Susceptibility of human enterotoxigenic *Escherichia coli* isolates to growth inhibition by porcine intestinal epithelial cells

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ETEC; IPEC-J2; cationic antimicrobial molecule; bacterial pathogenesis.

Abstract
Growth of human, but not porcine enterotoxigenic *Escherichia coli* (ETEC) isolates is inhibited during incubation with porcine intestinal epithelial cells and by a constitutively produced factor(s) present in unstimulated cell supernatants. The inhibitory factor(s) is heat stable, not produced by serum-starved cells, and is present in a diverse number of cultured epithelial cell lines of animal, but not of human origin. Susceptibility to porcine intestinal epithelial cells appears to be restricted to ETEC and not *E. coli* O157:H7 disease isolates.

Introduction
Diarrheal disease caused by enterotoxigenic *Escherichia coli* (ETEC) is a major contributor to total food-borne disease burden, a leading cause of travelers’ diarrhea and a major endemic health threat in underdeveloped nations, especially among children. The prototypical human strain, H10407 (O78:H11), expresses two different surface pili that serve as colonization factors (Cheney & Boedeker, 1983), and several enterotoxins, including the heat-labile (LT) and heat-stable (ST) toxins that induce water and electrolyte loss from the intestine of infected subjects (Nataro & Kaper, 1998).

Prior studies of human ETEC isolates in relevant animal models are very limited in scope. The gnotobiotic piglet model is considered to be the superior system for studying ETEC *in vivo*. Porcine ETEC naturally causes severe intestinal disease in pigs, manifested clinically by intestinal colonization, severe diarrhea, and death (Moxley *et al*., 1998). To begin to adapt human ETEC (hETEC) isolates for study in the gnotobiotic piglet, it was first attempted to quantify hETEC adherence to porcine intestinal epithelial cells (IPEC-J2). However, it was unexpectedly observed that hETEC growth is inhibited during incubation with IPEC-J2 cells and by a constitutively produced factor(s) present in the supernatants of IPEC-J2 cells and other epithelial cells of diverse origin.

Materials and methods

Bacterial strains and mammalian cell culture

The bacterial strains utilized in these studies are described in Table 1. IPEC-1 and IPEC-J2 cells are undifferentiated porcine intestinal epithelial cell lines derived from the jejunum and small intestine, respectively, of unsuckled day-old piglets (Rhoads *et al*., 1994, 1997; Kandil *et al*., 1995; Schierack *et al*., 2006). These cells were cultured in Dulbecco’s modified Eagle medium (DMEM)/F12 supplemented with 5% fetal bovine serum (FBS), insulin (5 μg mL⁻¹), transferrin (5 μg mL⁻¹), selenium (5 ng mL⁻¹), and epidermal growth factor (EGF, 5 ng mL⁻¹). The supplemented IPEC cell media are hereafter referred to as DMEM/F12 for brevity. Caco-2 (human colorectal adenocarcinoma cells; Laburthe *et al*., 1980) cells were cultured in DMEM supplemented with 10% FBS and nonessential amino acids. MDBK (bovine kidney cells; Nelson-Rees *et al*., 1966), Int407 (nontransformed human embryonic intestinal epithelial cells; ATCC#CCL-6; Mahajan & Rodgers, 1990), and ST (swine testes; Chu *et al*., 1982) cells were cultured in DMEM supplemented with 10% FBS. Cells were grown to > 80% confluency, when cells were washed and media were replaced. Cells were maintained in a humidified incubator in an atmosphere of 5% CO₂ at 37 °C.
Cell supernatants were collected for use in bacterial growth assays after a 48-h incubation in fresh media. Media bathing the eukaryotic cells were removed and centrifuged briefly to remove the dead cells, with the resultant media classified hereafter as 'cell supernatant'. Where indicated, cell supernatants were subjected to filtration (0.22 μm; Amicon) and/or heat treatment (100 °C, 10 min) before addition to growth assays.

**Bacterial adherence assays**

Approximately 5.0 × 10^5 IPEC-J2 cells were seeded in 24-well plates, grown overnight in DMEM/F12, and infected with 5 μL of ETEC cultures (~1.0 × 10^6 CFUs) grown statically overnight. Adherence assays were incubated for 4 h in 5% CO_2 at 37 °C. The supernatants were removed, serially diluted, and plated on Luria–Bertani (LB) for enumeration of CFUs. The geometric mean number of CFU in each treatment group was compared with a two-tailed Student’s t test. A P-value < 0.05 was considered to be significant.

**Bacterial growth assays**

Growth was monitored by measuring the OD_{600 nm} vs. time of bacterial cultures inoculated 1:100 into DMEM/F12 and subsequently incubated at 37 °C, with shaking at 200 r.p.m. Also included was the indicated amount of cell supernatants (% v/v) from the indicated mammalian cell lines. Control experiments were performed with static cultures grown in air and static cultures grown in 5% CO_2. Control experiments were also performed with inoculation of bacteria into DMEM (unsupplemented) and LB. Data were compared with a two-tailed Student’s t test, and a P-value < 0.05 was considered to be significant. Experiments were performed in triplicate on at least three separate occasions. Where indicated, growth assays were supplemented with twofold serial dilutions of EGF (0–20 ng mL⁻¹), lysozyme (0–3 μg mL⁻¹), FBS (0–25%), or trypsin (0–2%).

### Results

**IPEC-J2 cells inhibit hETEC growth**

While attempting to quantify the adherence of ETEC H10407 (s345) to porcine intestinal epithelial cells (IPEC-J2), it was unexpectedly observed that bacterial growth was inhibited during bacterial adherence assays. IPEC-J2 cells have been characterized extensively for their ability to serve as a relevant model of the pig intestine (Schierack et al., 2006). These cells are enterocyte-like, produce glycosalx-bound mucin, form microvilli, and form tight junctions and create a functional cell monolayer in vitro (Schierack et al., 2006). These cells have been used in numerous studies of Gram-negative bacterial–host interactions (McOrist et al., 1995; Skjolås et al., 2006, 2007; Brown & Price, 2007), and infection induces appropriate cytokine responses in these cells (Schierack et al., 2006).

Despite adding similar concentrations of porcine ETEC (pETEC) field isolates s372 and s403 vs. hETEC isolates s345, s417, and s419 to IPEC-J2 cells (Fig. 1a), the number of bacteria recovered from the supernatant of adherence assays after a 4-h incubation was greatly reduced for hETEC (Fig. 1b). To exclude the possibilities that the growth rates of the strains might be different, or that a component of the supplemented DMEM/F12 media might contain an inhibitory compound, we quantified bacterial growth of s345 and s372 in DMEM/F12, and yet observed that the growth rates were not significantly different (Fig. 1c). Because of the confounding growth inhibition of s345, we did not pursue precise quantification of human ETEC adherence to IPEC-J2 cells, but instead sought to characterize the source of inhibition.

To determine whether IPEC-J2 cells might secrete a substance inhibitory to hETEC, quantification was performed of bacterial growth in DMEM/F12 that had been supplemented with cell-free supernatants (1% v/v) obtained from media used to culture uninfected IPEC-J2 cells. As shown in Fig. 1d, the growth of hETEC was significantly inhibited in the presence of 1% supernatant (closed circles), whereas the supernatant did not inhibit the growth of pETEC (Fig. 1d, crosses). Both s345 and s372 grew robustly in DMEM/F12 lacking any IPEC-J2 supernatant (Fig. 1d, open circles and diamonds). Control experiments were also performed in which bacterial cultures were incubated statically in either air or 5% CO_2. Growth assays were also performed with bacterial inoculation into DMEM (unsupplemented) and LB. Because differences in bacterial growth as a function of culture aeration or culture media were minimal, only the DMEM/F12 data are shown here. Overall,

### Table 1. Bacterial strains used in these studies

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>s345</td>
<td>ETEC H10407 D78:H11, LT+</td>
<td>ATCC</td>
</tr>
<tr>
<td>s372</td>
<td>ETEC 3030-2 K88+, Stb+, LT+</td>
<td>Field isolate</td>
</tr>
<tr>
<td>s403</td>
<td>ETEC 1836-2 K88+, astA+</td>
<td>Field isolate</td>
</tr>
<tr>
<td>s414</td>
<td>ETEC 91.1283 O6:Sta+LT+</td>
<td>Chobi DebRoy</td>
</tr>
<tr>
<td>s415</td>
<td>ETEC 91.1626 O6:Sta+LT+</td>
<td>Chobi DebRoy</td>
</tr>
<tr>
<td>s417</td>
<td>ETEC 91.1261 O27:Sta+LT+</td>
<td>Chobi DebRoy</td>
</tr>
<tr>
<td>s419</td>
<td>ETEC 91.1353 O148:Sta+LT+</td>
<td>Chobi DebRoy</td>
</tr>
<tr>
<td>s422</td>
<td>E. coli O157:H7 86-24</td>
<td>Tzipori et al. (1995)</td>
</tr>
<tr>
<td>s426</td>
<td>E. coli O157:H7 2977</td>
<td>Anonymous (1994)</td>
</tr>
<tr>
<td>s428</td>
<td>E. coli O157:H7 3243-86</td>
<td>Ostroff et al. (1990)</td>
</tr>
<tr>
<td>s429</td>
<td>E. coli O157:H7 C9490</td>
<td>Anonymous (1993)</td>
</tr>
<tr>
<td>s432</td>
<td>E. coli O157:H7 C6183</td>
<td>Timothy J. Barrett</td>
</tr>
<tr>
<td>s440</td>
<td>E. coli O157:H7 2893</td>
<td>Anonymous (1994)</td>
</tr>
<tr>
<td>s443</td>
<td>E. coli B41 O101:K99/F41</td>
<td>Heuzenroeder et al. (1989)</td>
</tr>
</tbody>
</table>
these data suggested that IPEC-J2 cells might secrete a factor(s) inhibitory to hETEC, but not pETEC.

**Inhibitory factor (IF) is resistant to filtration and boiling**

To begin to characterize the macromolecular nature of the IF, IPEC-J2 cell supernatants were subjected to filtration (0.22 μm; Amicon) and heat treatment (100 °C, 10 min). These filtered, heat-treated supernatants were then compared with untreated supernatants in bacterial growth assays. As shown in Fig. 2a, filtration and boiling did not significantly affect the ability of IPEC-J2 supernatant to inhibit hETEC growth (compare closed circles with diamonds). It was considered that the presence of FBS in the cell supernatants might chaperone the stability of an otherwise heat-sensitive factor. Therefore, IPEC-J2 cells were grown in DMEM/F12 lacking FBS for 48 h and the extent to which the supernatant obtained from these cells could inhibit the growth of s345 was assayed. Unexpectedly, supernatants obtained from cells grown in the absence of FBS had no detectable inhibitory activity toward hETEC (Fig. 2b), suggesting that serum-starved cells do not produce the IF. However, this finding prevented direct assaying of the heat stability of the IF in the absence of FBS.

The extent to which trace compounds commonly present in mammalian cell culture media might affect bacterial growth rates was also considered. It was established in control experiments that hETEC and pETEC growth rates are insensitive to trace amounts of EGF, lysozyme, FBS, insulin, transferrin, selenium, or trypsin (data not shown).

**Prevalence and dose response of inhibitory activity**

To quantify the relative potency of the IF, the OD_{600 nm} of s345 cultures grown for 4 h (a timepoint at which significant differences in growth due to the IF are observed) was measured in DMEM/F12 supplemented with differing concentrations of IPEC-J2 supernatants. As shown in Fig. 2c, OD_{600 nm} was dependent upon the amount of supernatant added to the growth assay.

To determine whether other cell types might secrete a similar substance inhibitory to hETEC, s345 growth vs. time was quantified in DMEM/F12 supplemented with 1% supernatants obtained from several other commonly used human and animal cell lines. Inhibition of hETEC growth was observed with supernatants obtained from other cell types of porcine (IPEC-1, ST) and bovine origin (MDBK), but not from cell types of human origin (Caco-2, Int407; Fig. 2d).
pETEC was susceptible neither to supernatants from porcine nor from human origin, but was susceptible to MDBK supernatant (data not shown). A bovine ETEC isolate (s443) was not susceptible to any tested cell supernatant. Additionally, E. coli O157:H7 isolates from human infections and from colonized cattle were not susceptible to growth inhibition by IPEC-J2 supernatants (Fig. 3), suggesting that the activity of the IF produced by porcine epithelial cells may somehow be restricted to enterotoxigenic isolates.

Discussion

It is interesting to speculate on the identity of the IF(s). Of note are data suggesting its constitutive production in uninfected cells, its heat stability, and its lack of production in serum-starved cells. These data suggest that it is reasonable to consider an antimicrobial peptide as the source of the IF. Many host antimicrobial compounds are small cationic peptides known as cationic antimicrobial molecules (Muller et al., 2005), including the cathelicidins, defensins, lysozyme, the bactericidal/permeability-increasing protein (BPI), and group IIA phospholipase A2 (PLA2; Muller et al., 2005). In pigs, a large number of antimicrobial peptides have been identified, the majority of which are cathelicidins (Oswald, 2006). PR-39, which has activity against Gram-negative bacteria, has been isolated from porcine small intestines (Agerberth et al., 1991), but appears not to be produced by intestinal epithelia (Storici et al., 1994).

Cytotoxic T and NK cells of the porcine small intestine produce NK-lysin. However, its production by intestinal epithelial cells is also uncertain (Andersson et al., 1995). BPI is produced by neutrophils and human intestinal epithelial cells (Canny et al., 2002) and binds the lipid A component of LPS to destabilize the bacterial outer membrane (Mannion...
dependent upon NF-

**Fig. 3.** Susceptibility to IPEC-J2 cells is restricted to hETEC. The ratio of OD600 nm readings of cultures grown in DMEM/F12 containing IPEC-J2 cells vs. DMEM/F12 lacking IPEC-J2 cells is plotted vs. the indicated ETEC and EHEC strains.

**References**

Agerberth B, Lee JY, Bergman T, Carlquist M, Boman HG, Mutt V 

et al., 1990). BPI expression in porcine intestinal epithelial cells has not yet been described. Although α-defensins have been found in abundance in granules of polymorphonuclear cells and intestinal paneth cells in many mammalian species, they have not been identified in pigs (Oswald, 2006).

β-Defensins are expressed in epithelial cells of the human intestine (O’Neil et al., 1999), and several of these are constitutively expressed (Fahlgren et al., 2003). In pigs, only two members of this family have been characterized (Veldhuizen et al., 2006, 2007). While recombinant porcine β-defensin-1 has potent antibacterial activity against *E. coli* (Shi et al., 1999), transcripts encoding this protein are expressed more abundantly in tongue epithelia, rather than the digestive tract (Zhang et al., 1998). In humans, production of hBD-2 in response to infection is thought to be dependent upon NF-κB induction (O’Neil et al., 1999; Ogushi et al., 2001).

In a recent study of IPI-2I porcine intestinal epithelial cells, expression of both β-defensins-1 and -2 was upregulated by FBS components (Veldhuizen et al., 2006). The present findings parallel these observations where the IF(s) was either not produced or produced in minimal amounts in the absence of FBS. These observations support the notion that the IF(s) reported here might be one of the antimicrobial peptides constitutively produced by porcine intestinal epithelial cells. This possibility is further substantiated by a recent report (Sang et al., 2005) that demonstrated that IPEC-J2 cells constitutively express β-defensin-1 transcripts. β-Defensin-2 expression profiles in IPEC-J2 and IPEC-1 cells have yet to be described.

The present findings also indicate that an IF(s) is present in the culture supernatants of MDBK (bovine kidney) and ST (swine testes) cell lines. Human (Nitschke et al., 2002), canine (Wade et al., 2004), porcine (Zhang et al., 1998), and bovine (Aono et al., 2006) kidney epithelial cells do express many β-defensins. Similarly, both human (Zhao et al., 1996) and swine testes (Zhang et al., 1998) constitutively express β-defensins.

Human Caco-2 cells constitutively express many β-defensin mRNA species, but not human β-defensin-2. Caco-2 cells also upregulate the expression of many antimicrobial peptides upon infection with enteric pathogens (Ogushi et al., 2001; Hase et al., 2002; Begue et al., 2006). However, Int407 cells do not express many of the antimicrobial molecules expressed by other common human intestinal epithelial cell lines (Bernet-Camard et al., 1996). As culture supernatants from both Caco-2 and Int407 cells did not inhibit ETEC growth, interesting questions are raised as to the species specificity, identity, and mechanism of the IF(s) described in this study. Future studies will attempt to discern the identity of the inhibitory factor(s) and to describe the molecular basis for differential susceptibility of pathogenic *E. coli* isolates to its activity.

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