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Adherence factors of enterohemorrhagic *Escherichia coli* O157:H7 strain Sakai influence its uptake into the roots of *Valerianella locusta* grown in soil

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ABSTRACT

Increasing numbers of outbreaks caused by enterohemorrhagic *Escherichia coli* (EHEC) are associated with the consumption of contaminated fresh produce. The contamination of the plants may occur directly on the field *via* irrigation water, surface water, manure or fecal contamination. Suggesting a low infectious dose of 10 to 10^2 cells, internalization of EHEC into plant tissue presents a serious public health threat. Therefore, the ability of EHEC O157:H7 strain Sakai to adhere to and internalize into root tissues of the lamb's lettuce *Valerianella locusta* was investigated under the environmental conditions of a greenhouse. Moreover, the influence of the two adherence and colonization associated genes *hcpA* and *iha* was surveyed regarding their role for attachment and invasion. Upon soil contamination, the number of root-internalized cells of EHEC O157:H7 strain Sakai exceeded 10^2 cfu/g roots. Deletion of one or both of the adherence factor genes did not alter the overall attachment of EHEC O157:H7 strain Sakai to the roots, but significantly reduced the numbers of internalized bacteria by a factor of between 10 and 30, indicating their importance for invasion of EHEC O157:H7 strain Sakai into plant roots. This study identified intrinsic bacterial factors that play a crucial role during the internalization of EHEC O157:H7 strain Sakai into the roots of *Valerianella locusta* grown under the growth conditions in a greenhouse.

1. Introduction

Enterohemorrhagic *E. coli* (EHEC) O157:H7 strains can cause serious human diseases such as diarrhea, hemorrhagic colitis and the hemolytic-uremic syndrome (HUS) (Kaper, 1998). *Escherichia coli* O157:H7 strains produce an arsenal of pathogenicity factors that enable them to be competitive and cause serious human diseases and outbreaks. The most important ones are the production of one or more Shiga toxins and the expression of the locus of enterocyte effacement (LEE) (Kaper, 1998), enabling the bacteria to translocate type III effectors into the cytosol of target cells. *Escherichia coli* O157:H7 is mainly transmitted to humans by raw or undercooked meat and dairy products but during the last years infection sources of non-animal origin were increasingly reported representing ~20% of EHEC-caused infections (Greig and Ravel, 2009). The human infectious dose was estimated in a range of 10 to 10^2 cfu for ground beef (Tuttle et al., 1999).

So far, the biggest EHEC O157 outbreak occurred in Sakai, Japan, in 1996, with more than 9000 confirmed cases and 12 deaths. The identified agent were radish sprouts contaminated with EHEC O157:H7 strain Sakai (Michino et al., 1999). Several studies have already demonstrated that *E. coli* O157:H7 strains are able to colonize the leaves and roots of lettuce and other leafy greens (Erickson et al., 2010; Seo and Frank, 1999; Solomon et al., 2002), and to persist for several days to weeks (Chitarra et al., 2014; Wright et al., 2017). Surface structures such as pili, flagella, the type III secretion system (T3SS), as well as proteins involved in quorum sensing were found to be involved in successful adherence to spinach leaves and leaves of red oak lettuce (Macarisin et al., 2012; Nuebling et al., 2017; Saldana et al., 2011) under laboratory conditions. Various factors such as surface appendages, outer membrane proteins, extracellular polysaccharides (Frank, 2001), cell surface hydrophobicity and charge (Fletcher and Loeb, 1979) are supposed to be generally involved in attachment.

The capability of EHEC to colonize the roots of leafy greens such as lettuce, parsley and spinach was shown by recent studies (Erickson et al., 2014; Solomon et al., 2002; Wright et al., 2017). These studies were performed mostly in environmental growth chambers and focused on the influence of external factors. Solomon et al. (2002) investigated the impact of different inoculation strategies comparing contamination *via* manure or irrigation water on the internalization of *E. coli* O157:H7 into lettuce seedling grown in an environmental growth chamber. The

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different treatments had only little effects. The impact of the plant growth substrate on bacterial internalization was investigated multiple times leading to contradicting results (Franz et al., 2007; Hora et al., 2005; Macarisin et al., 2014; Sharma et al., 2009). Some studies showed that invasion of *E. coli* O157:H7 into spinach roots was enhanced when plants were grown in hydroponic medium compared to soil (Sharma et al., 2009). These authors hypothesized that a hydroponic solution provides better motility leading to increased internalization compared to soil (Sharma et al., 2009). By contrast, other studies demonstrated that the occurrence of internalization events into the roots of spinach was higher in soil-grown plants than in hydroponically grown plants (Franz et al., 2007; Macarisin et al., 2014). Presumably, this is due to augmented damage of the roots as this is more likely to occur when plants are grown in soil. For plants grown in hydroponic medium, damage of the plant roots was shown to act as promoting factor for internalization (Macarisin et al., 2014). In contrast, studies investigating the connection between root damage and frequency of internalization events that were performed under growth chamber conditions did not observe increased bacterial invasion on mechanically or biologically disrupted spinach plants (Hora et al., 2005). Bacterial internalization seems to be a complex process that needs further research. To our knowledge, it remains unclear which intrinsic factors of EHEC strains are important for colonization of plant roots.

Hence, the present study focused on the role of two adherence factors, Iha and HcpA, during root colonization. The IrgA homolog adhesion (Iha) is encoded by *iha* and functions as enterobactin siderophore receptor (Rashid et al., 2006). It was first described in *E. coli* O157:H7 (Tarr et al., 2000). Its expression is repressed by the ferric uptake regulation protein Fur (Rashid et al., 2006) and triggered by short-chain fatty acids (Herold et al., 2009). In contrast to other siderophore receptors, it harbors the unique feature of contributing to adherence to different human and animal cell lines (Johnson et al., 2005; Tarr et al., 2000; Yin et al., 2009). Introduction of *iha* into non-adhering *E. coli* strains is sufficient to confer attachment capability to these strains (Tarr et al., 2000). It is widely distributed among different *E. coli* pathotypes, such as enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and uropathogenic *E. coli* (UPEC) (Schmidt et al., 2001). For the latter and extraintestinal pathogenic *E. coli* (ExPEC), Iha was found to be a virulence factor during urinary tract infections (Johnson et al., 2000, 2005; Leveille et al., 2006). Interestingly, in Shiga-toxin producing *E. coli* (STEC) *iha* was found in 57,3% of the STEC strains isolated from food of animal origin (Slanec et al., 2009).

The *hcpA* gene encodes the pilin subunit of an adhesive type IV pilus called hemorrhagic coli pilus (HCP) (Xicohtencatl-Cortes et al., 2007) and was formerly called prepilin peptidase-dependent gene (*ppdD*) (Ledesma et al., 2010). The hemorrhagic coli pilus was shown to be involved in adherence to a variety of mammalian cell lines (Xicohtencatl-Cortes et al., 2007), and to leaf surfaces (Nuebling et al., 2017; Saldana et al., 2011). However, contradicting results were gained when investigating attachment to leaf surfaces. Upon deletion of *hcpA* decreased adherence was observed on spinach leaves (Saldana et al., 2011). Interestingly, its deletion resulted in enhanced attachment to red oak leaf lettuce leaves (Nuebling et al., 2017). As these two studies used different incubation periods, the obtained results may indicate that adherence is a time-dependent process and hinges on the target surface. Moreover, deletion of *hcpA* was shown to lead to decreased internalization into HT-29 cells (Xicohtencatl-Cortes et al., 2009). For the same cell line, it was demonstrated that HCP (HcpA) induces the activation of proinflammatory cytokines in polarized HT-29 cells (Ledesma et al., 2010). Hence, HCP can be considered as a virulence factor.

As both Iha and HcpA were shown to be involved in pathogenicity in mammalian model systems, we hypothesize that they may also play a role in successful colonization of plant roots. This is supported by observations of Schikora et al. (2011), who reported that for *Salmonella* there is a high degree of conservation of the infection mechanisms in plants and animals. Thus, the role of the adherence factors Iha and HcpA during root colonization was investigated using *Valerianella locusta*, also known as lamb's lettuce, as a host. *Valerianella locusta*, mostly cultivated in greenhouses during winter, is a fall and winter lettuce which stands out due to its short leaves that are predestinated for infections starting from the roots. According to the German Federal Ministry of Food and Agriculture, lamb's lettuce is one of the lettuces with the highest revenue in Germany (https://www.bmel.de/EN/). For analysis of the *in vivo* capacity of EHEC O157:H7 strain Sakai to adhere to and internalize into the roots of cultivated plants after irrigation with contaminated water, and whether selected typical adherence factors were involved, an experimental setup was chosen under environmental conditions in a biosafety greenhouse meeting the safety requirements for biosafety level 3 according to appendix 4 of the Swiss Containment Ordinance (ESV). In order to shed light on different aspects of colonization, plant roots were analyzed concerning adherence and internalization of EHEC O157:H7 strain Sakai.

2. Material and methods

2.1. Bacterial strains

All bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown in LB medium (10% (w/v) tryptone, 10% (w/v) NaCl, 5% (w/v) yeast extract, pH 7.0) at 37 °C with shaking at 180 rpm unless indicated differently. When needed, antibiotics were added to the following final concentrations: 100 µg/ml ampicillin, 50 µg/ml kanamycin and 20 µg/ml chloramphenicol.

2.2. Preparation of electrocompetent bacterial cells and electroporation

Electrocompetent bacterial cells were prepared, and electroporation was performed as described previously (Saile et al., 2016).

2.3. Plasmid construction

Plasmid pKEC2 was constructed by amplifying the *cat* gene plus 375 bp upstream using pCP20 as template, and cloning the PCR product into pWRG435 after digesting the PCR product and the backbone plasmid with PvuI. For PCR, restriction digestion, ligation, transformation and plasmid isolation standard protocols were applied as described by Maniatis et al. (1985). Plasmid DNA was isolated from *E. coli* DH5α using a QIAprep Spin Miniprep kit (Qiagen, Netherlands) following the manufacturer's instructions, and screened for the insert's identity and orientation by sequencing using the following primer: P-cat PvuI for, cat PuvI rev, cat 124 for and cat 225 rev (Table 2).

2.4. Construction of isogenic gene deletion mutants

Gene deletions were performed according to the method of Datsenko and Wanner as described previously (Datsenko and Wanner, 2000; Saile et al., 2016). The primers applied for mutagenesis are listed in Table 2. Verification of deletions was performed by PCR and sequencing.

2.5. Cloning of adherence factor genes

For plasmid-based complementation of the knock-out strains, genomic DNA (gDNA) of *E. coli* O157:H7 strain Sakai was isolated using DNeasy Blood & Tissue Kit (Qiagen, Netherlands) following the manufacturer's instructions. To amplify the genes *hcpA* and *iha* plus 400 bp upstream of the start codon, appropriate primers – hcpA-MscI-f and hcpA-BamHI-r for *hcpA*, and iha-HindIII-f and iha-XhoI-r for *iha* – as listed in Table 2 were used. The isolated gDNA served as template. After treatment with the restriction enzyme DpnI to cleave the parental