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Interactions of Typical and Atypical Enteropathogenic *Escherichia coli* Strains with the Calf Intestinal Mucosa Ex Vivo

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Enteropathogenic *Escherichia coli* (EPEC) can be found in healthy and diarrheic cattle; however, little is known about the role of attaching and effacing (A/E) lesion formation in colonization of bovine intestinal mucosa by such strains. We show that typical and atypical EPEC induce A/E lesions on calf intestinal explants independently of Tir tyrosine phosphorylation and TccP. Our data support the existence of conserved Tir- and TccP-independent mechanisms of A/E lesion formation in a range of hosts and reinforce the zoonotic potential of EPEC in cattle.

Enteropathogenic *Escherichia coli* (EPEC) and the closely related enterohemorrhagic *E. coli* (EHEC) are important human pathogens (28). EPEC strains are divided into typical EPEC and atypical EPEC (aEPEC) based on the presence or absence of the EAF plasmid, respectively (15). Typical EPEC strains, which belong mainly to 1 of 12 O serogroups, are further divided into EPEC lineage 1 (EPEC-1), which is characterized by expression of flagellar antigen H6 or H34 and intimin α, and EPEC-2, which commonly expresses H2 (or H7), intimin β, and the type III secretion system effector TccP2 (40). aEPEC strains are much more diverse and may belong to one of many serogroups.

EPEC, aEPEC, and EHEC are diarrheal pathogens capable of forming attaching and effacing (A/E) lesions (reviewed in references 8 and 9). A/E lesions are characterized by effacement of the brush border microvilli and intimate bacterial attachment to the host cell plasma membrane (23). The genes required for A/E lesion formation are carried on the locus of enterocyte effacement (27), which encodes transcriptional regulators, the adhesin intimin (20), a type III secretion system (19), chaperones, translocators, and several effector proteins (reviewed in references 9 and 11).

One of the major hallmarks of EPEC and EHEC strains is their ability to trigger actin polymerization at the site of bacterial attachment to cultured cells (23). The principal effector protein needed for A/E lesion formation on mucosal surfaces and actin polymerization in vitro is Tir (22, 33). Once translocated, Tir is integrated into the host cell plasma membrane in a hairpin loop topology (16), and the extra-cellular loop serves as an intimin receptor (reviewed in reference 10). In EPEC-1 (represented by strain E2348/69, O127:H6), actin polymerization in vitro is triggered by phosphorylation of a Tir tyrosine (Y) residue at position 474 (21), which recruits the adaptor protein Nck, leading to activation of the neuronal Wiskott-Aldrich syndrome protein (N-WASP) and actin polymerization via the actin-related protein 2/3 (Arp2/3) complex (reviewed in reference 7). Tir from E2348/69 can also trigger weak Nck-independent actin polymerization (5) via a universally conserved NPY Tir motif (4), which was recently shown to recruit insulin receptor tyrosine kinase substrate p53 (39) and/or insulin receptor tyrosine kinase substrate (36). In EHEC O157:H7, binding of insulin receptor tyrosine kinase substrate p53/insulin receptor tyrosine kinase substrate to Tir (which lacks an Y474 equivalent) leads to the recruitment of TccP (aka EspF1), which in turn activates N-WASP (6, 12, 36). Strains belonging to EPEC-2 (represented by strain B171, O111:NM) express both Tir containing a Y474 equivalent and TccP2 (24, 40), which is interchangeable with TccP of EHEC O157 (40).

aEPEC strains can trigger actin polymerization in vitro by diverse mechanisms involving Tir-Nck and/or Tir-TccP/TccP2 pathways. However, a significant proportion of aEPEC (represented by strain ICC223, O125:H6) strains cannot trigger actin polymerization in vitro, as they express Tir lacking a Y474 equivalent and TccP/TccP2 (3). However, these strains can trigger typical A/E lesions to form on human in vitro organ cultures (hIVOC) (33).

Fecal excretion of EPEC by healthy and diarrheic calves has been reported in the United States (18), Europe (2, 26), Australia (17), India (38), and Brazil (1); however, the zoonotic and pathogenic potential of such strains is ill defined. While A/E lesion formation is known to play a role in intestinal colonization of ruminants by EHEC O157 and O26 (35), not much is known about EPEC or aEPEC pathogenesis on bovine intestinal mucosa or what role actin nucleation may play in the efficiency of adherence. Here, we investigated the interactions of EPEC-1 strain E2348/69, EPEC-2 strain B171, and aEPEC strain ICC223 with the calf gut mucosa using a bovine IVOC (bIVOC) model. All the strains used in this study (listed in Table 1) were grown...
showing that strain E2348/69 tir\textsubscript{Y474S} induces typical A/E lesions to form on hIVOC, suggesting the existence of an alternative actin polymerization pathway in mucosal surfaces of both cattle and humans. Infection with wild-type E2348/69 but not with E2348/69 tir\textsubscript{Y474S} led to the recruitment of the mammalian adaptor protein Nck underneath adherent bacteria (data not shown), supporting the conclusion that A/E lesion formation by E2348/69 tir\textsubscript{Y474S} involved an Nck-independent mechanism.

In order to investigate how expression of TccP2 might impact on the interaction of EPEC with bIVOC, we infected bIVOC with B171 and B171 \textDelta tccP strain ICC216 (40). Staining of infected explants with anti-O111 antiserum revealed that ICC216 colonized the ileal mucosa less efficiently than the wild-type B171 strain, although the difference was not statistically significant ($P = 0.0554$) (Table 2). Moreover, the number of intercrypt mucosal epithelial regions with intimately adherent bacteria was significantly lower for ICC216 in the terminal rectum (the rectum had a $P$ value of 0.0437, unlike the ileum) (Fig. 2). Nevertheless, adherent ICC216 induced the formation of typical A/E lesions (Fig. 1), similar to that triggered by the wild-type strain; electron-dense material was visible underneath intimately adherent bacteria (Fig. 1). Taken together, our data show that EPEC can colonize and induce formation of A/E lesions independently of the Tir-Nck and Tir-TccP2 signaling complexes, although they appear to influence the binding efficiency.

Finally, we investigated if aEPEC can also colonize and induce formation of A/E lesions on bIVOC. We selected strain ICC223 for this analysis, which expresses an EHEC-O157-like Tir (lacking an equivalent of Y474) and is naturally \textit{tccP} or \textit{tccP2} gene negative. Accordingly, this strain cannot trigger actin polymerization in vitro (3). As a control for this infection, we have used EHEC O157:H7 strains 85-170 and TUV 93-0 and their respective isogenic \textit{tccP} mutant strains ICC185 and ICC203, which resemble ICC223. Although ICC223 was found in 92% of the infected

### TABLE 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUV 93-0</td>
<td>EHEC O157:H7, \textit{tir} and \textit{stx}\textsubscript{2} mutant</td>
<td>31</td>
</tr>
<tr>
<td>ICC185</td>
<td>TUV 93-0 \textDelta tccP::Kan\textsuperscript{r}</td>
<td>12</td>
</tr>
<tr>
<td>85-170</td>
<td>EHEC O157:H7, spontaneous \textit{stx}\textsubscript{1} and \textit{stx}\textsubscript{2} mutant, \textit{Nal}\textsuperscript{r}</td>
<td>34</td>
</tr>
<tr>
<td>ICC203</td>
<td>85-170 \textit{Nal}\textsuperscript{r} \textDelta tccP::Kan\textsuperscript{r}</td>
<td>37</td>
</tr>
<tr>
<td>ICC223</td>
<td>EPEC O127:H6</td>
<td>3</td>
</tr>
<tr>
<td>B171</td>
<td>EPEC-2 O111:H\textsuperscript{p}, \textit{tccP2}\textsuperscript{p}</td>
<td>30</td>
</tr>
<tr>
<td>ICC216</td>
<td>B171 \textDelta tccP::Kan\textsuperscript{r}</td>
<td>40</td>
</tr>
<tr>
<td>E2348/69</td>
<td>EPEC-1 O127:H6</td>
<td>25</td>
</tr>
<tr>
<td>E2348/69 \textit{tir}\textsubscript{Y474S}</td>
<td>EPEC-1 strain E2348/69 containing a \textit{tir}\textsubscript{Y474S} point mutation</td>
<td>33</td>
</tr>
</tbody>
</table>

### TABLE 2. Adherence of EPEC and EHEC to calf gut mucosa ex vivo

<table>
<thead>
<tr>
<th>Strain</th>
<th>Terminal ileum</th>
<th>Terminal rectum</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPEC</td>
<td>21/23 (91)</td>
<td>16/28 (57)</td>
</tr>
<tr>
<td>ICC216</td>
<td>12/19 (63)</td>
<td>13/24 (54)</td>
</tr>
<tr>
<td>E2348/69</td>
<td>13/16 (81)</td>
<td>18/21 (86)</td>
</tr>
<tr>
<td>ICC223</td>
<td>9/10 (90)</td>
<td>12/15 (80)</td>
</tr>
<tr>
<td>TUV 93-0</td>
<td>31/35 (89)</td>
<td>24/31 (77)</td>
</tr>
<tr>
<td>ICC185</td>
<td>21/27 (78)</td>
<td>18/30 (60)</td>
</tr>
<tr>
<td>85-170</td>
<td>32/38 (84)</td>
<td>28/39 (72)</td>
</tr>
<tr>
<td>ICC203</td>
<td>25/50 (83)</td>
<td>25/32 (78)</td>
</tr>
</tbody>
</table>

a Fisher's exact test was performed using commercially available GraphPad InStat version 3.06 software (GraphPad Software Inc., San Diego, CA). A $P$ value of $p<0.05$ was considered significant.

b The $P$ value was 0.0554 compared to B171.
ileal bIVOC, it was found on only 36% of the rectal explants (Table 2), which is lower than that seen for EPEC or wild-type and tccP mutant EHEC strains (Table 2). However, analysis of the number of intercrypt mucosal epithelial regions with intimately adherent bacteria did not show a significant difference between ICC223 and the EPEC and EHEC strains (Fig. 2 and 3). SEM and TEM analysis revealed that adherent ICC223 formed smaller foci of intimately adherent bacteria, left many bacterial footprints (Fig. 3), and was unable to trigger efficient accumulation of

FIG. 1. Adherence of EPEC strains E2348/69 (A) and B171 (B) to calf terminal ileal and rectal mucosa ex vivo. These results show that neither TirY474S (A) nor TccP2 (B) is required for adherence (arrows in immunofluorescence assay [IFA]) and development (arrowheads in SEM and TEM) of typical A/E lesions ex vivo. Representative micrographs are shown. IFA, Hoechst 33342 (blue, false color) staining of nuclei and bacteria; tetramethyl rhodamine isothiocyanate (red, false color) staining of O127- and O111-positive bacteria. Bar = 20 μm (IFA), 5 μm (SEM), or 0.5 μm (TEM).
electron-dense material underneath intimately adherent bacteria (Fig. 3). These results are consistent with the characteristics of the interaction of ICC223 with hIVOC (3). In contrast, wild-type EHEC and the EHEC ΔtccP mutants induced formation of typical A/E lesions, including the accumulation of an electron-dense material underneath intimately adherent bacteria (Fig. 3). A role for TccP in promoting efficient adherence of EHEC O157 to mucosal surfaces in infant rabbits and gnotobiotic pigs has been proposed (32); however, no significant differences in adherence to calf intercrypt epithelia were observed under the conditions used here (Fig. 3C). This may reflect the relative short-term nature of hIVOC studies, which are limited to ca. 8 h postinfection, owing to loss of tissue integrity after this time.

Our study shows that typical EPEC and aEPEC strains, at least those of the O125:H6 serotype, can induce A/E lesions on the calf intestinal mucosa using calf explants, and this, to some extent, correlates with the previous report by Pearson et al. (29). Formation of such lesions is known to be important in colonization of calves by EHEC O157 and O26, and the data imply that EPEC may also rely on this strategy when found in healthy or diarrheic calves. Induction of A/E lesions during EPEC infection can occur independently of the Tir-Nck and Tir-TccP complexes on both human and calf gut explants, although under some circumstances, such complexes may modulate the efficiency of adherence. The basis of pedestal formation in the absence of such complexes is ill defined, but one may infer from the data presented here that a conserved pathway is subverted by EPEC and EHEC in human and bovine enterocytes. The finding that EPEC strains are able to elicit the formation of A/E lesions associated with persistence and pathology in calves suggests that cattle might also be a reservoir for human EPEC infections. Further epidemiological studies are needed in order to assess the risk to human health posed by carriage of EPEC in ruminants.

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