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Evolution of STEC virulence: Insights from the antipredator activities of Shiga toxin producing *E. coli*

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) are a diverse group of strains that are implicated in over 270,000 cases of human illness annually in the United States alone. Shiga toxin (Stx), encoded by a resident temperate lambdoid bacteriophage, is the main STEC virulence factor. Although the population structure of *E. coli* O157:H7, the most common disease-causing STEC strain, is highly homogenous, the range of clinical illness caused by this strain varies by dramatically outbreak, suggesting that human virulence is evolving. However, the factors governing this variation in disease severity are poorly understood. STEC evolved from an O55:H7-like progenitor into a human pathogen. In addition to causing human disease, Stx released from STEC kill bacterivorous protist predators and enhance bacterial survival in the face of protist predation. Cattle are the primary reservoir for STEC and protists and bacteria occur together within the ruminant intestinal tract. Cattle associated STEC are not highly pathogenic to humans. These observations suggest that disease causing STEC strains evolved from cattle-associated "antipredator" STEC strains. To test this idea and to gain insight into the features that govern the evolution of STEC from a commensal strain of ruminants strain to virulent human pathogen, we compared the predation resistance of STEC strains isolated from asymptomatic infected cows and human patients. We find that STEC O157:H7 progenitor lineages and clades are more effective than human associated ones at killing the types of protist predators. In addition, our results indicate that the presence of Stx2c-containing bacteriophage is associated with more efficient amoeba killing. Also, these phage apparently also encode Q21-like version of the Q antitermination protein, the protein that controls expression of Stx.

1. Introduction

In order to understand the development of bacterial pathogens, it is critical to study the forces that drive the evolution of pathogenicity factors. Voracious predation by microfaunal predators, such as protozoa and nematodes is a major source of bacterial mortality (Berninger et al., 1991; Jurgens and Matz, 2002). Protozoan bacteriovory reduces bacterial numbers and is crucial in bacterial population control (Jousset, 2012). As such, in order to avoid eradication, bacterial prey have evolved a wide array of defenses against predation (Erken et al., 2013), including biofilm formation (Friman et al., 2013), toxin production (Lainhart et al., 2009), enhanced motility (Wanjugi and Harwood, 2014), among others. Pathways and factors that are the targets of antipredator defenses are common to all eukaryotes, including humans. By selecting for factors that protect bacteria from predation, predators drive evolution of bacterial "defenses", which consequently result in the ability to cause human disease (Erken et al., 2013; Matz and Kjelleberg, 2005). Therefore, the evolutionary arms race between microbial predators and their bacterial prey has forged and refined mechanisms that enhance the human pathogenicity of bacteria. Hence, despite the prevalence of disease caused by microbial pathogens, humans are likely nothing more than collateral damage in the evolutionary arms-race between bacteria and eukaryotic bacteriovores. Thus to better understand how bacterial pathogens evolve, it is important to look beyond the human-bacteria host-pathogen interaction.

A family of phages strongly related to the well-characterized coliphage λ has been identified as carriers of exotoxin encoding genes, including both those that affect humans e.g., cytolethal distending toxin

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(Boyd, 2012) and diphtheria toxin (Brüssow et al., 2004), and toxins that have no known effects on humans, e.g., botulinum C1 & D toxins, and an ADP ribosylating toxin that attacks the larvae of giant prawns (Munro et al., 2003; Oakey and Owens, 2000), All lambdoid bacteriophages, share a common developmental program. Upon infection of a bacterial cell, the lambdoid phages choose between two developmental fates. The phage can grow lytically, thereby killing the host. Alternatively, in lysogenic growth the phage chromosome inserts into the host genome where it is passively replicated until a signal that induces lytic growth is perceived by the prophage. The exotoxin genes in many lambdoid phages are expressed only during lytic growth and are thus not expressed by lysogens. In the case of temperate phages that carry toxin genes, most these toxins are only produced during lytic growth. As such, the individual bacterium that is responsible for production and release of the toxin is ultimately killed as a consequence of phage mediated lysis.

Shiga toxin (Stx) is one of the best known phage-encoded exotoxins. The Stx-encoding bacteriophages are found to be associated with a broad range of hosts, including several serotypes of *E. coli* (Beutin et al., 2004; Scheutz et al., 2012; Smith et al., 2014) *Enterobacter cloacae* (Paton and Paton, 1996), *Shigella flexneri* (Gray et al., 2014), *and Citrobacter freundii* (Schmidt et al., 1993) and *Enterococcus* (Casas et al., 2011). There are two classes of Stx encoded by phages, Stx1 and Stx2. Several Stx2 isoforms are known (Krüger and Lucchesi, 2015). Environmental studies suggest that in the presence of predators, bacteria gain a significant survival advantage when they are host to a phage that encodes this Stx (Arnold and Koudelka, 2014; Artz and Killham, 2002; Lainhart et al., 2009; Mauro and Koudelka, 2011, for reviews see Fremaux et al., 2008; Los et al., 2012). When found in humans, bacteria bearing Stx-encoding phages are responsible for causing such diseases as haemorrhagic colitis, infantile diarrhea and haemolytic uremic syndrome. Although both classes and all isoforms are functionally identical, only certain isoforms of Stx2 are commonly associated with more severe human disease. Nonetheless, the importance of the individual Stx classes and forms to human pathogenesis has not been firmly established.

The most commonly identified Shiga toxin producing *E. coli* (STEC) strain is *E. coli* O157:H7. STEC O157:H7 has evolved from an O55:H7-like progenitor (Feng et al., 1998; Lacher, 2011; Manning et al., 2008; Whittam et al., 1993; Zhou et al., 2010) (see Fig. 1). These strains can be divided into three major genetic lineages that differ in their distribution among humans and cattle (Yang et al., 2004; Zhang et al., 2007). Lineage I and I/II strains are commonly associated with severe human disease, and lineage II strains that are not associated with human disease and are found almost exclusively in bovine hosts (Eppinger et al., 2011a). Regardless, phylogenetic analyses suggest that STEC O157:H7 strains found worldwide are highly related and comprise a clone complex (Whittam et al., 1993). Consistent with this suggestion, genomic analysis of a comprehensive panel of STEC O157:H7 strains indicate that this strain's chromosomal backbone is highly conserved (Eppinger et al., 2013; Eppinger et al., 2011a,b). Despite the relative homogeneity of the population structure of STEC O157, the severity of human disease caused by this strain varies by outbreak. The finding that STEC O157 genomic variations occur primarily due to differences in phage carriage, alterations in phage gene content and the integration sites of the respective phage (Smith et al., 2012; Yin et al., 2015) suggests that phage-encoded factors may play a role in determining disease severity.

STEC O157:H7 outbreaks are becoming more numerous and are characterized by more severe disease (CDC, 2017). Unfortunately, it is not yet clear why outbreaks of EHEC O157 vary in the severity of illness and the frequency of the most serious complication of infection, hemolytic uremic syndrome (HUS). Nonetheless, this finding suggests that increased human virulence is evolving.

Cattle are considered to be the primary reservoir for STEC and the most common cause of STEC O157:H7 infection is ingestion of food or water contaminated with cattle feces (Armstrong et al., 1996). The primary site of *E. coli* O157:H7 colonization in ruminants is the rectoanal junction (Naylor et al., 2003). Bacterivorous protist predators are present in this region of these animals (Besser et al., 2011) and these gastrointestinal protozoa take up gastrointestinal bacteria at a high rate (Coleman, 1964). Given the antipredator activity of Stx, this predation would favor the carriage of the Stx-encoding prophage by gastrointestinal STEC O157:H7 (Steinberg and Levin, 2007). Together, these observations suggest that the 'cattle-only' strains may be intermediates in the evolution of STEC, bridging the ancestral STEC and human pathogens. To test this idea and to gain insight into the features that govern the evolution of virulence in STEC, we compared the abilities of a series of STEC O157:H7 strains isolated from infected cows or humans (Hartzell et al., 2011) to kill a eukaryotic bacteriovorous predator.

2. Materials and methods

2.1. Strains, plasmids and chemicals

Acanthamoeba castellanii strain ATCC 30234 MG1655 (ATCC 700926) and *E. coli* strain O157:H7 EDL933 (ATCC 700927) were obtained from the American Type Culture Collection (Manassas, VA). *E. coli* strain EDL933Δstx (Gobert et al., 2007), an EDL933 strain containing deletions for both *stx* genes, was a gift from Christine Miller, Institut National de la Recherche Agronomique. All other STEC O157:H7 strains (Table 1) used in this study were the gift of Dr. Ed Dudley and were obtained from the *E. coli* Reference Center (Penn State University, College Park, PA).

2.2. Cultivation and harvesting of A. castellanii

Acanthamoeba castellanii were grown in ATCC Medium 712: PYG w/ additives (2% proteose peptone, 0.1% yeast extract, 4 mM $MgSO_4$ $7H_2O$, 0.4 mM $CaCl_2$, 0.05 mM $Fe(NH_4)_2(SO4)2$ $6H_2O$, 2.5 mM Na_2HPO_4 $7H_2O$, 2.5 mM KH_2PO_4, 0.1% sodium citrate dihydrate, and 0.1 M glucose, pH 6.5) in 75-cm^2 tissue culture flasks, incubated at 28 °C without aeration. Amoebae were routinely subcultured every 7 days. After cultures reached saturation ($\sim 5 \times 10^5$ cells/ml), flasks were placed on ice for 10 min to release amoeba from the base of the flask, mixed well, and cells were removed for passaging or experimentation. Stocks of *Acanthamoeba castellanii* were stored at 4 °C as described (Axelsson-Olsson et al., 2009 B. 2009), and/or cryogenically (−196 °C) as described (Kilvington and White, 1991).

2.3. Effect of toxin encoding bacteria on A. castellanii viability

The effect of bacteria on amoeba viability was measured as described by (Arnold and Koudelka, 2014). Briefly, toxin encoding and control *E. coli* strains were grown with agitation to saturation overnight in Millers LB. *A. castellanii* were also grown to saturation and harvested as described above. Bacteria were harvested by centrifugation at 3000 *xg*, washed and re-suspended in equal volume of Pages Amoeba Saline [PAS] (per liter: 120 mg NaCl, 4 mg $MgSO_4$-$7H_2O$, 4 mg $CaCl_2$-$2H_2O$, 142 mg Na_2HPO_4, and 136 mg KH_2PO_4. *A. castellanii* were harvested from saturated cultures by centrifugation at 300 *xg* and washed twice with PAS. Re-suspended bacteria were used to re-suspend the washed *A. castellanii* pellet. Co-cultures were incubated at 30 °C for 2 h without agitation. After incubation, the viability of *A. castellanii* was determined using trypan blue exclusion assay. Each measurement was performed in triplicate and the data was averaged. The data shown represents the average of ≥9 sets of triplicate measurements.

3. Data analysis methods

Amoeba killing efficiencies were calculated from the fraction of amoebae killed in presence of STEC, relative to amount the killed by