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Reactogenicity and immunogenicity of live attenuated *Salmonella enterica* serovar Paratyphi A enteric fever vaccine candidates

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ABSTRACT

Eight *Salmonella enterica* serovar Paratyphi A strains were screened as candidates to create a live attenuated paratyphoid vaccine. Based on biochemical and phenotypic criteria, four strains, RKS2900, MGN9772, MGN9773 and MGN9779, were selected as progenitors for the construction of Δ*phoPQ* mutant derivatives. All strains were evaluated *in vitro* for auxotrophic phenotypes and sensitivity to deoxycholate and polymyxin B. All Δ*phoPQ* mutants were more sensitive to deoxycholate and polymyxin B than their wild-type progenitors, however MGN10028, MGN10044 and MGN10048, required exogenous purine for optimal growth. Purine requiring strains had acquired point mutations in *purB* during strain construction. All four mutants were evaluated for reactogenicity and immunogenicity in an oral rabbit model. Three strains were reactogenic in a dose-dependent manner, while one strain, MGN10028, was well-tolerated at all doses administered. All Δ*phoPQ* strains were immunogenic following a single oral dose. The *in vitro* profile coupled with the favorable reactogenicity and immunogenicity profiles render MGN10028 a suitable live attenuated Paratyphi A vaccine candidate.

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1. Introduction

Salmonella enterica serovar Typhi and *Salmonella enterica* serovars Paratyphi A and B are the causative agents of enteric fever. Estimates of the worldwide incidence of paratyphoid fever range from about one-tenth to one-fourth that of typhoid fever amounting to approximately 5 million cases a year [1]. However, there has been a recent sharp increase in the incidence of paratyphoid fever in a number of Asian countries [2,3], in particular China [4] and India [5]. Although typhoid and paratyphoid infections occur worldwide, they are considered rare in the developed world. The majority of infections in the developed world are in travelers returning from areas endemic with enteric fever [6–8]. Recently, a rise in the proportion of drug resistant *S.* Paratyphi strains has been reported [2,9,10]. Given the emergence of antibiotic resistant strains and an increase in the frequency of enteric fever caused by *S.* Paratyphi A, the need for an effective vaccine has become apparent [11].

There is currently no licensed vaccine to protect against *S.* Paratyphi A despite an obvious and growing need. A conjugated O-antigen vaccine candidate was tested in Phase 1 and Phase 2 studies and was found to be safe and immunogenic following one or two injections [12]. Interestingly, this conjugated O-antigen vaccine approach failed to elicit a booster response [12]. Although these results are encouraging, the preferred vaccine formulation in developing countries is oral, single-dose, for logistical reasons including ease of administration, acceptability, and compliance [13]. Orally delivered live attenuated vaccines are consistent with this goal and provide additional advantages, including inexpensive manufacture and stimulation of both systemic and local mucosal immune responses [14].

Live attenuated *phoP* and *phoPQ Salmonella* mutants have been extensively studied for their vaccine potential [15,16]. *In vitro* studies demonstrate that the PhoP/PhoQ proteins regulate the expression of more than 40 genes [17] including those involved in macrophage survival, as shown for *Salmonella enterica* serovar Typhimurium [18] and *S. enterica* serovar Typhi [19]. In addition, *phoP*-regulated genes affect sensitivity of *Salmonella* to a variety of antibacterial agents such as bile salts [20] and antimicrobial peptides including melittin [21] and polymyxin B [22]. PhoP is essential for *S.* Typhimurium virulence in the mouse typhoid fever model and *S.* Typhimurium *phoP* mutants are avirulent, immunogenic and protective against wild-type challenge [15,18]. It has also been shown

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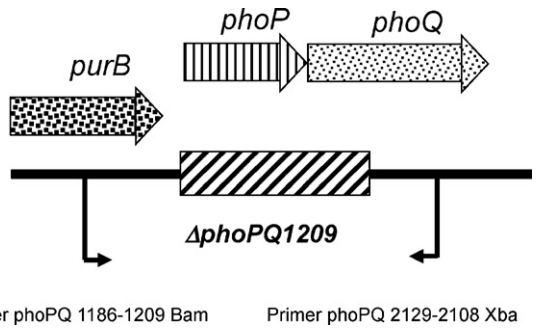

Fig. 1. Schematic diagram of the *phoPQ1209* deletion in *S.* Typhi strain Ty800. Arrows indicate the sites of primer binding for PCR cloning. The DNA between the arrows was cloned from Ty800 into plasmid pMEG-375 to create plasmid pMEG-2575.

that a Δ*phoP* mutant of the swine pathogen, *Salmonella enterica* serovar Choleraesuis is avirulent in pigs [23]. The *phoPQ* genes have also been identified as virulence regulators in a number of other bacteria [24,25].

Data obtained from preclinical studies, such as those described above, formed the basis for developing Δ*phoPQ Salmonella* strains for use as vaccines in humans. The typhoid vaccine candidate Ty800 is a derivative of *S.* Typhi strain Ty2 with a 1209-bp deletion removing all of *phoP* and part of *phoQ* (Fig. 1). In a Phase 1 clinical study, *S.* Typhi strain, Ty800, was safe and immunogenic following a single oral dose [26] and elicited anti-*S.* Typhi specific antibody responses in a higher percentage of volunteers than four oral doses of the licensed live attenuated typhoid fever vaccine, Ty21a [26]. Ty800 was recently evaluated in a larger Phase 1/2 trial in which 120 volunteers received escalating doses of vaccine [27] (Mitchell Cohen, personal communication). A Δ*phoPQ S.* Typhimurium vector strain, LH1160, has also been tested in volunteers and shown to be well-tolerated and immunogenic [28].

Given the clinical success of Ty800 and LH1160, we hypothesized that it is likely the *phoPQ* attenuation strategy will also be applicable to *S.* Paratyphi A. *S.* Paratyphi and *S.* Typhi cause similar diseases and interact with their human host through similar pathways. In addition, multi-locus enzyme analysis performed on the host-adapted serovars revealed a general tendency for clones of the host-restricted serovars, such as *S.* Typhi and *S.* Paratyphi A, to be less diverse in multi-locus genotype than the broad host range serovars [29]. The identification of unique genetic polymorphisms present in both *S.* Typhi and *S.* Paratyphi A [30] further supports the similarities observed in the epidemiology and pathogenicity of these serovars and supports the premise that vaccine development based on strategies that have been successful with *S.* Typhi will extend to *S.* Paratyphi A.

We have been developing a preclinical rabbit model to evaluate the reactogenicity and immunogenicity of orally administered live enteric disease vaccines derived from host-restricted pathogens including the cholera vaccine candidate *Vibrio cholerae* strain Peru-15 [31], its derivative, Peru-15pCTB [32] and the *S.* Typhi vaccine candidate, Ty800 [33]. Although the intranasal mouse model is well-established and is often used for evaluating the immunogenicity of *S.* Typhi vaccine candidates [34,35], the rabbit model involves oral dosing (i.e. the same route of administration used in humans) and may therefore generate a more analogous immune response and provide more relevant insights into vaccine/host interactions.

In this work, we characterized a series of wild-type *S.* Paratyphi A isolates to determine their innate suitability as to serve as vaccine parental strains. We described the introduction of the Δ*phoPQ* mutation into several parental strains, their subsequent characterization *in vitro* and preclinical evaluation of their reactogenicity and immunogenicity in an oral rabbit model.

2. Materials and methods

2.1. Bacterial strains, plasmids, antibiotics and growth media

The *S.* Paratyphi A parent strains used in this study (Table 1) were obtained from the *Salmonella* Stock collection at the University of Calgary, Alberta Canada. Mutant Δ*phoPQ* derivatives are described in Table 2. *S.* Typhi strain Ty800 is a Δ*phoPQ1209* derivative of Ty2 [26]. The *S.* Typhi strain Ty21a is a live attenuated typhoid vaccine [36]. *Escherichia coli* strain MGN617 was used as the donor strain for all conjugal transfer procedures [37]. Plasmid pMEG-375 is an R6K *pir* dependent suicide plasmid that encodes the *cat* and *sacB* genes [38]. Bacteria were routinely grown at 37 °C on LB-V, a vegetable-based rich medium, containing 20 g of HiVeg Luria Broth (HiMedia Laboratories, PVT. Ltd., Mumbai, India) and 5 g NaCl per liter. Chloramphenicol was added to 20 μg/ml when required. Agar (Difco, Detroit, MI) was added to a final concentration of 1.5% for making solid plate media. M9 minimal medium (Difco) containing 1% glucose and appropriate supplements was used to evaluate auxotrophic phenotypes. Adenine was added to a final concentration of 5 mM where indicated. Mueller–Hinton agar was purchased from Difco. Vaccine strains were grown in LB-V broth containing 5 mM adenine to an optical density at 600 nm (OD_{600}) of 1.0 prior to immunization of rabbits. Cultures were centrifuged, resuspended in phosphate-buffered saline, pH 7.0 (PBS), adjusted to the desired dose and administered on the same day. In one experiment, *S.* Typhi strain Ty800 cells were heat-killed by incubation at 56 °C for 1 h prior to administration to rabbits.

2.2. In vitro characterization of strains

Assays to determine sensitivity to deoxycholate [20] and polymyxin B [39] were performed essentially as described. Lipopolysaccharide profiles were assessed by SDS-PAGE [40].

Table 1
Preliminary characterization of *S.* Paratyphi A wild-type strains[a].

Strain #	LPS	Biochemical profile	Plasmid[b]	Antibiotic resistance[c]	Auxotrophy
RKS2900	Smooth	Typical	None	None	*trp*[d]
MGN9772	Smooth	Typical	None	None	None
MGN9773	Smooth	Typical	None	Streptomycin	*trp*
MGN9774	Smooth	Atypical	3.5 kb	None	Undefined[e]
MGN9775	Atypical	Typical	None	None	None
MGN9778	Not done	Atypical	None	None	Undefined
MGN9779	Smooth	Typical	3.5 kb	None	None
MGN9780	Rough	Atypical	None	Streptomycin	None

[a] All strains were positive for group A LPS by agglutination with anti-group A antiserum.
[b] Strains were evaluated for presence of both large and small plasmids.
[c] Antibiotics tested are listed in Section 2.2.
[d] "*trp*" indicates tryptophan is required for growth.
[e] "Undefined" refers to an unknown auxotrophic requirement.

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