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Avian pathogenic *Escherichia coli* Δ*tonB* mutants are safe and protective live-attenuated vaccine candidates

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ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) cause colibacillosis, a serious respiratory disease in poultry. Most APEC strains possess TonB-dependent outer membrane transporters for the siderophores salmochelin and aerobactin, which both contribute to their capacity to cause disease. To assess the potential of iron transport deficient mutants as vaccine candidates, the *tonB* gene was deleted in the APEC wild type strain E956 and a Δ*fur* (ferric uptake repressor) mutant of E956. The growth of the Δ*tonB* and Δ*tonB*/Δ*fur* mutants was impaired in iron-restricted conditions, but not in iron-replete media. Day old chicks were exposed to aerosols of the mutants to assess their efficacy as live attenuated vaccines. At day 18, the birds were challenged with aerosols of the virulent parent strain E956. Both mutants conferred protection against colibacillosis; weight gains and lesion scores were significantly different between the vaccinated groups and an unvaccinated challenged control group. Thus mutation of iron uptake systems can be used as a platform technology to generate protective live attenuated vaccines against extraintestinal *E. coli* infections, and potentially a range of Gram negative pathogens of importance in veterinary medicine.

1. Introduction

Colibacillosis is a systemic disease of poultry caused by a group of extraintestinal pathogenic *Escherichia coli* (ExPEC) referred to as avian pathogenic *E. coli* (APEC) (Barnes et al., 2008). Infection with APEC is responsible for considerable economic losses in the poultry industry worldwide and is often the most frequently cause of carcass condemnation at processing (Barnes et al., 2008; Yogaratnam, 1995). The most common lesions associated with colibacillosis are airsacculitis, perihepatitis and pericarditis. Chickens with airsacculitis have lower body weights, more faecal contamination and higher levels of *Campylobacter* spp. contamination (Russell, 2003). In addition, the zoonotic potential of APEC raises food safety concerns (Chanteloup et al., 2011; Mora et al., 2009; Tivendale et al., 2010). ExPECs that can cause neonatal meningitis, urinary tract infections and septicaemia in humans share high levels of genomic similarity and some key virulence factors with APECs (Johnson et al., 2008; Mora et al., 2011; Moulin-Schouleur et al., 2006, 2007; Olsen et al., 2012). These include iron-uptake systems (Ike et al., 1992; Johnson et al., 2006; Vidotto et al., 1990).

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In Gram negative organisms, ferric iron is imported as Fe^{3+}/siderophore complexes through specific cognate receptors. Although pathogenic and non-pathogenic *E. coli* can produce various siderophores and their receptors, only some of these uptake systems appear to play an effective role during infection of the host, where free iron is scarce. Two virulence-related siderophores, namely the salmochelin and aerobactin, are commonly produced by APECs. These siderophores are thought to escape the innate defence mechanisms that neutralise other common *E. coli* siderophores such as the enterochelin, thus facilitating scavenging of iron from the host (Fischbach et al., 2006).

The cognate receptors specific for Fe^{3+}/siderophore complexes, including Fe^{3+}/salmochelin and Fe^{3+}/aerobactin, are powered by the ExbB/ExbD/TonB system, which obtains energy from the proton motive force. Excess iron can be deleterious for the cell and the genes encoding TonB and the TonB-dependent iron-uptake systems are repressed by the Fur protein in iron-replete conditions. The *fur* regulon also includes a number of virulence genes in *E. coli* and other bacteria (Carpenter et al., 2009).

Previous studies have shown that TonB is required for virulence in a range of bacterial species, including APEC, uropathogenic *E. coli*, *Shigella dysenteriae*, *Klebsiella pneumoniae* and *Haemophilus influenzae* type b (Holden et al., 2012; Hsieh et al., 2008; Jarosik et al., 1994; Reeves et al., 2000; Torres et al., 2001). In the APEC strain E956, which possesses a large virulence plasmid encoding the production of aerobactin and salmochelin (Tivendale et al., 2009a), TonB was shown to be essential for virulence; a derivative of E956 lacking this protein could not cause clinical signs in experimentally infected chickens, despite being able to colonise their respiratory tract (Holden et al., 2012). This suggests that *tonB* mutants may have potential as live-attenuated vaccines that could be administered as aerosols to protect chickens from colibacillosis. Moreover, a strain in which the expression of iron transport systems is deregulated, such as a *fur* mutant, would be expected to strongly and persistently express a number of protective antigens.

In this study, we investigated the potential of mutants derived from the APEC strain E956 that lack TonB as live-attenuated vaccines to protect chickens from a homologous APEC infection. We also sought to evaluate the impact, if any, of an added mutation in *fur* to modulate the vaccinal protection conferred by the *tonB* mutant.

2. Materials and methods

2.1. Bacterial strains, plasmids, primers, culture conditions and reagents

The bacterial strains, plasmids and primers are described in Table 1. *E. coli* strains were cultured in Mueller-Hinton (MH, Oxoid) broth or agar, or nutrient broth (NB) or Luria-Bertani (LB) broth at 37 °C unless stated otherwise. Iron-depleted M9 minimal medium was prepared in glassware deferrated by acid-washing (Maniatis, 1982). All cultures for pKD46 temperature sensitive plasmid maintenance were incubated at 30 °C. Antibiotic concentrations were: chloramphenicol (Cm) 12 μg/ml, kanamycin (Km) 50 μg/ml and ampicillin (Ap) 100 μg/ml.

2.2. DNA manipulation and analysis methods

Genomic DNA was extracted using the Wizard-SV Genomic DNA Purification System, following Promega's eNotes AP0051 (Promega). Plasmid DNA was extracted using the Wizard-Plus-SV DNA Purification System (Promega). PCR was performed using BIOTAQ™ (Bioline), Platinum *Taq* (Invitrogen) or High Fidelity Platinum *Taq* (Invitrogen). PCR-amplified DNA was treated using Wizard-PCR-preps DNA purification system (Promega). All cloning constructs were confirmed by DNA sequencing at Applied Genetic Diagnostics (Department of Pathology, The University of Melbourne, Parkville, Australia). The nucleotide sequences of *cirA*, *fepA*, *fhuA* and *fhuE* from *E. coli* strain K12 (NC_000913), and *iroN*, *iutA* and *fyuA* from *E. coli* strain S88 (NC_011747) were downloaded from Genbank and analysed with the BlastN program (Altschul et al., 1997), using non-redundant nucleotide databases restricted to *E. coli* (taxid:562) as source organism. Sequences similar to each query were compiled into multiple alignments using the Multalin program (Corpet, 1988) and group-specific primers were designed in the conserved regions of each gene by the program FastPCR (Kalendar et al., 2014). All PCR products were amplified using the same conditions with an annealing temperature of 60 °C and an elongation time of 1.5 min.

2.3. Cloning and mutagenesis

Mutants were obtained by lambda red recombination as described before (Holden et al., 2012; Murphy and Campellone, 2003). The *tonB* mutant, Δ*tonB*::Cm, was previously constructed in APEC strain E956 (Holden et al., 2012). The *tonB*/*fur* double mutant, E956 Δ*tonB*::Cm-Δ*fur*::Km, was constructed by inserting a kanamycin resistance gene in the *fur* gene of E956 Δ*tonB*::Cm. A 1296 bp fragment containing *fur* was PCR-amplified from APEC strain E3 using primers furF1 and furR1 and cloned into pGEM-T easy (Promega), to generate the 4313 bp construct pKL2, which was amplified by inverse-PCR with primers invfurF1+ and invfurR1+ to introduce a *Xho*I site and a 320 bp deletion within the *fur* coding region. This amplicon was digested with *Dpn*I and *Xho*I, then self-ligated to generate the 3993 bp construct pinvKL2. The kanamycin resistance gene was amplified from pKmK5 and inserted into the *Xho*I site of pinvKL2, generating the 5042 bp construct pKL2Km. A 1795 bp PCR fragment was amplified from pKL2Km using the primers furF1 and furR1, purified and diluted to 500 ng/μL in water.

Electrocompetent APEC E956 Δ*tonB*::Cm cells carrying pKD46 were transformed with 2.5 μg of PCR fragment DNA and plated on MH agar with kanamycin as described previously (Holden et al., 2012; Murphy and Campellone, 2003; Sambrook and Russell, 2001). The *tonB*/*fur* double mutant was confirmed by PCR using the primer pair fldAΔfurF1 and ybfMΔfurR1 (Table 1).

To construct the single *fur* mutant, E956 Δ*fur*::Km, a 1795 bp PCR fragment was amplified from pKL2Km using