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Colonization of the meat extracellular matrix proteins by O157 and non-O157 enterohemorrhagic *Escherichia coli*

Caroline Chagnot [a,b], Nelly Caccia [a], Estelle Loukiadis [c,d], Sarah Ganet [c,d], Alexandra Durand [a], Yolande Bertin [a], Régine Talon [a], Thierry Astruc [b], Mickaël Desvaux [a,*]

[a] INRA, UR454 Microbiologie, F-63122 Saint-Genès Champanelle, France
[b] INRA, UR370 Qualité des Produits Animaux, F-63122 Saint-Genès Champanelle, France
[c] Université de Lyon, VetAgro Sup, LMAP Laboratory, National Reference Laboratory for E. coli (including STEC), F-69280 Marcy l'Etoile, France
[d] Université de Lyon, UMR 5557 Ecologie Microbienne, Université Lyon 1, CNRS, VetAgro Sup, Research Group on Bacterial Opportunistic Pathogens and Environment, F-69622 Villeurbanne, France

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ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) are anthropozoonotic agents that range third among food-borne pathogens respective to their incidence and dangerousness in the European Union. EHEC are Shiga-toxin producing *E. coli* (STEC) responsible for foodborne poisoning mainly incriminated to the consumption of contaminated beef meat. Among the hundreds of STEC serotypes identified, EHEC mainly belong to O157:H7 but non-O157 can represent 20 to 70% of EHEC infections per year. Seven of those serogroups are especially of high-risk for human health, i.e. O26, O45, O103, O111, O121, O145 and O104. While meat can be contaminated all along the food processing chain, EHEC contamination essentially occurs at the dehiding stage of slaughtering. Investigating bacterial colonization to the skeletal-muscle extracellular matrix (ECM) proteins, it appeared that environmental factors influenced specific and non-specific bacterial adhesion of O157 and non-O157 EHEC as well as biofilm formation. Importantly, mechanical treatment (i.e. shaking, centrifugation, pipetting and vortexing) inhibited and biased the results of bacterial adhesion assay. Besides stressing the importance of the protocol to investigate bacterial adhesion to ECM proteins, this study demonstrated that the colonization abilities to ECM proteins vary among EHEC serogroups and should ultimately be taken into consideration to evaluate the risk of contamination for different types of food matrices.

1. Introduction

The enterohemorrhagic *Escherichia coli* (EHEC) are anthropozoonotic agents responsible for foodborne poisoning incriminating contaminated animal food products, vegetables and watery drinks (Bavaro, 2012). EHEC are one of the six pathotypes of intestinal pathogenic *E. coli* (InPEC) (Kaper et al., 2004; Nataro and Kaper, 1998). Worldwide, EHEC infection is predominantly a pediatric disease frequently implicated in severe clinical illness, which is characterized by bloody diarrhea and, in most acute forms, can degenerate into hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP) (Karch et al., 2005; Tarr, 2009). While the involvement of contaminated fruits and vegetables is increasing, EHEC infections are most often linked to the consumption of beef meat and are predominantly attributed to the *E. coli* O157:H7 (Berger et al., 2010). *E. coli* O157:H7 is only one of the over 300 distinct serotypes for Shiga toxin-producing *E. coli* (STEC) isolated so far (Karmali et al., 2010), i.e. the previously called verotoxin-producing *E. coli* (VTEC) (Griffin and Tauxe, 1991; Paton and Paton, 1998; Schmidt and Karch, 1996). However, only a very limited number of serotypes appear to be associated with the majority of human diseases; all EHEC are pathogenic STEC but all STEC are not systematically EHEC (or InPEC) (Karmali et al., 2003; Naylor et al., 2005). Respective to their incidence, EHEC range third in the European Union (EU) among food-borne pathogens after *Campylobacter* and *Salmonella* (EFSA and ECDC, 2013), in terms of dangerousness, however, it is the worst of the three. While these serotypes vary in frequency with the country and year, non-O157 represents between 20 and 70% of overall EHEC infections. The latest north American and EU reports, from the USDA (United States Department of Agriculture) and EFSA (European Food Safety Authority) respectively, indicated that the serogroups O26, O45, O103, O111, O121 and O145 are by far the most commonly isolated, the so-called "big six" (EFSA and ECDC, 2013; USDA, 2012). Since 2012, the USA (Unites States of America) legislation incorporates those non-O157 serogroups, which are considered as

* Corresponding author at: INRA Clermont-Ferrand Research Centre, UR454 Microbiology, Site of Theix, F-63122 Saint-Genès Champanelle, France. Tel.: +33 4 73 62 47 23; fax: +33 4 73 62 45 81.
E-mail address: mickael.desvaux@clermont.inra.fr (M. Desvaux).

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high-risk for human health and requiring high vigilance for food safety. In EU, the serogroup O104 is watched closely since the last major outbreaks in 2011 (EFSA and ECDC, 2013).

In any cases, EHEC/STEC contamination of food products originates from fecal contamination, the ruminants being the main natural reservoir (Chase-Topping et al., 2008; Nguyen and Sperandio, 2012). The respect of good hygienic slaughtering practices reduces the risk of contamination of carcasses but cannot guarantee the absence of EHEC/STEC from meat (Buncic et al., 2014; Rhoades et al., 2009). It has been shown that *E. coli* O157:H7 could adhere to the muscle tissue, most certainly to the extracellular matrix (ECM) (Auty et al., 2005; Cabedo et al., 1997; Chen et al., 2007; Li and McLandsborough, 1999; Medina, 2001; Rivas et al., 2006). While binding to collagen I and laminin of the cell surface of *E. coli* O157:H7 was evidenced (Medina, 2001, 2002; Medina and Fratamico, 1998), a later study could not report any significant attachment to any of the tested immobilized ECM proteins (Zulfakar et al., 2012). Using a non-toxigenic *E. coli* O157:H7 EDL933 Δ*stx* strain, i.e. *E. coli* O157:H7 CM454 (for Dr Christine Martin at UR454) (Gobert et al., 2007), bacterial adhesion to muscle ECM proteins was re-investigated (Chagnot et al., 2013a,b) by taking a great care to the types of ECM proteins to ensure the highest relevance to the skeletal muscle tissue (Chagnot et al., 2012). It was evidenced that environmental factors such as the growth medium, temperature and pH had a strong influence on bacterial adhesion. Non-specific adhesion occurred when bacteria were grown in bovine small intestine content, with similar trend in DMEM (Dulbecco's modified Eagle's medium), whereas specific adhesion to some ECM fibrillar proteins occurred when bacteria were grown in ruminal content, as well as in LB (lysogenic broth) where maximal specific adhesion to collagens I and III occurred at pH 7 and 25 °C (Chagnot et al., 2013a,b). Besides the use of different environmental conditions, it was hypothesized that the discrepancies with previous investigations for the attachment of the *E. coli* O157:H7 to ECM proteins (Zulfakar et al., 2013) could be linked to bacterial-surface protein determinants potentially involved in adhesion to ECM that were damaged by mechanical shaking–centrifugation–vortexing treatment. Altogether, this prompted us to test this hypothesis using *E. coli* O157:H7 wild type (Chagnot et al., 2013a,b) as well as studying the specific and non-specific adhesion of the major non-O157 EHEC serogroups to some ECM proteins in relevant environmental conditions.

2. Material and methods

2.1. Bacterial strains and culture conditions

EHEC strains investigated in this study are listed in Table 1. One strain for each of the major EHEC serogroups (recognized at higher risk by the USDA and EFSA) was considered. Bacterial strains were cultured in different relevant nutrient media as previously determined (Chagnot et al., 2013a,b), either rich chemically defined, i.e. DMEM (Dulbecco's modified Eagle's medium, Gibco, 31885) (Dulbecco and Freeman, 1959; Eagle, 1955), or complex undefined, i.e. LB (lysogeny broth) (Bertani, 1951, 2004). From −80 °C stock culture previously grown in the respective medium, strains were plated on the relevant agar medium and incubated overnight at 39 °C (bovine temperature) (Bertin et al., 2011; Desvaux et al., 2007). A preculture was set up from one bacterial colony grown in the respective nutrient broth medium at 39 °C in an orbital shaker till stationary phase as determined using the logarithmic growth curve of the $OD_{600\ nm}$ as previously described (Chagnot et al., 2013a,b).

2.2. Virulence profile

The presence of *stx* (encoding the Shiga-toxins), *eae* (encoding the intimin), and *ehxA* (encoding the enterohemolysin) genes and serogroup was determined by real-time PCR as previously described (Bugarel et al., 2010; Nielsen and Andersen, 2003; Perelle et al., 2004). Briefly, DNA was extracted using the InstaGene matrix kit (Biorad) from single colonies of each EHEC strain grown overnight on LB agar (Perelle et al., 2004). Pall GeneDisc Technologies' kits (GSTEHEC106006; GTOP6EC106006; and GECO104106006) and GeneDisc cycler were used according to the manufacturer's instructions (Pall GeneDisc Technologies) (Beutin et al., 2009; Bugarel et al., 2010). Notably, the oligonucleotide primers and probes included within these discs for detection of stx_1, stx_2, *eae*, $rfbE_{O157}$, wzx_{O26},wzx_{O103}, $wbd1_{O111}$, and $ihp1_{O145}$ genes were described in the ISO/TS 13136:2012 standard (Nielsen and Andersen, 2003; Perelle et al., 2004).

2.3. Coating of microtiter plates with ECM proteins

96-well polystyrene microtiter plates (Falcon) were surface-coated with ECM proteins as previously described (Chagnot et al., 2013a,b). The ECM proteins consisted of collagen I (Millipore, 08-115), collagen III (Millipore, CC078) and a reconstituted ECM, i.e. MaxGel ECM (Sigma, E0282), including collagens, laminin, fibronectin, and elastin. BSA (bovine serum albumin (Sigma, A3803)) was used as a control for specific adhesion to muscle ECM proteins. Basically, ECM proteins were solubilized in a 0.1 M carbonate coating buffer (pH 9.6), 250 μl was dispatched at a saturating concentration of well surface (50 μg ml^{-1}) and incubated overnight at 4 °C (Chagnot et al., 2013a,b; Hennequin and Forestier, 2007). The wells were washed 3 times with Tryptone Salt (TS) at room temperature for subsequent bacterial adhesion or biofilm formation assays.

2.4. Bacterial adhesion assay

Preculture was diluted 1:100 and grown as described above. The two media LB and DMEM were adjusted with NaOH (0.1 M) to reach a pH of 7 at the time of sampling, i.e. during the exponential growth phase at an $OD_{600\ nm}$ of 0.5 (about 10^8 CFU ml^{-1}). Chloramphenicol (90 μg ml^{-1} final) was added to prevent de novo protein synthesis and bacterial growth during the time of the assay (Chagnot et al., 2013a,b). Prior to the crystal violet adhesion assay per se, bacterial cells were sampled and handled in two different ways, i.e. (i) a minimal mechanical

Table 1
List of O157 and non-O157 EHEC bacterial strains studied.

Strain	Somatic antigen	Flagellar antigen	Origin	Virulence profile[a]	Reference
EDL933	O157	H7	Clinical	*stx1*, *stx2*, *eae*, *ehxA*	Riley et al. (1983)
ED180	O26	H11	Clinical	*stx2*, *eae*, *ehxA*	This study
12047(Fred5)	O45	H2	Clinical	*stx1*, *eae*, *ehxA*	Voitoux et al. (2002)
CH087	O103	H2	Clinical	*stx1*, *eae*, *ehxA*	Pradel et al. (2008)
ED191	O111	H2	Clinical	*stx1*, *stx2*, *eae*, *ehxA*	This study
32316	O121	H19	Clinical	*stx2*, *eae*, *ehxA*	This study
PH27	O145	H28	Clinical	*stx2*, *eae*	Posse et al. (2008)
CB13348	O104	H4	Clinical	*stx2*	This study

[a] Virulence profile of each strain was determined by real-time PCR as described in the Material and methods section.