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Secretion of the Shiga toxin B subunit (Stx1B) via an autotransporter protein optimizes the protective immune response to the antigen expressed in an attenuated E. coli (rEPEC E22Δler) vaccine strain

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ABSTRACT

We previously developed attenuated rabbit enteropathogenic *E. coli* (rEPEC) strains which are effective oral vaccines against their parent pathogens by deleting *ler*, a global regulator of virulence genes. To use these strains as orally administered vectors to deliver other antigens we incorporated the B subunit of shiga-like toxin 1(Stx1) into the passenger domain of the autotransporter EspP expressed on a plasmid. Native EspP enters the periplasm where its passenger domain is exported to the bacterial surface through an outer membrane channel formed by its translocator domain, then cleaved and secreted. Since antigen localization may determine immunogenicity, we engineered derivatives of EspP expressing Stx1B- passenger domain fusions: 1. in cytoplasm 2. in periplasm, 3. surface-attached or 4. secreted. To determine which construct was most immunogenic, rabbits were immunized with attenuated O103 *E. coli* strain (E22 Δler) alone or expressing Stx1B in each of the above four cellular locations. IgG responses to Stx1B, and toxin-neutralizing antibodies were measured. Animals were challenged with a virulent rabbit Enterohemorrhagic *E. coli* (EHEC) strain of a different serogroup (O15) than the vaccine strain expressing Stx1 (RDEC-H19) and their clinical course observed. IgG responses to Stx1B subunit were induced in all animals vaccinated with the strain secreting Stx1B, in some vaccinated with surface-expressed Stx1B, but in not animals immunized with periplasmic or cytoplasmic Stx1B. Robust protection was observed only in the group immunized with the vaccine secreting Stx1B. Taken together, our data suggest that secretion of Stx1B, or other antigens, via an autotransporter, may maximize the protective response to live attenuated oral vaccine strains.

1. Introduction

The development of live, attenuated strains of enteric pathogens such as *Salmonella* and *Vibrio cholera* as vaccines has led to many attempts to utilize these strains as vaccine vectors to deliver antigens from other important pathogens in the gut (Boedeker et al., 1995; Zhang et al., 2008) (Keller et al., 2010).

As our vector, we have previously reported the development of live attenuated strains of attaching effacing (A/E) *E. coli*, by deleting the gene encoding the LER global regulator (Zhu et al., 2006a). These strains have decreased secretion of virulence factors encoded on the locus of enterocyte effacement. They are immunogenic, and are effective oral vaccines against the A/E pathogens from which they are derived, although they do not provide cross-serotype protection (Ahmed et al., 2013; Zhu et al., 2007). The A/E *E. coli* have an advantage over other attenuated enteric pathogens such as Salmonella, in maintaining a balance between immunogenicity and reactogenicity since they are non-invasive pathogens which induce protective immunity via interaction with mucosal inductive sites in the intestine (Cantey and Inman, 1981).

To express foreign antigens in our attenuated A/E vector strains we have explored the incorporation of a target antigen into autotransporter proteins (Ruiz-Perez et al., 2002; Ruiz-Perez and Nataro, 2014). The autotransporter proteins are comprised of three domains, a signal peptide, a passenger domain and the beta or translocator domain (Fig. 1). The signal peptide of the autotransporter targets the protein into the periplasmic space by the *sec*-dependent general secretory pathway. The C-terminal translocator domain of the autotransporter forms a transmembrane β-barrel through which the N-terminal (passenger) domain travels through the outer membrane (Henderson et al.,

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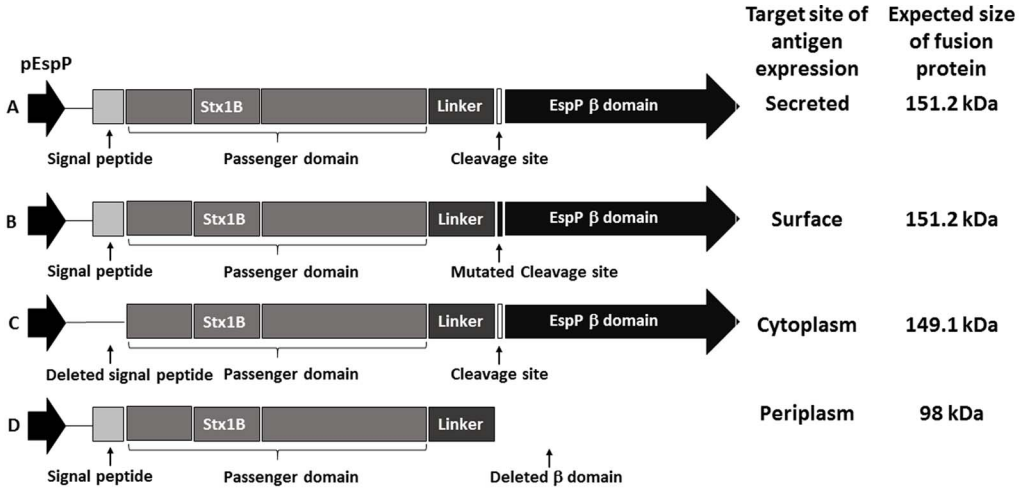

Fig. 1. Schematic diagram of EspP-Stx1B fusion proteins designed to target antigen expression in different subcellular compartments. Stx1b (7.7 kDa) was inserted into a 5.3 kDa deletion (49 amino acids from 242 to 291) of the passenger domain in the N-terminal region of the native EspP, which included the serine protease active site. This construct (A) yielded a 98 kDa secreted product in the supernatant after cleavage from the β domain. This initial EspP-Stx1B construct was then modified to deliver the antigen in different subcellular compartments by mutating the cleavage site (B, surface retention), deleting the signal peptide (C, cytoplasmic retention) or the b-domain (D, periplasmic retention). The expected size of antigen-containing fragments in each target compartment is shown in the table.

Target site of Stx1B antigen expression	Size in whole cell (Fig 2)	Size in periplasm (Fig 3)	Size in supernatant (Fig 4)	Size in outer membranes (Fig 5)
Secreted	152 kDa or less	152 kDa or less	98 kDa or less	0
Surface	152 kDa or less	152 kDa or less	0	151 kDa or less
Cytoplasm	149 kDa or less	0	0	152 kDa or less
Periplasm	98 kDa or less	98 kDa or less	0	0

2000, 1998). Once translocated to the bacterial surface, the passenger domain can remain attached or be cleaved and released into the external milieu depending on the integrity of a proteolytic cleavage site immediately upstream of the β-domain.

The aim of the current study is to evaluate the expression of foreign antigens and the immunogenicity of the target antigen expressed in our attenuated rEPEC by autotransporter proteins. To determine the bacterial compartment for expression of target antigen/passenger domain fusions which induces the optimal protective immune response, we developed strategies for directing the expressed Stx1B target antigen to be secreted into the external milieu, attached to the bacterial surface, retained in the periplasmic space, or expressed only in the cytoplasm. These strategies are illustrated in Fig. 1. Insertion of the target antigen into the passenger domain of the native autotransporter (Fig. 1A) will result in its secretion into the external milieu. Deletion of the proteolytic cleavage site proximal to the passenger domain (Fig. 1B) will result in antigen retention on the bacterial surface. Deletion of the signal peptide (Fig. 1C) will confine antigen expression to the cytoplasm. Deletion of the entire β-barrel (Fig. 1D) will result in antigen expression in the periplasm.

To test the efficacy of our attenuated Attaching/Effacing (A/E) rEPEC strains as vectors to deliver a protective antigen, we incorporated Shiga toxin B subunit (Stx1B), into the passenger domain of the autotransporter EspP serine protease of O157:H7 (Weiss and Brockmeyer, 2013). Shiga toxin-producing strains of *E. coli* induce hemorrhagic colitis, which may progress to fatal hemolytic uremic syndrome (HUS) (Paton and Paton, 1996, 1998; Pongpech et al., 1989; Voss et al., 1998). Disease-associated strains are collectively referred to as enterohemorrhagic *E. coli* (EHEC), a subset of Shiga-toxin producing *E. coli* (STEC) (Kaper et al., 2004; Paton and Paton, 1998). Shiga-toxins are broadly classified as Stx1 and Stx2 (Creydt et al., 2006; Paton et al., 1995, 1993, 1992; Paton and Paton, 1996). Both Stx1 and Stx2 are multi-subunit AB toxins composed of one enzymatically-active A subunit (approx mw. 32 kDa) in noncovalent association with a pentamer of receptor-binding B subunits (approx mw.7.7 kDa). Both Stx1 and Stx2 variants are encoded on lysogenic bacteriophages which can be induced to enter a lytic phase in conditions of stress (such as sub-inhibitory concentrations of antibiotics) with resulting toxin production and release. Immunization with Stx1 or Stx2 toxoid provided both homologous and heterologous protection from target tissue uptake of both Stx1 and Stx2 (Smith et al., 2006). Immunization with the B subunits of Stx (Stx1B or Stx2B) provides protection against challenge by the homologous toxin (Bentancor et al., 2009; Marcato et al., 2005).

We have selected Shiga toxin producing strains of rabbit enteropathogenic *E.coli* (rEPEC) (Sjogren et al., 1994) whose administration to rabbits induces hemorrhagic colitis (HC) and has provided a reproducible animal model for human EHEC disease (Tarr, 1994). The strain RDEC-H19 is a derivative of the O15 rEPEC strain RDEC-1 (Cantey and Inman, 1981) which has acquired the H19 Stx1 encoding bacteriophage of a human EHEC strain. This strain has been used by our laboratory in studies of vaccines against STEC infection (Zhu et al., 2006a, 2007, 2008). In the present study, we have used it as a challenge strain to test the relative protective efficacy of our O103 E22Δler vaccine vector strain expressing Stx1B subunit in different bacterial compartments. Since previous studies have shown that the O103 E22Δler vaccine vector did not provide cross-serogroup protection against infection with the O15 RDEC-H19 strain (Ahmed et al., 2013), protection observed in the present challenge studies should be primarily attributable to vaccine-induced immunity to the Stx1B antigen.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains, plasmids and primers used in this study are listed in Table 1. The vaccine O103 strain rEPEC E22Δ*ler* (Zhu et al., 2006a)was used as a vector to deliver Stx1B in the passenger domain of the autotransporter EspP when transformed with plasmids designed to express the antigen in different cellular compartments. Another rEPEC strain RDEC-H19 (Sjogren et al., 1994) expressing Stx1 was used to challenge animals. Luria Broth (Difco Laboratories, Detroit, MI, USA) was used for liquid cultures (LB) and Luria Broth Agar (LBA) was used for solid media when culturing *E. coli* strains. O15 *E. coli* strain RDEC-H19 (Sjogren et al., 1994) expressing Stx1 was used as the challenge strain. The plasmids used in this study were propagated in *E. coli* DH5α, grown in LB broth supplemented with Ampicillin (Amp), 250 μg/ml. Stocks used for the animal inoculations were made fresh from bacteria cultured in LB, and viable bacteria were quantified by plating serial dilutions on LBA plates. Bacterial strains were stored at −80 °C in LB containing 20% glycerol.