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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Running Title: EHEC factors influence colonizing of lettuce roots

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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.

Keywords

Enterohemorrhagic *E. coli* O157:H7; lamb's lettuce; internalization; *hcpA*; *iha*; 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 9,000 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 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 methylated gDNA, the PCR products and the vector pOKD4, a gift from Prof. Hao Wu (Harvard Medical School; Addgene plasmid #17214; https://www.addgene.org/), were digested with the corresponding restriction enzymes, ligated and transformed into chemically competent *E. coli* DH5α. The resulting plasmids pKEC4 and pKEC5 were prepared from *E. coli* DH5α using a QIAprep Spin Miniprep kit (Qiagen, Netherlands) following the manufacturer's instructions and verified by sequencing using the following primer combinations: hcpAdel-rev/hcpA-BamHI-r/hcpA-50-rev and iha-I/iha-II/iha-HindIII-f/iha-XhoI-r/iha-134up-for/iha-1754-rev/iha-1598-for (Table 2).

2.6 Plasmid stability in EHEC strain Sakai grown in soil

The stability of pKEC2 in EHEC strain Sakai was examined in the same manner as persistence experiments published by Fornefeld et al. (2017) with minor modifications. Briefly, EHEC strain

Sakai/pKEC2 was grown overnight in LB medium supplemented with 20 µg/ml chloramphenicol at 37 °C with aeration. Cells were pelleted at 6,000 × *g* at 4 °C for 8 min and resuspended in 10 mM $MgCl_2$. Inoculation was conducted by thoroughly mixing of soil and bacterial suspension to a final inoculum level of 10^8 cfu per g soil. As a control, soil was mixed only with 10 mM $MgCl_2$. Inoculated samples were incubated at 22 °C for 14 days and analyzed 0, 2, 4, 7, and 14 days post infection (dpi). EHEC cells were recovered from soil for quantification by the addition of 9 ml 0.5 × Murashige-Skoog (MS) medium (2.165 g/L Murashige & Skoog Medium, Duchefa Biochemie, Netherlands, pH 5.8) and subsequent extensive mixing. Serial decimal dilutions were plated on TBX chromogenic agar (Roth, Germany) and on TBX agar supplemented with 20 µg/ml chloramphenicol. After incubation overnight at 37 °C, the cfu per gram soil were calculated. Three independent experiments were performed.

2.7 Propagation of *Valerianella locusta* from seeds

For the propagation of seeds, seed trays (50 × 30 × 5 cm) with 150 slots were used. Seeds of *Valerianella locusta* (L.) „Verte á coeur plein“ (Select, Wyss Seed and Plants AG, Switzerland) were first grown in Floradur® A potting soil (Floragard, Germany) for approximately two weeks until reaching the second leaf stage (first leaf rosette). The plants were then carefully excavated and freed of soil before being repotted in plant pots (9 cm in diameter) containing diluvial sand soil, kindly provided by Dr. Rita Grosch (Leibniz Institute of Vegetable and Ornamental Crops, Großbeeren, Germany). The DS soil (diluvial sand) was described as an Arenic-Luvisol with less silty sand and 5.5 % clay (silty sand) (Ruehlmann and Ruppel, 2005; Schreiter et al., 2014). Each pot then contained three plants. After a two-day adaption phase, plants were ready-to-use for infection experiments.

2.8 Experimental setup

Preliminary experiments with the non-pathogenic gfp-expressing *E. coli* strain DH5α/pWRG435 were conducted to test the overall experimental set-up before performing the actual plant infection experiments with EHEC O157:H7 strain Sakai in the greenhouse. Lettuce plants were inoculated with 10 ml bacterial suspension ranging from 1.0×10^5 cfu/ml to 1.0×10^9 cfu/ml. Plants were then incubated at 21 °C with 12 h day-/night-cycle and 20% relative humidity for 4 days and subsequently analyzed qualitatively by fluorescent microscopy, where at least 20 microscopic fields were surveyed per root. The number of observed bacteria varied from 0 to 4 bacteria per microscopic field.

2.9 Determination of inoculum level and incubation time

To determine the inoculum level and incubation time suitable for the experimental set-up, experiments were conducted with *E. coli* O157:H7 strain Sakai/pKEC2 in duplicate. Plant pots were inoculated by dispensing 20 ml of bacterial suspensions into the soil, followed by incubation in a biosafety level 3 greenhouse at 21 °C with a 12 h day-/night-cycle for 2, 3, and 4 days. Bacterial suspensions contained either 5.0×10^7 cfu/ml or 5.0×10^8 cfu/ml. Plant pots were irrigated with 20 ml of a 10 mM $MgCl_2$ solution prior to inoculation in order to prevent the soil from drying out throughout the experiment.

2.10 Inoculation of *Valerianella locusta* plants

For inoculation of the *Valerianella locusta* plants, the following strains were used: *E. coli* O157:H7 Sakai/pKEC2, *E. coli* O157:H7 Sakai/pKEC2/pOKD4, *E. coli* O157:H7 Sakai *Δiha*/pKEC2, *E. coli* O157:H7 Sakai *Δiha*/pKEC2/pOKD4, *E. coli* O157:H7 Sakai *Δiha*/pKEC2/ pKEC4, *E. coli* O157:H7 Sakai *ΔhcpA*/pKEC2, *E. coli* O157:H7 Sakai *ΔhcpA*/pKEC2/pOKD4,

223 *E. coli* O157:H7 Sakai *ΔhcpA*/pKEC2/pKEC5, *E. coli* O157:H7 Sakai *ΔhcpAΔiha*/pKEC2. The
224 bacterial strains were grown overnight (~18 h) in LB medium supplemented with 20 µg/ml
225 chloramphenicol or 20 µg/ml chloramphenicol and 50 µg/ml kanamycin at 37 °C with aeration at
226 180 rpm. Prior to inoculation, cells were harvested at 6,000 × *g* at 4 °C for 8 min and resuspended
227 in 10 mM $MgCl_2$. OD_{600} was measured and the samples were adjusted to an OD_{600} of 1.0
228 (corresponding to ~ 5.0×10^8 cfu/ml). Plants were inoculated with 20 ml of bacterial suspension
229 by careful pipetting in order to avoid contamination of the leaves. To guarantee sufficient
230 humidity of the soil throughout the experiment, plant pots were irrigated with 20 ml of a 10 mM
231 $MgCl_2$ solution prior to inoculation. Subsequently the plants were incubated in the biosafety level
232 3 greenhouse at 21 °C with 12 h day-/night-cycle for 4 days. As negative control, plants irrigated
233 solely with 10 mM $MgCl_2$ were used.
234
235 **2.11 Analysis of roots**
236 After incubation, the plants were carefully excavated using sterile tweezers. The plants were then
237 washed for 10 min on a rotary shaker with 50 rpm in 0.5 × MS medium to remove soil particles.
238 In case of internalization experiments, the plants were surface-sterilized by washing in 0.5 × MS
239 medium supplemented with 50 µg/ml gentamicin for 20 min prior to analysis. To validate
240 efficient disinfection, the surface-sterilized roots of the first experiment were placed onto an agar
241 dish containing TBX agar with the corresponding antibiotics for approximately 10 s before
242 further processing. These plates, called “imprint plates”, were then incubated at 37 °C overnight.
243 In case of adherence assays, the root systems were aseptically removed from the plant using
244 sterile scissors directly after washing. The roots were investigated by fluorescence microscopy
245 for qualitative analysis as well as by homogenization and spread plating in order to determine the
246 number of adherent or internalized bacteria, respectively. For microscopic analysis, the root

systems were aseptically removed from the plants and mounted on an object slide with 30 µl 0.5 × MS medium. After applying the cover slip, the microscopic preparation was sealed with nail polish and analyzed using an inverted fluorescence microscope Axio Vert.A1 (Zeiss, Germany) equipped with a Axiocam 105 color camera. Pictures were taken with 100 × / 1.25 oil N-Achroplan objective and the filter set 43 (AF 546; Zeiss, Germany) for detection of RFP signals. Pictures were processed with ZEN 2 lite software (Zeiss, Germany) and analyzed using ImageJ (Schindelin et al., 2012, 2015). Per field of microscopy, a maximum of two bacterial cells could be detected and formation of microcolonies was not observed. For quantitative analysis, the root systems were aseptically removed from the plants after washing or disinfection and transferred in a reaction tube containing ~ 15 glass beads (1-3 mm in diameter) and 500 µl PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2.0 mM KH_2PO_4, pH 7.4). The reaction tubes were weighed before and after adding the roots to calculate the roots' weight. Subsequently the roots were homogenized at 25 Hz for 5 min using a mixer mill (MM200, Retsch, Germany). For adherence experiments, 4 µl of serial decimal dilutions were spotted on TBX agar with appropriate antibiotics and 100 µl of appropriate dilutions were spread plated in duplicates on TBX agar with antibiotics. For internalization experiments, 200 µl of the homogenate were spread plated in duplicates directly on TBX agar with appropriate antibiotics. The plates were incubated at 37 °C overnight and the cfu per gram of root were calculated the next day. For each strain three independent experiments were performed in triplicates.

2.12 Statistical analysis

Data were analyzed with Brown–Forsythe test for variance homogeneity, followed by either Welch's one-way analysis of variance (ANOVA) ($\alpha = 0.05$) and two-tailed Welch *t*-test with Bonferroni correction ($\alpha_{corrected}$ = 0.05/number of comparisons), or by one-way ANOVA followed

271 by two-tailed unpaired Student's *t*-test with Bonferroni correction. $p < \alpha_{corrected}$ was considered
272 significant.
273

3. Results

3.1 Construction of isogenic deletion mutants

To investigate the influence of Iha and HcpA on adherence to and internalization into the roots of lamb's lettuce, isogenic deletion mutants in the respective genes were constructed as described. For this purpose, EHEC strain Sakai was transformed with plasmid pKD46, which promotes recombination of linear DNA as it carries an inducible λ Red recombinase system. PCR products for deleting the target genes carry the kanamycin resistance gene flanked by FRT sites and 40 – 50 nt homologous to the gene of interest. For the deletion of *hcpA*, the primer pair G70 hcpA-P1 and G71 hcpA-P2 was used as reported by Nuebling et al. (2017), and for *iha* primers deliha-for and deliha-rev were used (Table 2). In case of successful mutagenesis, application of these primer pairs led to deletion of 78 % and 93 % of *hcpA* and *iha*, respectively. Transformation of purified PCR products resulted in intermediate deletion mutants where the genes of interest were substituted by a kanamycin resistance cassette flanked by FRT sites. This antibiotic resistance gene was removed by transformation of the plasmid pCP20 encoding for thermal inducible FLP recombinase. FLP targets the FRT sites and leads to double-crossover at those positions and thereby eliminates sequences between these two target sites. The resulting isogenic deletions mutants were verified by PCR applying primers that are specific for the corresponding genomic context (Table 2). Moreover, gene deletions were confirmed by sequencing the respective target sites (Table 2). Consequently, EHEC strain Sakai Δ*iha* and EHEC strain Sakai Δ*hcpA* were successfully generated (Table 1).

3.2 Complementation of deletion mutants

In order to complement the single deletion mutants, plasmids were constructed that contain the functional genes under the control of their native promotors, which were knocked out in the

298 chromosome. The native promotors were chosen since induction was not applicable considering
299 the experimental set-up and as the aim was to investigate the influence of the HcpA and Iha at
300 natural conditions. Therefore, the genes *hcpA* and *iha* plus 400 bp and 378 bp upstream of the
301 start codon, respectively, were amplified with the corresponding primers listed in Table 1, using
302 genomic DNA as template. For plasmid-based complementation it was necessary to select a
303 plasmid that is not incompatible with the RFP-encoding plasmid pWRG435 and the plasmids that
304 EHEC strain Sakai carries naturally. Hence, the vector pOKD4 was used as backbone (Fig. 1A).
305 After cloning, the resulting plasmids pKEC4, coding for Iha, and pKEC5, coding for HcpA (Fig.
306 1B), were verified by sequencing the target sites. Subsequently, the plasmids were transformed
307 into the corresponding single deletion mutants, resulting in EHEC strain Sakai Δ*iha*/pKEC4 and
308 EHEC strain Sakai Δ*hcpA*/pKEC5 (Table 1).
309

310 **3.3 Red fluorescence protein (RFP)-encoding plasmid pKEC2 is stable in EHEC strain**
311 **Sakai grown soil**

312 In the general experimental set-up, the microscopic and the numeric detection of the investigated
313 EHEC strains relied on the stability of the RFP-encoding plasmid pKEC2. Therefore, it was
314 important that this plasmid was not lost in EHEC O157:H7 strain Sakai throughout the time scale
315 of the experiment. To analyze this, EHEC O157:H7 strain Sakai/pKEC2 was inoculated in soil,
316 incubated for up to 14 days at room temperature and recovered therefrom. Samples were plated
317 on TBX agar with and without 20 µg/ml chloramphenicol to determine the percentage of EHEC
318 cells that lost plasmid pKEC2. Since bacterial colonies were not found on TBX agar without any
319 antibiotics after recovery treatment of the uncontaminated soil, all detected colonies were
320 considered as EHEC O157:H7 strain Sakai and/or EHEC O157:H7 strain Sakai/pKEC2. The total
321 viable counts of EHEC O157:H7 strain Sakai declined over time, starting at 1.1×10^8 cfu/g soil

and 9.6×10^7 cfu/g soil (Fig. 2), respectively. After 14 days, only 8.9×10^3 cfu/g soil and 8.7×10^3 cfu/g soil could be detected (Fig. 2), respectively. This result is in line with findings that reported similar degression patterns for other EHEC O157:H7 strains and a variety of *Salmonella* strains (Fornefeld et al., 2017; Gurtler et al., 2013). The viable counts for chloramphenicol-resistant bacteria behaved the same as the total counts and were not significantly different (Fig. 2). These results indicate that pKEC2 is largely stable in EHEC O157:H7 strain Sakai in soil throughout the duration of the experiments.

3.4 EHEC O157:H7 strain Sakai and *E. coli strain* DH5α adhere both to the root surface of *Valerianella locusta*

Before conducting plant infection experiments with the EHEC O157:H7 strain Sakai, which is level 3** in Germany according to the German Ordinance on Biological Substances, a preliminary experiment was carried out with the RFP-labelled non-pathogenic *E. coli* laboratory strain DH5α/pWRG435 to test the overall experimental set-up. For this purpose, *Valerianella locusta* plants were inoculated with bacterial suspensions of different concentrations between 1.0×10^5 cfu/ml to 1.0×10^9 cfu/ml. Microscopic analysis showed that *E. coli* DH5α/pWRG435 could be detected upon inoculation with 10^8 cfu/ml or with 10^9 cfu/ml (Fig. S1). When inoculated with 10^8 cfu/ml, cells were mainly detected as single cells (Fig. S1A). In contrast, upon inoculation with 10^9 cfu/ml it was also possible to detect several bacterial cells in close proximity to each other and between the rhizodermal cells (Fig. S1B).

As this initial experiment showed that the overall set-up is applicable for plant infection experiments carried out with *E. coli*, experiments with EHEC O157:H7 strain Sakai were performed to determine suitable growth and infection parameters. Therefore, *Valerianella locusta* plants were first contaminated with two different inocula of EHEC O157:H7 strain Sakai and

grown for up to four days post infection. Samples were investigated on days 2, 3, and 4, and EHEC strain Sakai was found to be root-associated in all cases (Fig. 3A). For both inocula the number of cfu per gram root decreased between day 2 and 3, and increased again at day 4. The highest numbers of adherent *E. coli* Sakai cells were detected at an inoculum level of 5.0×10^8 cfu/ml after 4 days of incubation (1.1×10^6 cfu/g root, Fig. 3A). Microscopic analysis verified adherence to roots (Fig. 3B). Hence, plants were incubated with 5.0×10^8 cfu/ml for 4 days for all subsequent experiments.

3.5 Deletion of *iha* and *hcpA* did not affect adherence to roots

Similar to other enteric bacteria, *E. coli* is known to possess a variety of molecular and physiological mechanisms that facilitate effective survival and colonization within the plant environment (Quilliam et al., 2012). In the current study, we investigated the influence of *iha* and *hcpA* deletions on the adherence to and internalization into lamb's lettuce roots using strains containing RFP-encoding plasmids for inoculation. Experiments with the wildtype strain and mutants were performed as described below and the viable counts were determined. As expected, investigating the native washed roots by fluorescence microscopy showed no red fluorescent bacteria (Fig. 4A-C). After contamination with *E. coli* O157:H7 Sakai, bacteria could be detected at the roots (Fig. 4D-F). Neither deletion of *iha* or *hcpA* nor deletion of both genes led to diminished appearance of EHEC at the roots (Fig. 4G-I, M-O, S-U). By using fluorescent microscopy no differences in adherence could be detected as all tested strains were found to be root-associated (Fig. 4 and S2 Fig.). Formation of microcolonies was not observed for any tested strain.

For quantitative analysis, the root systems were separated from the plants after washing, homogenized and appropriate decimal dilutions of the homogenate were spread plated (see

370 below). The viable counts per gram root varied slightly between the strains (Fig. 5). After
371 inoculation with Sakai wildtype 1.3×10^6 cfu/g root could be found (Fig. 5). Wildtype strain
372 Sakai/pOKD4 and Sakai *ΔhcpA* showed the same counts of adherent bacteria (1.4×10^6 cfu/g
373 root and 1.5×10^6 cfu/g root, respectively, Fig. 5). A slightly higher capability in adherence was
374 detected for the *hcpA* knock-out strain with the empty vector control and for the complementation
375 strains of the single deletion mutants (2.4×10^6 cfu/g root for Sakai *ΔhcpA*/pOKD4, 2.5×10^6
376 cfu/g root for Sakai *ΔhcpA*/pKEC5, and 2.3×10^6 cfu/g root for Sakai *Δiha*/pKEC4, Fig. 5). The
377 lowest counts were found for the double deletion mutant (8.9×10^5 cfu/g root, Fig. 5), while the
378 *iha* deletion mutant (4.4×10^6 cfu/g root, Fig. 5) and the *iha* deletion mutant carrying the empty
379 vector (5.8×10^6 cfu/g root, Fig. 5) were the most adherent. To test for significance, Welch's
380 one-way analysis of variance (ANOVA) was applied as described below, which indicated
381 significant differences between the strains. Further statistical analysis was performed assuming
382 that if the strains that seem to differ from each other the most – in this case Sakai *Δiha*/pKEC4
383 and Sakai *ΔhcpAΔiha* – did not show significance, all differences observed between every strain
384 were insignificant. Hence, the data from Sakai *Δiha*/pKEC4 and Sakai *ΔhcpAΔiha* were analyzed
385 by two-tailed Welch *t*-test with Bonferroni correction ($\alpha_{corrected} = 0.00139$). The obtained *p* value
386 was 0.00372 and therefore 2.6 fold higher than the corrected α value. Thence the observed
387 differences can be considered as insignificant. Unlike results from cell culture experiments
388 performed by other authors (Johnson et al., 2005; Ledesma et al., 2010; Tarr et al., 2000;
389 Xicohtencatl-Cortes et al., 2007; Yin et al., 2009), Iha and HcpA do not seem to play a role in
390 adherence to the roots of lamb's lettuce under the conditions tested.
391

3.6 Deletion of *iha* and *hcpA* leads to reduced internalization

As the absence of the genes *iha* or *hcpA* did not result in reduced adherence, it was further investigated whether these adherence factors are involved in the internalization of EHEC strain Sakai into the root tissues. Washing of the roots was followed by surface disinfection using gentamicin to eliminate all bacteria trapped on the root surface as described below. Disinfection was regarded successful as no viable counts were detected on the imprint plates. Analysis by fluorescence microscopy again did not reveal any differences between the strains concerning internalization (Fig. 6). After surface sterilization, all tested strains could be detected within the roots. However, bacteria did not localize in distinct patterns. They were found within the plant cells (Fig. 6D-F, M-O, S-U; S3G-I Fig.) as well as in the grooves between the cells (Fig. 6G-I, J-L, P-R; S3A-C Fig., S3D-F Fig.). With this technique, it is difficult to define if there are less root-associated bacteria after surface disinfection compared to adherence experiments. Therefore, the roots were homogenized after gentamicin treatment and the homogenate was spread plated directly to determine the number of internalized bacteria. Quantitative analysis confirmed that internalized bacteria could be found for all tested strains (Fig. 7). In contrast to adherence assays, the differences between the strains were more prominent. For the wildtype strain 2.4×10^2 cfu/g root could be found, while only 1.8×10^1 cfu/g root were detected for the *hcpA* deletion mutant (Fig. 7). This reduction in internalization was almost restored to wildtype level by plasmid-based complementation (1.8×10^2 cfu/g root, Fig. 7). Decrease in internalization was even higher when *iha* was lacking (1.1×10^1 cfu/g root, Fig. 7). As shown for the *hcpA* knock-out mutant, the complemented *iha* deletion mutant displayed wildtype-like behavior (2.8×10^2 cfu/g root, Fig. 7). Interestingly, the double knock-out mutant showed slightly higher counts of internalized bacteria than the single deletion mutants (2.8×10^1 cfu/g root, Fig. 7). The highest number of internalized

415 bacteria was found for Sakai/pOKD4 (3.6×10^2 cfu/g root, Fig. 7). In contrast, the lowest counts
416 were detected for Sakai *Δiha*/pOKD4 (6.2×10^0 cfu/g root, Fig. 7).
417 Taken together, the *E. coli* O157:H7 Sakai wildtype, its derivative carrying the empty control
418 vector and the complemented single deletion mutants, showed similar numbers of internalized
419 bacteria between 1.8×10^2 and 3.6×10^2 cfu/g root, whereas the knock-out mutants were
420 significantly ($p_{corrected} < 0.05$) hampered in internalization (6.2×10^0 to 2.8×10^1 cfu/g root).
421 These results demonstrate that EHEC O157:H7 strain Sakai is able to internalize into root tissue
422 resulting in viable counts similar or higher to the human infectious dose. Therefore, we conclude
423 that both Iha and HcpA are involved in the internalization of root tissue during plant colonization.
424

425 **4. Discussion**

426 Cultivated plants can serve as host for EHEC bacteria and fresh produce generated from such
427 plants is mainly consumed raw. Irrigation water, surface water and manure are some possible
428 routes of pathogen contamination that can occur directly on the field. As a consequence,
429 contamination of plant-based food represents a serious threat to public health. Recent studies on
430 EHEC-plant interactions focused on the overall ability of EHEC to adhere, internalize, and persist
431 in plant tissue (Sharma et al., 2009; Solomon et al., 2002; Wright et al., 2017) on laboratory
432 scale. However, it remained unknown which intrinsic factors are important for EHEC in order to
433 be able to internalize into the tissue of plants.
434 Conceivably, effective adherence is a prerequisite for successful internalization. In this study, we
435 focused on two adherence factor genes, *hcpA* and *iha*, and their roles during colonization of roots
436 of *Valerianella locusta*. In contrast to previous studies published for plant leaves, animal tissue
437 and human cell lines (Johnson et al., 2005; Nuebling et al., 2017; Saldana et al., 2011; Tarr et al.,
438 2000; Xicohtencatl-Cortes et al., 2007; Yin et al., 2009), the present study showed that deletion

of *hcpA* or *iha* did not decrease the ability of *E. coli* O157:H7 strain Sakai to adhere to, in this case, plant roots but rather led to a slight increase in adherence. Also, the deletion of both genes did not significantly alter the strains' attachment behavior and resulted in only slightly reduced attachment. These results indicate that neither HcpA nor Iha did specifically interact with the roots of *Valerianella locusta,* which is different from results published for leaf surfaces of commercially available baby spinach (Saldana et al., 2011). In contrast to our study, in the mentioned study the object of investigation was leaves that were cut in equal pieces and contaminated by incubation in EHEC suspension resulting in a higher inoculation density. Moreover, plant leaves are covered by a waxy layer called cuticle which is not present at the roots. Hence, roots and leaves have different overall surface structures with distinct characteristics. As EHEC carries a variety of adherence-conferring factors, *e.g.* pili, flagella, intimin (Jaglic et al., 2014), it sounds reasonable that EHEC requires distinct adherence factors depending on its target host and its environment. Attachment to plant roots may follow different mechanisms than attachment to plant leaves or mammalian cell lines as their surface exhibit distinct properties.

We further investigated the involvement of *hcpA* and/or *iha* in internalization into the plant roots. Single deletion mutants as well as the double deletion mutant demonstrated significantly reduced levels of internalization. For *ΔhcpA*, the number of internalized EHEC per gram root decreased by more than a factor 10 from 2.4×10^2 cfu/g root (wildtype) to 1.8×10^1 cfu/g root. This finding is in accordance with *in vitro* cell culture studies conducted by Xicohtencatl-Cortes et al. (2009) demonstrating that the deletion of *hcpA* results in decreased internalization into HT-29 cells. The same study showed that HCP is involved in biofilm formation indicating a possible link between biofilm formation and internalization. The reduction of internalization was even more prominent upon deletion of the siderophore receptor gene *iha* by a factor > 30 (6.2×10^0 cfu/g root). For

463 EHEC O157:H7 strain 86-24 and for *E. coli* strain UPEC76 it is known that the absence of Iha
464 hampers successful colonization of pig ileal loops (Yin et al., 2009) and of the murine urinary
465 tract (Johnson et al., 2005). It is currently not known how Iha mediates adherence and contributes
466 to internalization. However, Iha participates in iron uptake (Leveille et al., 2006). Accordingly,
467 there is a chance that Iha does not directly facilitate attachment and internalization, but rather acts
468 indirectly by either inducing adhesive and/or invasive proteins or by repressing counteracting
469 factors resulting in a better capability of adherence and internalization..
470 Surprisingly, deletion of both adherence-associated genes did not lead to further reduction of
471 internalization and subsequently did not reveal synergistic effects as the double knock-out
472 showed results similar to the *hcpA* single deletion mutant. If HcpA and Iha were the only proteins
473 involved in internalization into roots, the *hcpA* and *iha* double negative mutant would not be able
474 to internalize. Subsequently, no EHEC would be recovered from surface-disinfected roots.
475 Hence, these results indicate that both investigated proteins are not the only factors involved in
476 internalization and do not act synergistically. Presumably, the observed effects are not derived
477 from specific interactions between these proteins and plant surface structures but are rather
478 unspecific. Factors potentially contributing to these unspecific interactions are cell surface
479 hydrophobicity and cell surface charge. Numerous studies investigated the effect of these two
480 surface properties with respect to attachment. It was found that there is no general correlation
481 between cell surface hydrophobicity and cell surface charge and adherence, as most studies
482 gained contradicting results. Dickson and Koohmaraie (1989) as well as Li and McLandsborough
483 (1999) investigated the relationship between surface hydrophobicity and attachment to beef. The
484 former authors showed that hydrophobicity is involved in adherence in fat tissue whereas in lean
485 beef muscle cell surface charge is more important (1989). On the contrary, the latter authors did
486 not observe any correlation between surface charge, hydrophobicity and adhesion to beef muscle

(Li and McLandsborough, 1999). Ukuku and Fett (2002) showed that adhesion of *E. coli*, *Salmonella* and *Listeria monocytogenes* to cantaloupe rind is influenced by cell surface hydrophobicity and charge. Zita and Hermansson (1997) demonstrated that cell surface hydrophobicity is important for attachment to activated sludge flocs. Boyer and colleagues (Boyer et al., 2007, 2011) detected that surface proteins such as curli fibers and the O-antigen of *E. coli* O157:H7 influence cell surface hydrophobicity and charge. However, deletion of the curli fibers did not alter attachment (Boyer et al., 2007) to lettuce leaves while reduced adherence to the same surface was observed in the absence of the O-antigen (Boyer et al., 2011). Notably, during these studies different *E. coli* O157:H7 strains were used and cultivation conditions were altered, which makes comparisons difficult. Thus, no generally valid statement can be made concerning adherence and cell surface properties. Conceivably the impact of cell surface charge and hydrophobicity to attachment depends on various factors like the bacterial strain, cultivation conditions, target host, and environment. It should be noted that none of the mentioned studies investigated the potential connection between internalization and cell surface properties. Hence, a possible role of surface properties in internalization cannot be excluded. Bacteria are supposed to use either natural plant openings for internalization, such as sites of lateral roots emergence, or plant lesions that either occurred naturally or are caused by plant pathogens (Deering et al., 2012). Contingently these potential plant entry points hold surface properties that are different from the residual root area and thus provide better access for the bacteria. Jozefaciuk et al. (2014) already observed that the surface charge density of roots may be more heterogeneous than expected and reported. This could explain why the adherence of *E. coli* O157:H7 strain Sakai is not affected upon deletion of the tested genes whereas its internalization is. For clarification of this question, experiments investigating the cell surface hydrophobicity as well as the cell surface

510 charge of the used strains and further strains lacking additional surface proteins should be
511 performed followed by attachment and internalization experiments.
512 Taken together, the results of this study demonstrate that invasion of EHEC O157:H7 strain Sakai
513 into root tissue of *Valerianella locusta* can occur under the tested conditions. The overall viable
514 counts of EHEC O157:H7 strain Sakai found in the present study are comparable or even above
515 the human infectious dose determined for ground beef. In this experimental set-up the so-called
516 adherence factors HcpA and Iha do not act as adhesins during root colonization but are important
517 internalization factors. Therefore, intrinsic factors which play a crucial role in the internalization
518 of EHEC into plant roots were identified. In conclusion, further research is needed to fully
519 understand the underlying mechanisms in order to develop countermeasures.
520

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541

542 **Conflict of interest**

543 The authors declare that there is no conflict of interest.

544

545 **References**

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735

736 **Tables**

737 **Table 1: *E. coli* strains & plasmids used in this study**

Strain or plasmid	Characteristics	Origin
Strains		
E. coli DH5α	Laboratory strain	Hanahan, 1983
E. coli DH5α/pWRG435	Labelled with RFP, amp^R	this study
E. coli O157:H7 Sakai	Wildtype O157:H7 isolate from 1996 outbreak associated with white radish sprouts, *stx*+	Outbreak strain from Japan 1996, (Hayashi et al., 2001)
E. coli O157:H7 Sakai/pKEC2	Labelled with RFP, referred to as wildtype strain, cam^R	this study
E. coli O157:H7 Sakai/pKEC2/pOKD4	Labelled with RFP, carries pOKD4, cam^R, kan^R	this study
E. coli O157:H7 Sakai *ΔhcpA*/pKEC2	Labelled with RFP, deletion of *hcpA*, cam^R	this study
E. coli O157:H7 Sakai *ΔhcpA*/pKEC2/pOKD4	Labelled with RFP, deletion of *hcpA*, carries pOKD4, cam^R, kan^R	this study
E. coli O157:H7 Sakai *ΔhcpA*/pKEC2/pKEC5	Labelled with RFP, deletion of *hcpA*, complemented, cam^R, kan^R	this study
E. coli O157:H7 Sakai	Labelled with RFP, deletion	this study

Δiha/pKEC2	of *iha*, cam^R	
E. coli O157:H7 Sakai *Δiha*/pKEC2/pOKD4	Labelled with RFP, deletion of *iha*, carries pOKD4, cam^R, kan^R	this study
E. coli O157:H7 Sakai *Δiha*/pKEC2/pKEC4	Labelled with RFP, deletion of *iha*, complemented, cam^R, kan^R	this study
E. coli O157:H7 Sakai *ΔhcpAΔiha*/pKEC2	Labelled with RFP, deletion of *hcpA* and *iha*, cam^R, kan^R	this study

Plasmids

pKD46	Encodes for red recombinase, temperature-sensitive, amp^R	Datsenko and Wanner, 2000
pKD4	Carries kan^R flanked by FRT sites	Datsenko and Wanner, 2000
pCP20	Encoding for FLP recombinase, temperature-sensitive, cam^R/amp^R	Cherepanov and Wackernagel, 1995
pKEC2	pWRG345 backbone with cam^R instead of amp^R, encoding for RFP	this study
pOKD4	Expression plasmid, kan^R, p15A origin of replication	Dzivenu et al., 2004

pKEC4	pOKD4 backbone with iha plus 400bp upstream	this study
pKEC5	pOKD4 backbone with *hcpA* plus 400bp upstream	this study
pWRG435	P_{rpsM}:*tagRFP-T*, amp^R, pBR322 origin of replication	Bender et al., 2013

738

739

740 **Table 2: Oligonucleotides used in this study**

Name	Sequence (5' – 3')	Function	Reference
G70 *hcpA*-P1	**TGGACAAGCAACGCGGTTTTACA CTTATCGAACTGATGGTGGT**GTA GGCTGGAGCTGCTTCG	Mutagenesis	Nuebling et al., 2017
G71 *hcpA*-P2	**GTCATCAAAGCGGAAGACATCTT CGCAGGCTTGCTGCAATGC**CATA TGAATATCCTCCTTAG	Mutagenesis	Nuebling et al., 2017
del*iha*-rev	**ATGCGAATAACCACTCTGGCTTC CGTAGTCATTCCCTGTCT**GTAGG CTGGAGCTGCTTCG	Mutagenesis	this study
del*iha*-for	**CATATCCTGTTGTTGATGATCCC GTCTGGAAGTAATCACC**CATATG AATATCCTCCTTAG	Mutagenesis	this study
*hcpA*del-for	ATCTCAATACGTTTGGTGG	Confirmation of mutagenesis	Nuebling et al., 2017
*hcpA*del-rev	CGAAATAAAAAACCTCGG	Confirmation of mutagenesis	Nuebling et al., 2017
iha-I	CAGTTCAGTTTCGCATTCACC	Confirmation of mutagenesis	this study
iha-II	GTATGGCTCTGATGCGATG	Confirmation of mutagenesis	this study
hcpA-MscI-f	CTC*TGGCCA*ATCATGCTGGATAACT	Complementation	this study

	T		
hcpA-BamHI-r	TAG*GGATCC*TTAGTTGGCGTCATCAA	Complementation	this study
iha-HindIII-f	GGG*AAGCTT*GGTCGACTGAATAAGGT	Complementation	this study
iha-XhoI-r	CGG*CTCGAG*GATAGCGTTTTGTTATTA	Complementation	this study
iha-134up-for	GCCGAGGCAGTCGTTATTTATA	Confirmation of complementation	this study
iha-1754-rev	AAATACCGACCAGCTTTTCTGC	Confirmation of complementation	this study
iha-1598-for	GGAATCGAACCTTATCCTGAATTC	Confirmation of complementation	this study
hcpA-50-rev	CCAATAACCACCATCAGTTCGA	Confirmation of complementation	this study
P-*cat* PvuI for	ATA*CGATCG*AGCGCTGATGTCCGGC	Exchange of resistance	this study
cat PuvI rev	ATA*CGATCG*TTACGCCCCGCCCTGCCA	Exchange of resistance	this study
cat 124 for	GGCCTTTTTAAAGACCG	Confirmation of exchange	this study
cat 225 rev	CATACGGAATTCCGGATG	Confirmation of exchange	this study

741 The homologous regions for recombineering are highlighted in bold, letters in italics indicate
742 restriction sites.

743 **Figure captions**

744

745 **Fig. 1. Construction of plasmids for complementation.** The genes iha and *hcpA* including 378
746 bp and 400 bp of the corresponding upstream region (up_{iha} and up_{hcpA}) were amplified by PCR
747 using the primer pairs iha-HindIII-f/iha-XhoI-r and hcpA-MscI-f/hcpA-BamHI-r, respectively.
748 (A) The resulting PCR products were cloned into pOKD4, resulting in (B) the respective
749 complementation plasmid pKEC4 and pKEC5.

750

751 **Fig. 2. Stability of EHEC O157:H7 strain Sakai/pKEC2 in soil.** Number of bacteria in cfu per
752 gram soil. Soil was inoculated with 1.0×10^8 cfu/g soil and incubated for up to 14 days. Samples
753 were taken 0, 2, 4, 7 and 14 dpi, investigated concerning total counts of *E. coli* cfu, *i.e.* EHEC
754 O157:H7 strain Sakai (dark grey), and chloramphenicol (cam) resistant *E. coli* counts, *i.e.* EHEC
755 O157:H7 strain Sakai/pKEC2 (light grey). Data are means ± standard deviations of three
756 independent experiments.

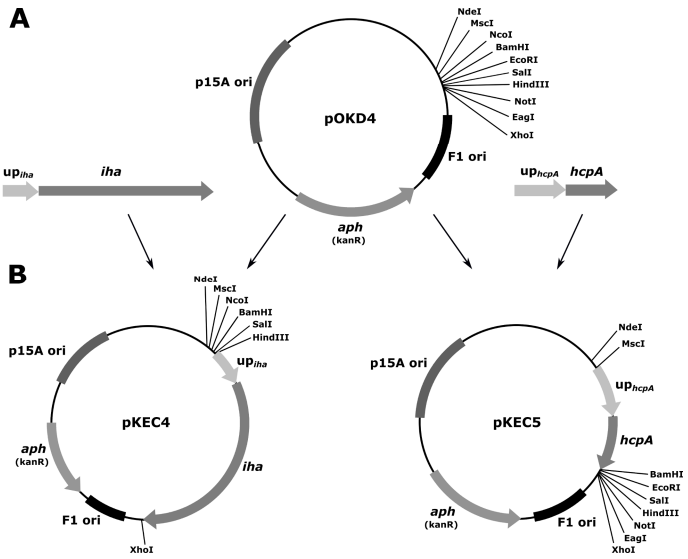
757

758 **Fig. 3. Analysis of roots-association of EHEC.** (A) Number of root-associated bacteria in cfu
759 per gram root. Plants were inoculated with 5.0×10^7 cfu/ml (light grey) or 5.0×10^8 cfu/ml (dark
760 grey) and incubated for 2, 3 and 4 days. Data are means ± standard errors of the experiment
761 performed in duplicate. (B) Microscopic analysis of roots after 4 days of incubation performed
762 with non-contaminated control roots (ctrl, upper panel) and roots contaminated with 5.0×10^8
763 cfu/ml of EHEC strain Sakai (lower panel). Shown are brightfield (left), and rfp signals (middle)
764 and the overlays of both channels (right). Bars are 10 µm. Magnification is 100-fold.

765

766 **Fig. 4. Microscopic analysis of fluorescent bacteria at the roots of lamb's lettuce after 4 days**
767 **of incubation.** Microscopy was performed after 10 min of washing in 0.5 × MS medium of
768 untreated roots (A-C) and roots contaminated with several bacterial strains: EHEC Sakai
769 wildtype (D-F), Sakai *Δiha* (G-I), Sakai *Δiha*/pKEC4 (J-L), Sakai *ΔhcpA* (M-O), Sakai
770 *ΔhcpA/*pKEC5 (P-R) and Sakai Δ*hcpA*Δ*iha* (S-U). Shown are brightfield (left), and rfp signals
771 (middle) and the overlays of both channels (right). Bars are 10 µm. Magnification is 100-fold.
772
773 **Fig. 5.** Number of adherent bacteria at the roots of lamb's lettuce in cfu per gram root. Plants
774 were inoculated with 5.0×10^8 cfu/ml of the strains indicated and incubated for 4 days. No
775 bacteria could be detected at the non-contaminated roots (ctrl). Data are means ± standard errors
776 of three independent experiments performed in triplicates.
777
778 **Fig. 6. Microscopy of fluorescent bacteria after root surface disinfection**. Analysis was
779 conducted after 10 min of washing in 0.5 × MS medium and 20 min of surface disinfection in 0.5
780 × MS medium supplemented with 50 µg/ml gentamicin, with non-contaminated roots (A-C) and
781 roots contaminated with several bacterial strains: EHEC Sakai wildtype (D-F), Sakai *Δiha* (G-I),
782 Sakai *Δiha*/pKEC4 (J-L), Sakai *ΔhcpA* (M-O), Sakai *ΔhcpA/*pKEC5 (P-R) and Sakai Δ*hcpA*Δ*iha*
783 (S-U). Shown are brightfield (left), and rfp signals (middle) and the overlays of both channels
784 (right). Bars are 10 µm. Magnification is 100-fold.
785
786 **Fig. 7. Internalization of EHEC O157:H7 strain Sakai into the roots of lamb's lettuce.**
787 Number of internalized bacteria in cfu per gram root. Plants were inoculated with 5.0×10^8
788 cfu/ml of the strains indicated and incubated for 4 days. No bacteria could be detected at the non-
789 contaminated roots (ctrl). Data are means ± standard errors of three independent experiments

790 performed in triplicates. * $p_{corrected} < 0.05$ compared to wildtype-like strains (Welch's one way
791 ANOVA followed by two-tailed Welch *t*-test with Bonferroni correction).



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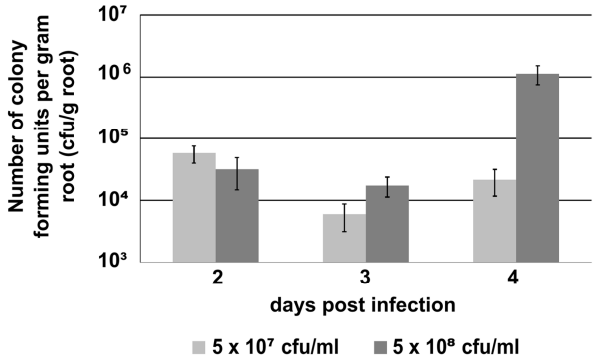

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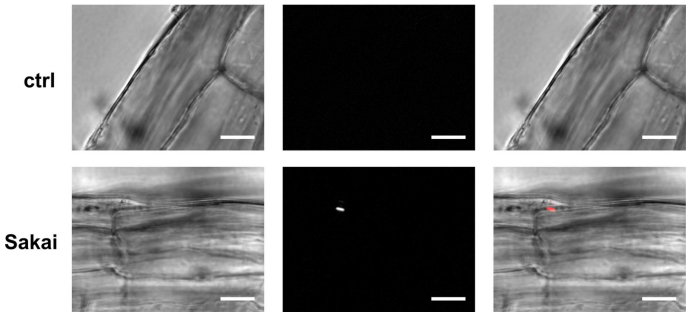

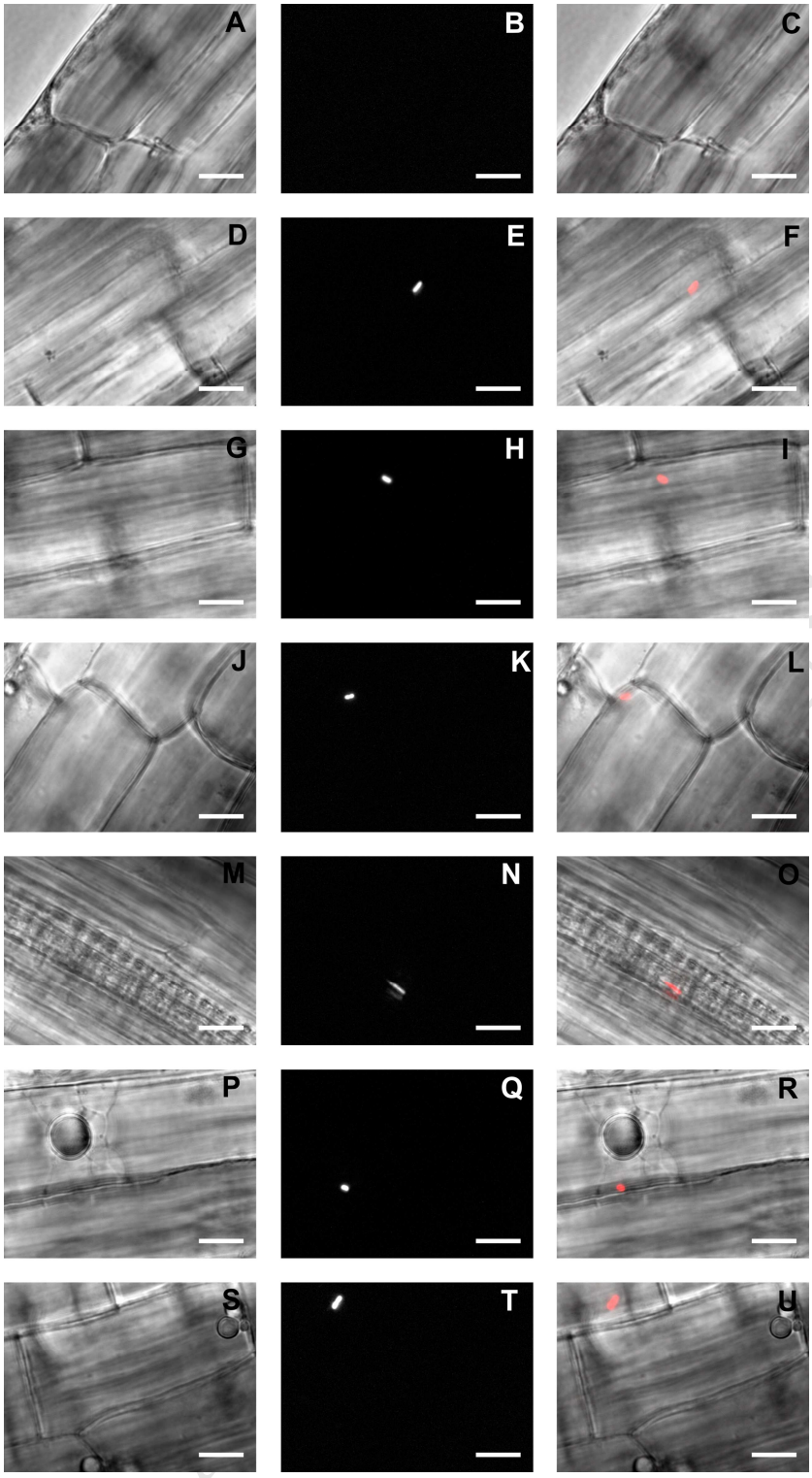

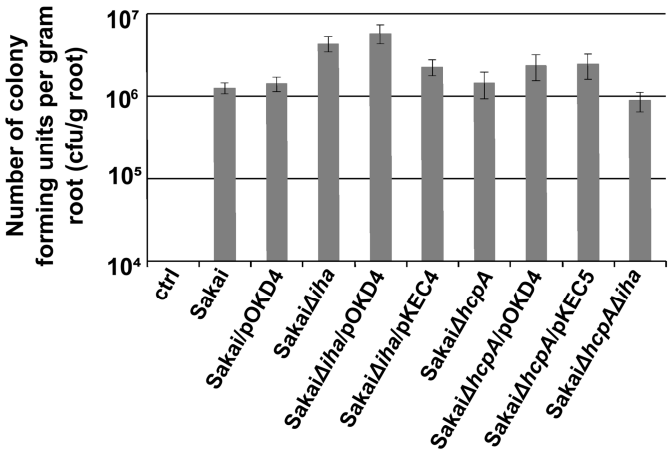

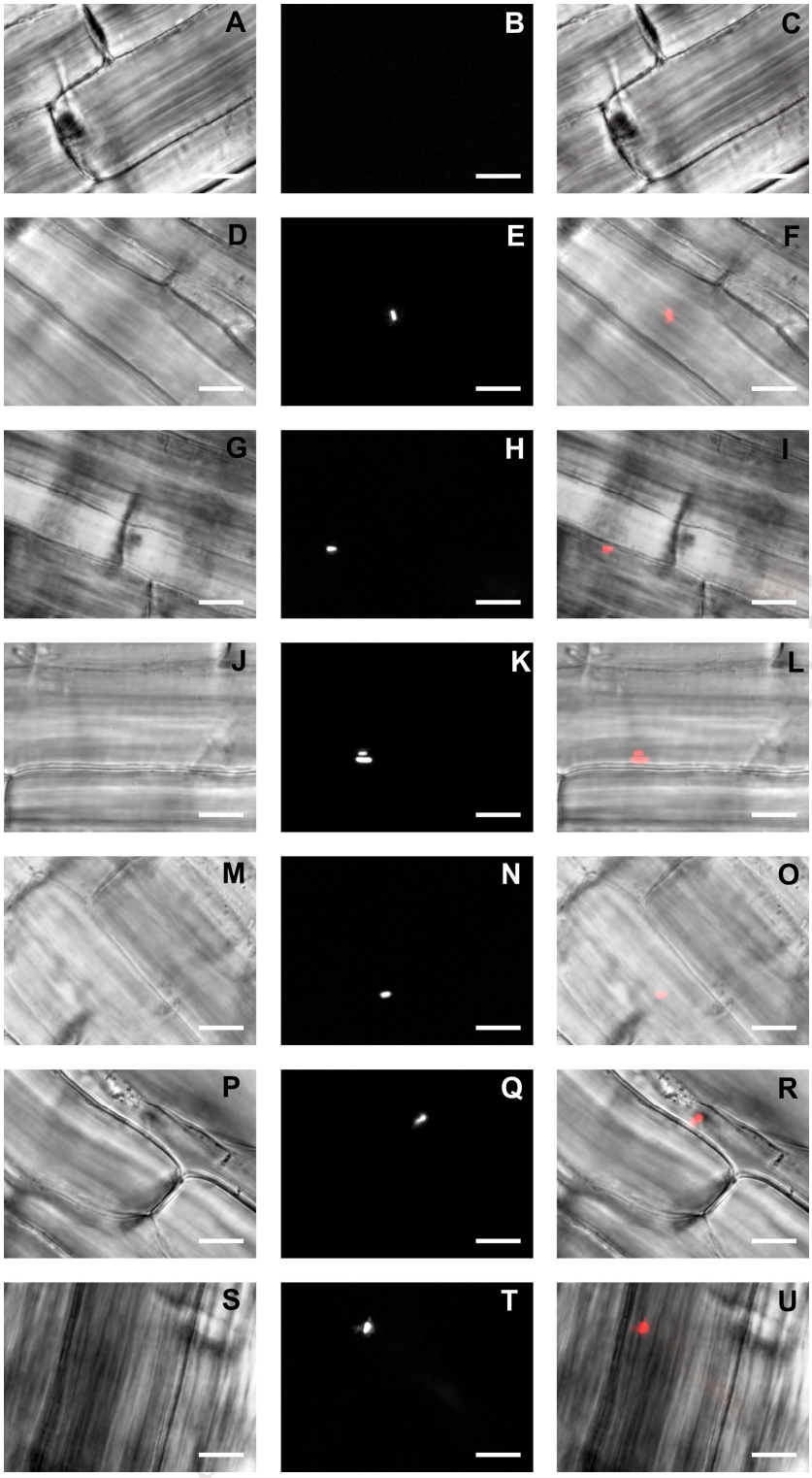

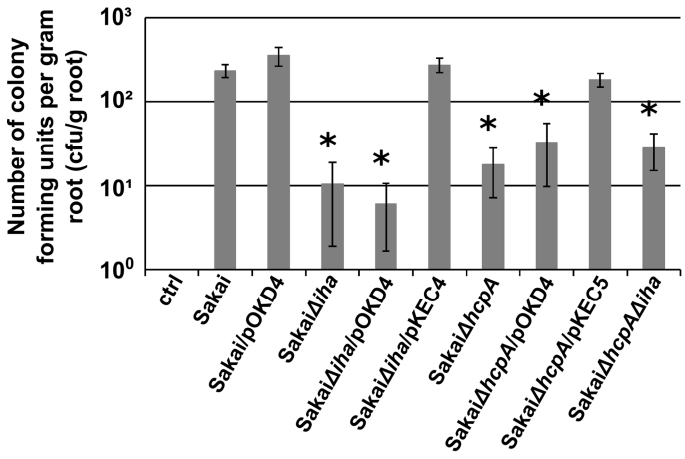

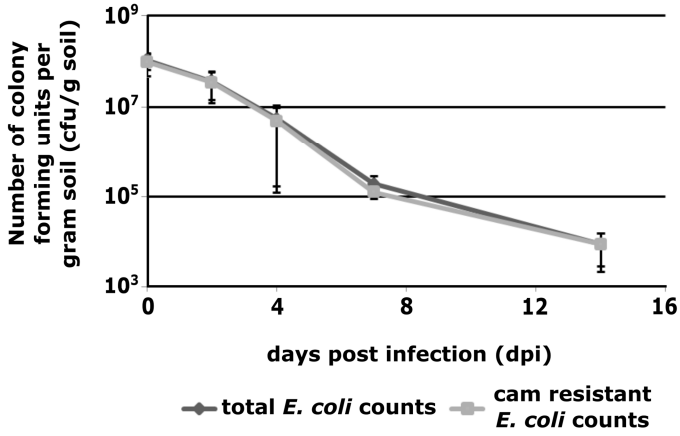

Highlights

- *E. coli* O157:H7 strain Sakai can indeed colonize the roots of lamb's lettuce
- The numbers of internalized bacteria exceeded the infectious dose for human disease
- The virulence factors *hcpA* and *iha* are crucial for internalization into root tissue