wt
	<i>yrdB</i>	Conserved hypothetical	wt
	STM4198	Conserved hypothetical	—
	STM4199	Hypothetical	wt
	STM4302	Hypothetical	wt
	STM4316	Hypothetical	—
	pSLT026	Hypothetical	wt

*The STM and pSLT prefixes indicate gene name/number on the chromosome and virulence plasmid as assigned by *S. Typhimurium* strain LT2 complete genome sequencing project.

†wt indicates that the mutant exhibited no apparent defect in intestinal colonization of pigs despite previous attenuation in calves and/or chicks (Morgan *et al.*, 2004); — denotes mutants showing attenuated intestinal colonization in pigs; wt/— indicates both attenuated and wild-type phenotypes observed for different mutants of the same gene.

‡Indicates multiple mutants isolated.

§Indicates mutants rescreened.

||Indicates insertion site upstream of gene.

and encodes a group of functionally unrelated proteins (Amavisit *et al.*, 2003; Blanc-Potard *et al.*, 1999). *sugR* lies within the phylogenetically variable region of SPI-3 and encodes a putative ATP-binding protein (Blanc-Potard *et al.*, 1999). Very little is known about *sugR* and this is the first evidence of a role *in vivo* for this gene. *misL* encodes an autotransporter protein, similar to the AIDA-I adhesin of enteropathogenic *E. coli*, which plays a role in the intestinal colonization of chickens and mice (Blanc-Potard *et al.*, 1999; Dorsey *et al.*, 2005; Morgan *et al.*, 2004). The exact function of MisL *in vivo* is unclear; however, recently Dorsey *et al.* (2005) identified fibronectin as a host ligand for MisL, suggesting that MisL may be involved in bacterial attachment during colonization. The *mgtC* gene encodes an integral membrane protein of unknown function that is required for systemic virulence in mice (Blanc-Potard & Groisman, 1997). *mgtC* forms part of an operon with *mgtB*, which encodes a P-type ATPase that mediates the influx of Mg^{2+} (Snively *et al.*, 1991). It has been suggested that, due to its genetic association with *mgtB*, MgtC might function as a Mg^{2+} transporter but it has since been shown that MgtC is neither required for, nor influences, MgtB transport activity (Blanc-Potard & Groisman, 1997; Moncrief & Maguire, 1998). Recent studies suggest that serovar Typhimurium MgtC may be involved in regulating host or bacterial cell membrane potential and therefore may play a role in influencing cellular ion homeostasis during infection (Gunzel *et al.*, 2006). The attenuation of the *mgtC* mutant in our screen is the first *in vivo* role assigned to this gene using an intestinal colonization model.

A further three mutants with insertions in SPI-3 genes (*rmbA*, *slsA* and STM3784) were known to be present in the mutant bank used in this study, but these mutants did not appear to be attenuated in pigs. This result mirrors those obtained with previous screens in calves and chickens in that different SPI-3-encoded genes do not consistently play a role in colonization or host-specificity (Morgan *et al.*, 2004).

In previous studies SPI-4 was found to be required for colonization of calf ileum but not caecal colonization of chickens (Morgan *et al.*, 2004). The present study suggests that SPI-4 does not play an important role in intestinal colonization in pigs as only 1 of the 11 SPI-4 insertion mutants in the library was attenuated. A total of eight mutants with defects in *siiE* (which encodes a secreted ~600 kDa protein; Morgan *et al.*, 2007) were screened, of which seven colonized well and one was attenuated. As the SPI-4 mutants tested were present in different pools it is possible that variations in competition dynamics between mutants in a given pool may have dictated why only one of the *siiE* mutants was lost during colonization of pigs. Taken together, the data support the notion that SPI-4 is specifically required for colonization of bovine intestines (Morgan *et al.*, 2004).

One of the mutants attenuated in this screen had a transposon insertion in the *pipC* gene (also known as *sigE*). *pipC* is one of six genes found on SPI-5, a 7.6 kb island known to play a role in enteropathogenesis of *Salmonella* (Wood *et al.*, 1998). *pipC* was originally identified in an

in vitro screen for invasion genes and encodes a chaperone specifically required for the stability and/or secretion of SPI-1 effector protein SopB (also known as SigD) also encoded on SPI-5 (Darwin *et al.*, 2001; Galyov *et al.*, 1997; Hong & Miller, 1998). SopB is an inositol phosphate phosphatase and modulates cell signalling to induce enteritis in concert with other effectors (Galyov *et al.*, 1997; Norris *et al.*, 1998). Interestingly the *pipC* mutant was not previously attenuated for colonization in calves (Morgan *et al.*, 2004). An additional mutant with an insertion in the SPI-5 *pipB* gene was also present in the mutant bank used in this study. PipB is an effector protein secreted by T3SS-2, required for colonization of chickens but not calves (Morgan *et al.*, 2004). In the pig model this mutant was not attenuated, suggesting that this effector may not play a role in the colonization of swine.

The role of LPS in virulence has been well documented in STM-based studies of Gram-negative pathogens in food-producing animals (Dziva *et al.*, 2004; Morgan *et al.*, 2004; van Diemen *et al.*, 2005). As expected, several *S. Typhimurium* mutants predicted to be impaired in LPS biosynthesis were found to be attenuated in pigs. The precise role of LPS in colonization is difficult to determine as in addition to its predicted role in providing protection against host defence mechanisms LPS also plays an important role in ensuring the correct insertion, folding and stability of membrane proteins and other surface-anchored molecules involved in colonization (Raetz & Whitfield, 2002).

Three of the attenuated mutants (*pagO*, *pagK* and STM1864) identified in our screen had insertions in genes located within a putative phage remnant known to contain the T3SS-1 effector gene *sopE2* (McClelland *et al.*, 2001). *pagO* and *pagK* were originally identified as PhoP-PhoQ-activated genes by *TnphoA* mutagenesis; however, little is known about their role during infection (Belden & Miller, 1994). The *Salmonella* PhoP-PhoQ system is a two-component regulatory system known to regulate, positively and negatively, a number of genes involved in virulence, growth at low Mg^{2+} concentrations, and resistance to antimicrobial peptides, bile salts and acid pH (Garcia Vescovi *et al.*, 1996; Groisman, 2001; van Velkinburgh & Gunn, 1999). The *pagO* gene encodes a predicted membrane protein which shows homology to PagO from *Klebsiella pneumoniae* and a product of the *Yersinia* virulence plasmid and is a member of the drug/metabolite exporter (DME) superfamily (Jack *et al.*, 2001). In contrast, *pagK* shows no homology to any publicly available database sequences. STM1864, which is also regulated by PhoP-PhoQ, encodes a predicted membrane protein containing a conserved domain common to proteins involved in processes requiring disulphide bond formation (Monsieurs *et al.*, 2005). Such proteins are often involved in processes such as post-translational modification, protein turnover and protein stability. Interestingly this mutant was also attenuated in a calf colonization model (Morgan *et al.*, 2004).

Other PhoP-PhoQ-regulated genes were identified in this screen, including the SPI-3 *mgtC* gene, *pcgL*, *sseK1* which encodes a T3SS-2 secreted effector protein, pSLT026 and a number of SPI-2 encoded genes (Bijlsma & Groisman, 2005; Monsieurs *et al.*, 2005). The identification of PhoP-PhoQ-regulated genes in our model raises the possibility that this system may be required for colonization of pigs by *Salmonella*.

Assessment of secreted proteins as subunit vaccine candidates

Based on the finding that numerous secreted and surface-anchored molecules contribute to intestinal colonization of pigs by *S. Typhimurium* and the fact that vaccines based on extracellular and type III secreted proteins have shown promise against other pathogens, including *Y. pestis* (Leary *et al.*, 1995) and *E. coli* O157 : H7 (Potter *et al.*, 2004), we sought to determine if vaccines based on secreted components of *S. Typhimurium* are protective in pigs. In order to evaluate the protective effect of these proteins in reducing faecal shedding of *S. Typhimurium* we vaccinated pigs with secreted protein vaccines from wild-type ST4/74 Nal^R or a mock vaccine as described in Methods. To evaluate the role of T3SS-1-secreted proteins in protection a further cohort of pigs was vaccinated in parallel with secreted proteins prepared from a *prgH* : : mini-Tn5Km2 T3SS-1 mutant under identical conditions and normalized for protein content to FliC and SiiE (Fig. 1). PrgH is an inner-membrane protein which forms part of the T3SS-1 needle complex required for injection of effector proteins

Fig. 1. SDS-10% PAGE gel of secreted protein vaccine preparations from ST4/74 Nal^R (lane 2) and ST4/74 Nal^R *prgH* : : mini-Tn5Km2 (lane 3). Proteins were separated by SDS-10% PAGE using Mark 12 pre-stained standards (Invitrogen; lane 1) and stained with Gelcode Blue (Pierce). Representative type III secreted Sip proteins, FliC and SiiE are indicated.

Fig. 2. Course of faecal excretion of ST4/74 Nal^R in pigs vaccinated with wild-type secreted protein vaccine (■), *prgH* mutant secreted protein vaccine (●) and mock vaccine (□). The error bars indicate SEM. (*) indicates significant differences ($P < 0.05$) between vaccinated and mock-vaccinated pigs.

into host cells (Kubori *et al.*, 2000). The *prgH* mutant used for vaccine preparation was shown to be attenuated in our pig model of infection (Table 1).

All pigs were challenged orally with wild-type ST4/74 Nal^R following vaccination. Post-challenge, all animals displayed clinical signs of salmonellosis and shed ST4/74 Nal^R from 24 h post-infection (Fig. 2). Clinical signs of infection, primarily general demeanour, were more pronounced in the mock-vaccinated group with two of the pigs from this group reaching their humane end point at day 39 (2 days post-infection). Faecal shedding in all groups peaked 2 days post-infection (day 39) and although pigs from both the wild-type-vaccine and *prgH*-vaccine groups shed lower numbers of ST4/74 Nal^R when compared with the mock-vaccinated animals at this time point ($P = 0.0325$ and $P = 0.0615$ respectively) only the reduction displayed by wild-type-vaccinated pigs was statistically significant. Wild-type-vaccinated and *prgH*-vaccinated pigs also shed lower numbers of ST4/74 Nal^R when compared with

mock-vaccinated pigs on days 1, 3, 4 and 6 post-challenge; however, no statistically significant differences were observed (Fig. 2).

At post mortem examination ST4/74 Nal^R was recovered from selected ileal, caecal and colonic sites (Fig. 3). There was no significant difference in the number of bacteria recovered from mucosal tissues and lymph nodes between all groups; however, animals vaccinated with the wild-type vaccine and *prgH* vaccine had lower numbers of ST4/74 Nal^R in ileal, caecal and colonic contents when compared with the mock-vaccinated animals (Fig. 3). The difference in ST4/74 Nal^R recovered from ileal and colonic contents from wild-type vaccinated animals when compared with mock-vaccinated animals was statistically significant ($P < 0.05$; Fig. 3).

Although the present study indicated that our secreted protein vaccines did not confer long-term protection we were able to demonstrate a significant reduction in faecal shedding during the acute phase of infection and a lessening of disease severity. As the *prgH* vaccine also reduced shedding during this phase of infection [albeit that this was not significant at the 95 % confidence interval ($P = 0.0615$)], it is likely that factors unrelated to T3SS-1 secreted proteins contributed to this effect.

Serum IgG response

Serum IgG responses induced by secreted protein vaccines were analysed by ELISA and are summarized in Fig. 4. Vaccination by the intramuscular route resulted in the induction of primary and secondary serum antibody responses to proteins secreted by both ST4/74 Nal^R and the *prgH* mutant. Using the wild-type secreted protein vaccine as antigen, serum from pigs vaccinated with wild-type vaccine or *prgH* vaccine showed significantly higher reactivity than serum from mock-vaccinated pigs ($P < 0.01$; Fig. 4a). Similarly using the *prgH* vaccine as antigen produced comparable levels of reactivity against serum from both wild-type and *prgH* vaccinated pigs (Fig. 4b).

Fig. 3. Recovery of ST4/74 Nal^R from intestinal lymph nodes, mucosa and contents of pigs vaccinated with wild-type secreted protein vaccine (grey bars), *prgH* mutant secreted protein vaccine (hatched bars) and mock vaccine (open bars) followed by oral challenge with ST4/74 Nal^R. (*) indicates significant differences ($P < 0.05$) between vaccinated and mock-vaccinated pigs. The error bars indicate SEM.

Fig. 4. Serum IgG responses in pigs vaccinated with proteins from wild-type ST4/74 Nal^R and ST4/74 Nal^R *prgH* :: mini-Tn5Km2. Serum samples from pigs vaccinated with ST4/74 Nal^R secreted protein preparation (grey bars), *prgH* mutant secreted protein preparation (hatched bars) and mock vaccine (open bars) were collected on day 0 (pre-immune), day 22 (pre-boost), day 37 (pre-infection) and day 43 (post mortem) diluted 1 : 100; and subjected to ELISA. ELISA plates were coated with (a) ST4/74 Nal^R secreted protein vaccine or (b) *prgH* mutant secreted protein vaccine. IgG immune responses induced by the vaccines were determined. The error bars indicate SEM.

Serum from mock-vaccinated pigs showed minimal reactivity with both wild-type and *prgH* mutant secreted protein vaccines (Fig. 4).

To define further the serum IgG responses, wild-type and *prgH* mutant vaccine preparations were separated by 10 % SDS-PAGE, transferred to nitrocellulose and probed with sera taken from vaccinated animals over the course of this study (Fig. 5). Vaccination with wild-type vaccine resulted in the induction of antibody response to known T3SS-1 secreted proteins (Fig. 5b); however, such responses were absent in pigs vaccinated with *prgH* mutant and mock vaccine (Fig. 5a, c).

Vaccines based on secreted components of other Gram-negative pathogens have shown promise. For example, vaccination of calves using secreted proteins from *E. coli* O157 : H7 generated responses against type III secreted proteins and was protective against experimental challenge (Potter *et al.*, 2004) and in the field (A. Potter, personal communication). Further studies to dissect the basis of protection and optimize the vaccine dose, adjuvant and route of delivery of the vaccine used in this study are therefore warranted. It is also possible that additional factors combined with this vaccine preparation may be

Fig. 5. Immunoblot analysis of sera from pigs vaccinated with ST4/74 Nal^R secreted protein preparation, *prgH* mutant secreted protein preparation or mock vaccine. Each blot contains kaleidoscope pre-stained standards (Bio-Rad; lane 1), secreted proteins from wild-type ST4/74 Nal^R (lane 2) and secreted proteins from ST4/74 Nal^R *prgH* :: mini-Tn5Km2 (lane 3). Proteins were separated by SDS-10 % PAGE, transferred to nitrocellulose and probed with representative sera from one animal from each group taken on days 0, 22 and 37 of the study (pre-vaccination, pre-boost and pre-challenge respectively). (a) Mock-vaccinated group, (b) wild-type vaccinated group, (c) *prgH*-mutant vaccinated group.

required to improve levels of protection, as suggested by the multi-factorial nature of colonization in pigs highlighted by the STM screen.

In conclusion, this study has provided a better understanding of the host-specific requirements for establishment of *S. Typhimurium* infection in a key reservoir host and identified numerous genes with previously unrecognized roles in colonization. Inert vaccines that exploit the pivotal role of secreted components conferred limited protection against intestinal colonization and the basis of the protective effect remains to be elucidated.

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