Colonization of barley (*Hordeum vulgare*) with *Salmonella enterica* and *Listeria* spp.

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Abstract

Colonization of barley plants by the food-borne pathogens *Salmonella enterica* serovar *typhimurium* and three *Listeria* spp. (*L. monocytogenes*, *L. ivanovii*, *L. innocua*) was investigated in a monoxenic system. *Herbaspirillum* sp. N3 was used as a positive control and *Escherichia coli* HB101 as a negative control for endophytic root colonization. Colonization of the plants was tested 1–4 weeks after inoculation by determination of CFU, specific PCR assays and fluorescence *in situ* hybridization (FISH) with fluorescently labelled oligonucleotide probes in combination with confocal laser scanning microscopy (CLSM). Both *S. enterica* strains were found as endophytic colonizers of barley roots and reached up to 2.3×10^6 CFU per g root fresh weight after surface sterilization. The three *Listeria* strains had 10-fold fewer cell numbers after surface sterilization on the roots and therefore were similar to the results of nonendophytic colonizers, such as *E. coli* HB101. The FISH/CSLM approach demonstrated not only high-density colonization of the root hairs and the root surface by *S. enterica* but also a spreading to subjacent rhizodermis layers and the inner root cortex. By contrast, the inoculated *Listeria* spp. colonized the root hair zone but did not colonize other parts of the root surface. Endophytic colonization of *Listeria* spp. was not observed. Finally, a systemic spreading of *S. enterica* to the plant shoot (stems and leaves) was demonstrated using a specific PCR analysis and plate count technique.

Introduction

In recent years, the increase in food-borne infections has become an important public health concern worldwide. The epidemiology of microbial food-borne illnesses has changed, not only because the human population is increasingly susceptible to diseases and because of changing eating styles, more convenient foods and less time devoted to food preparation, but also because of the emergence of newly recognized microbial pathogens and ever-evolving technologies for food production, processing and distribution (Meng & Doyle, 1998).

Salmonellosis has been linked to tomatoes, apple or orange juice, and seed shoots (Beuchat, 2002). Moreover, *Salmonella* species have been identified in soils that were treated with cattle manure and cropped to grain such as spring wheat and barley (De Freitas *et al.*, 2003). *Salmonella* serovariations cause severe diarrhoea in humans and are estimated to cause approximately 1.3 million cases of food-borne infection each year in the United States, with 15 000 hospitalizations and 500 deaths (Mead *et al.*, 1999). Outbreaks of Listeriosis have been linked to, for example, cabbage and lettuce. *Listeria monocytogenes* has been linked to potentially severe food-borne diseases characterized by meningitis, encephalitis or septicaemia in immunocompromised patients, infants and the elderly (Farber & Peterkin, 1991). The ubiquity of *Listeria monocytogenes* in nature and its acknowledged presence in food-processing environments (Gravani, 1999) have focused attention on cross-contamination of processed foods from environmental sources and explain the difficulty in producing minimally processed foods free of pathogenic bacteria. By contrast, preharvest contamination with food-borne pathogens such as *Salmonella* sp. can occur via (improperly treated) manure application rather than chemical fertilizers, untreated sewage or irrigation water, or use of contaminated seed (Beuchat & Ryu, 1997). Bacterial colonization and biofilm development can occur and result in spoilage of food sources by bacterial pathogens (Burnett & Beuchat, 2000; Beuchat, 2002).

Although much is known about the ecology of bacterial pathogens in foods of animal origin such as meat and dairy

products, the behaviour of these bacteria in association with cereal plants in the field environment is less well known. The persistence of food-borne pathogens such as *Salmonella enterica* servovar *typhimurium* in soils has been reported (De Freitas *et al*., 2003). Colonization of different parts of dicotyledoneous plants such as *Medicago sativa* and *Medicago truncatula* (Dong *et al*., 2003), *Arabidopsis thaliana* (Vasse *et al*., 1995), tomato (Guo *et al*., 2002) or radish sprouts (Itoh *et al*., 1998) by human pathogens, e.g. *Escherichia coli* O157:H7, *Salmonella typhimurium* and *Listeria monocytogenes*, and by a variety of other enteric bacteria has been investigated. However, monocotyledoneous plants such as Gramineae have not been studied at all in this respect. In Gramineae, colonization of roots by plant growth-promoting bacteria has been well studied (e.g. Rothballer *et al*., 2003). These investigations demonstrated that not only the root surface but also the root interior and finally the whole plant can be colonized systemically by certain rhizosphere bacteria, e.g. *Azoarcus* sp. BH72 (Reinhold-Hurek & Hurek, 1998a, b), *Herbaspirillum* spp. (James & Olivares, 1998) or *Gluconacetobacter* spp. (Sevilla *et al*., 2001).

Gramineae, for example barley, may be colonized starting from the rhizosphere by food-borne pathogenic bacteria. In this respect, the roots of gramineous plants could function as a host for better survival of these bacteria in soils or to scavenge these bacteria and thus prevent further spreading. However, colonization may also lead to propagation of these pathogenic bacteria in agricultural production and food-processing systems. Because of the abundance of genomic information and micro-array systems for barley, specific bacteria–root interactions and even systemic colonization and spreading of the plant are able to be tested.

With the increase in reports of food-borne infections, considerable attention has been given to the development of methods for detecting microbial pathogens; these methods include culture isolation, serological tests, DNA probes and PCR assays (Rahn *et al*., 1992; Wagner *et al*., 1998; Schmid *et al*., 2003). In real agricultural production systems, numerous interactions of human pathogenic bacteria occur with the vast diversity of resident microflora present in plant roots in the complex soil and rhizosphere environment. To study the potential of selected strains of typical food-borne pathogens with roots of potential host plants, colonization and interaction studies performed in an axenic system are necessary.

Therefore, we investigated the colonization behaviour of different food-borne pathogenic bacteria of roots and shoots of barley plants in a monoxenic model system. We selected two strains of *Salmonella enterica* serovar *typhimurium* (LT2 and S1) and pathogenic as well as apathogenic strains of the genus *Listeria* (*L. monocytogenes*, *L. ivanovii* and *L. innocua*) for these investigations.

Materials and methods

Bacterial strains and culture conditions

Details of the strains used in this study are summarized in Table 1. *Herbaspirillum* sp. N3 and *Escherichia coli* HB101 served as reference strains for positive and negative control of root colonization, respectively. Two *Salmonella enterica* serovar *typhimurium* strains were used: *S. typhimurium* LT2, a widely used strain for laboratory experiments, and *S. typhimurium* S1, originally isolated from an organic waste fermentation plant. To compare different (a) pathogenic strains among one genus, three *Listeria* species were chosen: *Listeria monocytogenes* sv4b, as a representative human pathogen, *Listeria ivanovii*, a pathogen for sheep and cattle, and *Listeria innocua*, an apathogenic species.

Salmonella enterica strains were grown aerobically at 37 °C in liquid standard Luria Bertani (LB) broth (tryptone, 10.0 g L^{-1}; yeast extract, 5.0 g L^{-1}; NaCl, 10.0 g L^{-1}; pH = 7.0) or on solid XLD agar (xyline–lysine–desoxycholate; Merck, Darmstadt, Germany) as selective medium for plate count experiments. *Listeria* strains were grown aerobically at 37 °C in brain heart infusion broth (BHI; Difco Laboratories, Franklin Lakes, NJ) or on OXFORD selective agar plus

Table 1. All strains used in this study and their colonization behaviour

Strain	Serovar	Reference	Rhizosphere colonization (surface)	Root colonization (endophytic)
Salmonella enterica	*typhimurium* LT2	TUMSal8	+++	+++
Salmonella enterica	*typhimurium* DT104h	Isolate, Schmid, M., unpublished	+++	+++
Listeria monocytogenes	4b	SLCC4013	+	–
Listeria ivanovii		ATCC19119	++	–
Listeria innocua	6a	NCTC11288	+	–
Escherichia coli		Invitrogen, Karlsruhe, Germany	–	–
Herbaspirillum sp. N3		Isolate, Klein, I., unpublished	+++	+++

SLCC, Special Listeria Culture Collection, Institute of Hygiene and Microbiology, University of Würzburg, Würzburg, Germany; ATCC, American Type Culture Collection, University Boulevard, Manassas, USA; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK.

selective supplement (Fluka, Buchs, Switzerland). *Herbaspirillum* sp. N3 and *E. coli* HB101 were grown aerobically at 37 °C in/on LB medium.

Surface sterilization and germination of barley seeds

Barley seeds (*Hordeum vulgare* var. Barke) were obtained from 'Saatzucht Josef Breun GdbR' (Herzogenaurach, Germany). The seeds were surface sterilized with ethanol and sodium hypochlorite. After seed incubation in 1% [volume in volume (v/v)] Tween 20 (Sigma-Aldrich, Steinheim, Germany) in sterile deionized water for 2 min the seeds were immersed in 70% ethanol for 5 min followed by three washes in sterile deionized water. Subsequently, the seeds were incubated in sodium hypochlorite solution with 6–14% active chloride for 20 min (Riedel-de-Häen, Seelze, Germany) and washed five times with sterile deionized water. Seeds were then allowed to swell in sterile water for 2–4 h and treated again with sodium hypochlorite solution for 10 min followed by five washes with sterile deionized water. Sterility of the seeds was tested by placing them on NB medium agar plates (meat extract, 1 g L^{-1}; peptone, 5 g L^{-1}; NaCl, 5 g L^{-1}; yeast extract, 2 g L^{-1}; pH = 7.1 ± 0.2; Sigma-Aldrich). After incubation in a growth chamber at 30 °C for several days, seedlings showing no visible bacterial contamination on the agar plate were then transplanted into sterile test tubes (see below).

Monoxenic plant growth conditions

As an axenic system, large glass tubes (3 cm wide, 20 cm long) filled with washed, dried (overnight at 105 °C) and autoclaved quartz sand were used. The tubes were filled about 5 cm of quartz sand of particle size 2–3.5 mm, overlaid with 2 cm of quartz sand of particle size 1.0–2.5 mm. The glass tubes were filled under sterile conditions with 10 mL Hoagland medium (Terry, 1980), and germinated axenic seedlings were planted in the sand layer. Glass tubes were covered with a cap to prevent contamination. During the inoculation period the tubes were incubated at room temperature under normal light conditions. After 1 week the tubes were extended with another sterile test tube placed upside down on the first tube and fixed with parafilm. In this system the plants grow up to a length of 30 cm.

After the appropriate experimental period (1–4 weeks), the plants were harvested under sterile conditions. For PCR experiments samples were taken every week. Samples for microscopic analysis and for CFU studies were taken after 2 weeks. The plants were carefully removed from the glass tubes and sand with tweezers to avoid damaging the roots. Adherent particles of sand and loosely adhesive bacterial cells were washed away with sterile phosphate-buffered saline (PBS) solution; the remaining shoot part of the plants was treated in the same way.

Inoculation of barley plants

Prior to inoculation bacterial strains were grown in the respective medium described above. Cell numbers were determined by dilution series and plate counting. Cells were harvested, and cell suspensions containing 1×10^8 CFU mL^{-1} PBS (pH 7.2–7.4) were used for inoculation.

Barley plants were inoculated by pipetting 1 mL of the suspension onto the surface of the quartz sand 1–2 days after planting the seedling into the axenic system. To control for sterility of the system, several plants were inoculated with 1 mL of sterile PBS solution only.

Determination of CFU

To determine the number of CFU after the inoculation period (both attached and endophytic bacteria), roots and shoots were first air dried for 10 min at room temperature. Cell count determinations of root colonization were made in triplicate. For each replicate the roots of three plants were sampled and pooled to obtain sufficient root material. One part of each pooled root sample remained untreated for the CFU determination. The other part was surface sterilized with 1% chloramine T solution (Sigma-Aldrich) for 10 min at room temperature (Baldani *et al.*, 1986). Following five washes with sterile PBS solution, to determine the efficiency of surface sterilization, roots and shoots were placed on the respective solid media. The roots and shoots were then removed and the plate was further incubated at the given temperature overnight. After this procedure surface contamination could no longer be detected.

The samples were then homogenized manually with a pestle and mortar for about 5 min until a homogeneous suspension was produced. For each 100 mg of root and shoot fresh weight material, 1 mL PBS solution was added. The suspension was serially diluted and CFU counts were determined on NB agar (*E. coli* HB101, *Herbaspirillum* sp. N3), XLD agar (*Salmonella enterica*) or OXFORD medium with selective supplement (*Listeria* spp.) after 1 day of incubation (Miles & Misra, 1938).

Determination of colonization behaviour using fluorescence *in situ* hybridization

Radial slices were prepared over the whole length of the root to identify possible preferred colonization sites. Freshly harvested roots were placed between two small blocks of Styrofoam and radial slices of about 50–100 µm thickness were cut off with a razor blade. These slices were transferred on to an eight-well adhesive Teflon-coated slide (Paul Marienfeld, Bad Mergentheim, Germany) immobilized by

incubation at 70 °C for 30 min. After heat immobilization, root slices were fixed in 4% (w/v) paraformaldehyde solution (gram-negative bacteria) or in 50% (v/v) absolute ethanol (gram-positive bacteria) for 1.5 h at 4 °C as described by Amann *et al.* (1990). For the culture-independent detection of inoculated bacteria we used fluorescence *in situ* hybridization (FISH) with phylogenetic fluorescently labelled oligonucleotide probes. All probes used in this study, the strength of the deionized formamide used in the hybridization buffer and probe specificity are given in Table 2. The probes were synthesized and purchased from Thermo Electron, Division Interactiva (Ulm, Germany). Hybridization with fluorochrome-labelled oligonucleotide probes (Cy3, Cy5) was performed according to the standard FISH protocol as described previously (Amann *et al.*, 1992; Manz *et al.*, 1992).

For three-dimensional microscopic analyses, confocal laser scanning microscopy (CLSM; LSM-510-META, Zeiss, Oberkochen, Germany) was used. Two He–Ne lasers provided excitation wavelengths of 543 nm (for Cy3 excitation) and 633 nm (for Cy5 excitation) with LP 560 and LP 650 long-pass filters, respectively. The Cy5 fluorescent dye emits in the far-red spectrum but blue colour is assigned for illustration, whereas Cy3 is shown with its red fluorescence colour. The remaining third colour channel (Ar ion laser, 488 nm excitation wavelength, with BP 500–550 band-pass filter) was used to show autofluorescence and thus the structure of plant and roots.

DNA isolation

For preparation of high-molecular-weight DNA, plants were harvested and adhering sand particles were removed from the roots. Then, homogenates were prepared from surface-sterilized roots and shoots separately. Chromosomal DNA of bacterial cultures as well as from plant material was isolated using the FastDNA SPIN Kit for Soil (Bio101, MP-Biomedicals, Heidelberg, Germany) according to the manufacturer's instructions.

PCR assay for the detection of *Listeria* and *Salmonella* sp.

All oligonucleotides used in this study (Table 2) were purchased from Thermo Electron. PCR assays were performed in a total reaction mix volume of 50 µL, Reaction mix contained 1 × PCR/*Taq* buffer, $MgCl_2$ (2.5 mM), dNTPs (200 µM each), primers (50 pM of each forward and reverse primer), *Taq* polymerase (0.5 U; MBI Fermentas, Vilnius, Lithuania) and DNA of the respective sample (100 ng). PCR was performed in a programmable thermal cycler (Primus 96 or 25, MWG Biotech, Ebersberg, Germany). The cycling programme starts with a primary denaturation at 94 °C for 4 min, followed by 35 (for primer pairs MonoA/MonoB, Siwi2/Lis1B and S139/S141) or 30 cycles (for primer pair Ino2/Lis1B) of 94 °C for 1 min, annealing at 50–65 °C for 30–60 s (depending on the primer set used; see Table 2), and elongation at 72 °C for

Table 2. All probes (a) and primers (b) used and their specificities and references

(a)

Probe	Specificity	Target [rRNA]	Binding position in *Escherichia coli* †	Sequence 5′–3′	% FA‡	Reference
EUB-338	*Bacteria* (except * and **)	16S	338–355	GCTGCCTCCCGTAGGAGT	35	Amann *et al.* (1990)
EUB-338-II	**Planctomycetales*	16S	338–355	GCAGCCACCCGTAGGTGT	35	Daims *et al.* (1999)
EUB-338-III	***Verrumicrobiales*	16S	338–355	GCTGCCACCCGTAGGTGT	35	Daims *et al.* (1999)
Lis-1255	Genus *Listeria*, *Bacillus thermosphacta*, *Bacillus campestris*	16S	1255–1272	ACCTCGCGGCTTCGCGAC	35	Wagner *et al.* (1998)
Salm-63	*Salmonella* spp., *Plesiomonas shigelloides*	23S	1742–1760	TCGACTGACTTCAGCTCC	35	W. Ludwig, unpublished

(b)

Primer	Specificity	Target gene	Sequence 5′–3′	Size of PCR product (bp)	Anealing (°C)/(s)	Reference
MonoA	*Listeria*	*iap*	CAAACTGCTAACACAGCTACT	396	50/30	Bubert *et al.* (1992)
MonoB	*Listeria*	*iap*	GCACTTGAATTGCTGTTATTG	396	50/30	Bubert *et al.* (1992)
Siwi2	*Listeria ivanovii*	*iap*	TAACTGAGGTAGCAAGCGAA	1200	58/45	Bubert *et al.* (1992)
Lis1B	*Listeria monocytogenes*	*iap*	TTATACGCGACCGAAGCCAA	850	58/45	Bubert *et al.* (1992)
Ino2	*Listeria innocua*	*iap*	ACTAGCACTCCAGTTGTTAAAC	870	62/60	Bubert *et al.* (1992)
S139	*Salmonella*	*invA*	GTGAAATTATCGCCACGTTCGGGCAA	284	65/30	Rahn *et al.* (1992)
S141	*Salmonella*	*invA*	TCATCGCACCGTCAAAGGAACC	284	65/30	Malorny *et al.* (2003)

†Position in rRNA of *E. coli* (Brosius *et al.*, 1981).
‡% FA, formamide concentration (v/v) used in the hybridization buffer.

30 s (primer pairs MonoA/MonoB and S139/S141), 45 s (for primer pair Ino2/Lis1B) or 60 s (for primer pair Ino2/Lis1B). A final extension at 72 °C for 10 min completed the reaction. Results and success of the PCR were analysed after horizontal agarose electrophoresis and ethidium bromide staining according to standard techniques.

The primers specific for *Salmonella* DNA detection target the *invA* gene, which is essential for the pathogenicity of all *Salmonella enterica* strains. Because it encodes a component of the type III secretion system located on the 40-kb pathogenicity island (SPI-1) in the *S. enterica* chromosome, this system provides a species-level detection of *Salmonella* organisms (Rahn *et al.*, 1992; Malorny *et al.*, 2003).

All primer sets used for the detection of *Listeria* DNA in this study target the *iap* gene, coding for the invasion-associated protein. This is an important virulence factor present in all *Listeria* species. The *iap* gene contains variable and conserved regions, which allows the design of primers for the specific detection of each *Listeria* spp. (Bubert *et al.*, 1992). For the detection of *Listeria monocytogenes*, primers MonoA and MonoB were chosen. *Listeria ivanovii* was verified through primer pair Siwi 2/Lis1B and *Listeria innocua* through primers Ino2/Lis1B.

The primer sets for *Salmonella* and *Listeria* (Table 2) were tested on DNA extracted from uninoculated barley seedlings (roots and shoots), grown for 2 weeks in the axenic system as a negative control. With none of the primer sets was an amplification product obtained with root or shoot DNA (data not shown).

Results

Colonization of barley roots and shoots

For inoculation experiments we used *Herbaspirillum* sp. N3 (positive control), *Escherichia coli* HB101 (negative control), *Salmonella enterica* serovar *typhimurium* strains LT2 and S1, *Listeria monocytogenes* sv4b, *Listeria ivanovii* and *Listeria innocua*. Together with noninoculated control plants, the inoculated barley plants were grown under monoxenic conditions on quartz sand with Hoagland's solution for 2 weeks. Roots and shoots were harvested, and CFU of each strain were determined in triplicate before and after surface sterilization. Cell counts of between 2.4×10^7 and 2.7×10^8 CFU per g root fresh weight were found without surface sterilization. After surface sterilization bacterial cell numbers decreased to between 4.6×10^6 and 2.5×10^5 CFU per g root fresh weight (Fig. 1). Highest levels of reduction were found for all *Listeria* strains and for *E. coli*. The lowest reduction after surface sterilization, only a 10-fold decline, was found for *Herbaspirillum* sp. N3 as the inoculant. With *Salmonella typhimurium* strains about 100-fold reductions of cell numbers were detected.

Cell counts of surface-sterilized shoots of plants inoculated with the *Salmonella typhimurium* strains ranged from 4.3×10^4 (strain LT2) to 5.2×10^6 CFU per g shoot fresh weight (strain S1).

In situ analysis of root colonization

Because the plate count method provides no detailed information about the colonization behaviour of the inoculated strain on barley roots, we applied a microscopic

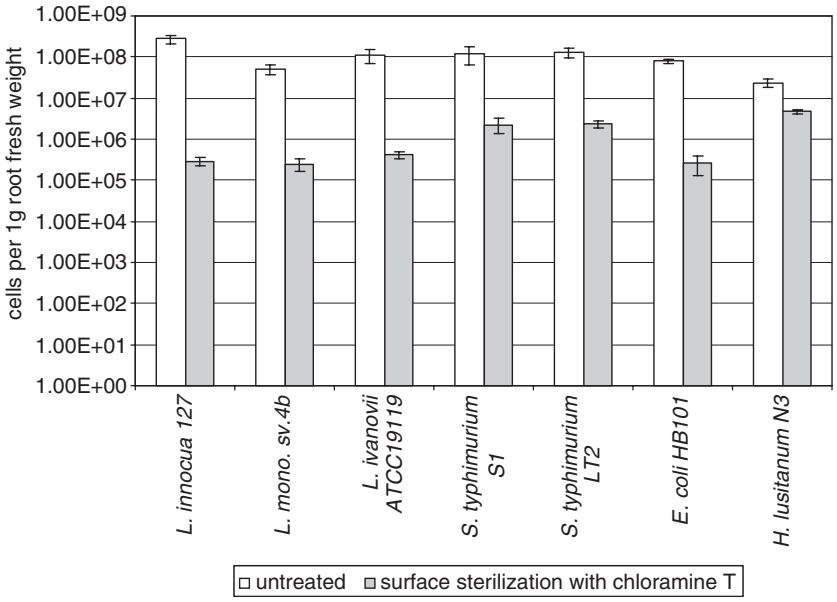

Fig. 1. CFU determination of root-associated bacterial strains per gram root fresh weight of monoxenically grown barley plants before (white columns) and after (shaded columns) surface sterilization. Incubation period: 2 weeks after inoculation.

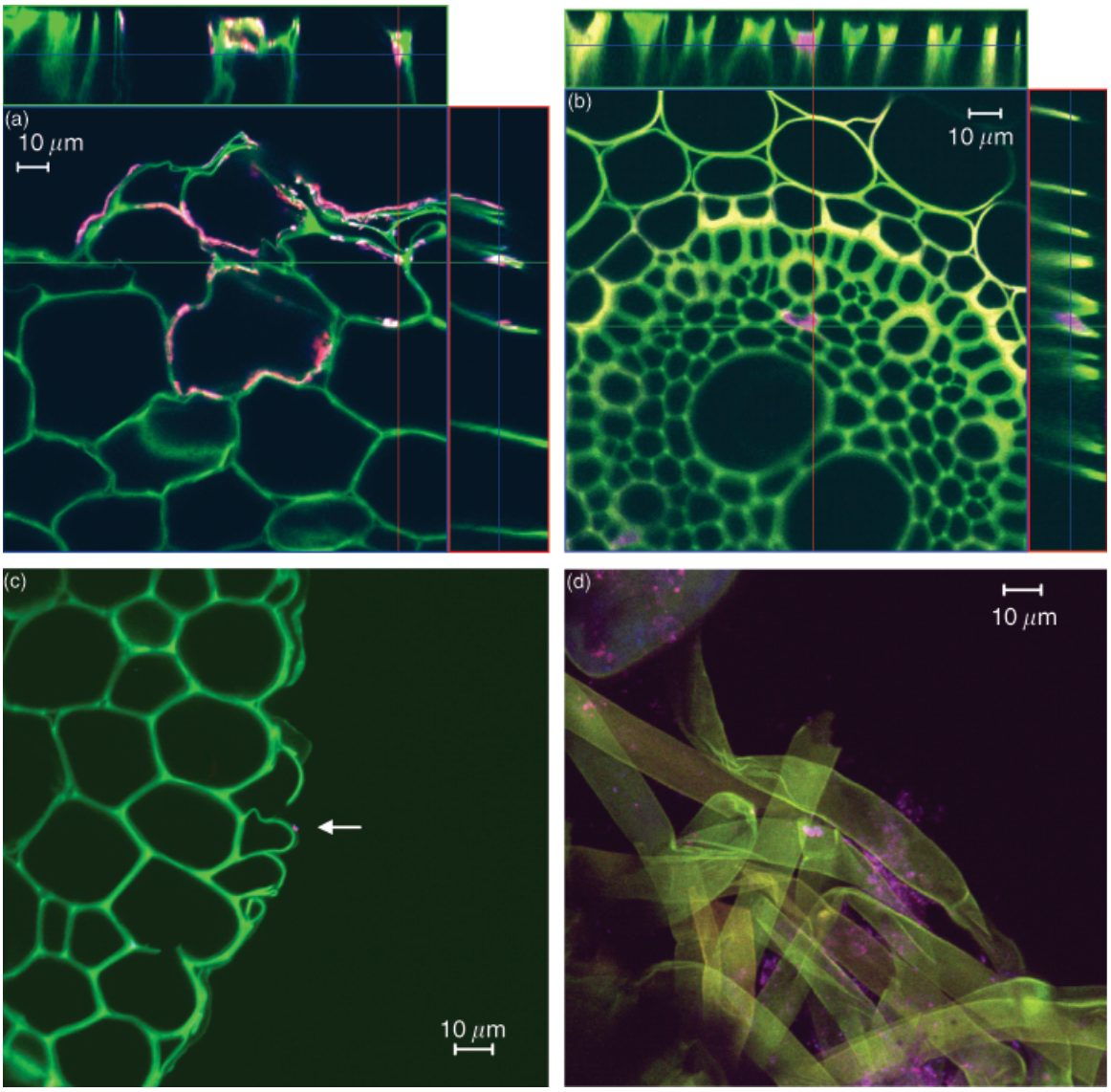

Fig. 2. *In situ* detection of *Salmonella typhimurium* LT2 by fluorescence *in situ* hybridization analysis after hybridization with probe EUB-338-mix-Cy5 (blue) and SALM-63-Cy3 (red) in root slices. Orthogonal views of a three-dimensional confocal image created from a z-stack of xy-scans. Thirty stacks of 1 μm thickness were taken to create the orthogonal image. (a) Radial slice from the lower part of the root obtained 2 weeks after inoculation (between root tip and the root hair zone). *Salmonella typhimurium* LT2 cells are stained in magenta, (combination of both of the fluorescent signals). Arrows indicate positive hybridization signals within the root cortex. (b) Radial slice from the root inoculated with *Salmonella typhimurium* LT2 3 weeks after inoculation. *Salmonella* cells (magenta) are colonizing the central cylinder of the root endophytically at high density. (c,d) Confocal laser scanning microscopy images after inoculation of barley roots with *Listeria ivanovii* after 2 weeks of incubation and hybridization with the probes EUB-338-mix-Cy5 (blue) and Lis-1255-Cy3 (red). *Listeria* cells are stained pink (mixture of red and blue).

approach together with FISH analysis. It was not possible to design a specific probe to target the 16S rRNA gene because of the high similarity between *Salmonella* species and serovariations and other related members of the *Enterobacteriaceae*. Therefore, we designed a probe that detects all members of the genus *Salmonella* with the 23S rRNA coding gene as target site. This probe, SALM-63, is suitable for the specific *in situ* detection of all *Salmonella enterica* strains used in this study under stringent hybridization conditions. After inoculation to barley roots, *S. enterica* strains LT2 (Fig. 2a,b) and S1 (data not shown) could be detected with this probe in great numbers colonizing the surfaces of the main root (Fig. 2a), side roots and root hairs in the monoxenic system. Furthermore, it was noticeable that colonization of subjacent rhizodermis cell layers also occurred at high cell density (Fig. 2a). The *Salmonella* cells formed microcolony-

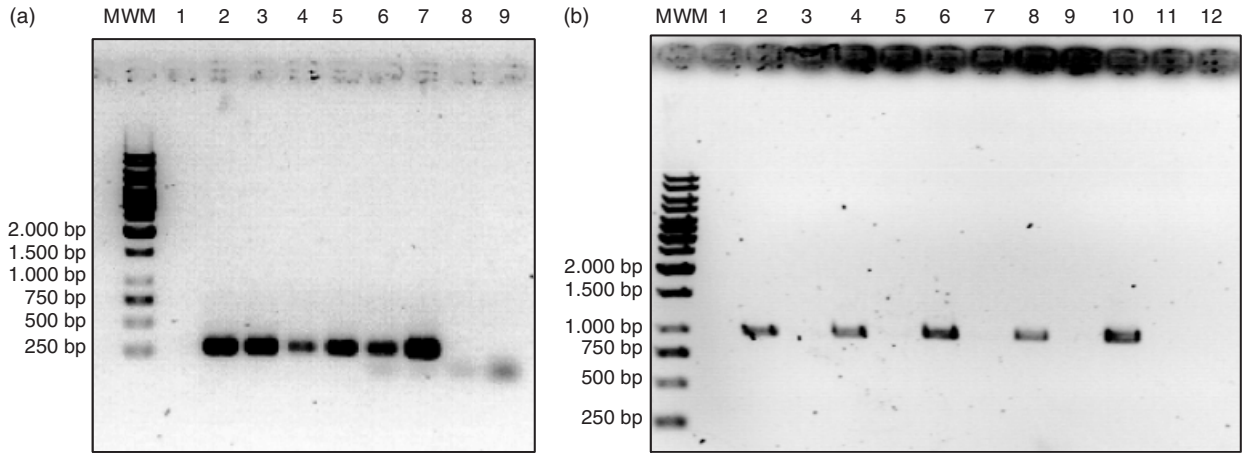

Fig. 3. (a) Detection of *Salmonella typhimurium* LT2 DNA on inoculated barley seedlings. Plants were grown for 1-4 weeks. PCR with primer set S139/S141. MWM, molecular weight marker. Lane 1, empty; lanes 2–5, roots inoculated for 1, 2, 3 or 4 weeks, respectively; lane 6, shoot from plant inoculated for 4 weeks; lane 7, positive control with *S. typhimurium* LT2 DNA; lane 8, negative control with *Escherichia coli* HB101 DNA; lane 9, control with deionized water (no DNA). (b) Detection of *Listeria innocua* 127 DNA on inoculated barley seedlings. Plants were grown for 1–4 weeks. PCR with the primer set Ino2/Lis1B was used to identify *Listeria* in different parts of the plant. MWM, molecular weight marker. Lane 1, empty; lanes 2, 4, 6 and 8, roots inoculated for 1, 2, 3 and 4 weeks, respectively; lanes 3, 5, 7 and 9, shoots from plants inoculated for 1, 2, 3 and 4 weeks, respectively; lane 10, positive control with *L. innocua* 127 DNA; lane 11, negative control with *E. coli* HB101 DNA; lane 12, control with deionized water (no DNA).

like structures. Numerous *S. enterica* cells colonized root cells, probably after dispersion through the intercellular spaces (i.e. apoplast). It appeared that penetration of epidermis cell walls had occurred. Furthermore, numerous microcolonies could be found in the inner cortex of the central cylinder (Fig. 2b). Preferential colonization of a specific root area was not be observed. We found the same colonization pattern on all parts of the root.

For the specific detection of the *Listeria* strains we applied the previously described FISH probe Lis-1255 (Wagner *et al.*, 1998). Root colonization patterns were also examined for the different *Listeria* strains, but in contrast to *Salmonella*, no endophytic colonization could be detected after the same incubation time for any of the three strains (data for *L. ivanovii* are shown in Figs 2c,d). Colonization of root hairs appeared at the same high cell density as for *Salmonella*, and these appeared to be the preferred area of settlement (Fig. 2d). Colonization of the root surface by *Listeria* strains occurred only rarely and only by a few single cells throughout all scanned samples (Fig. 2c). No colony formation was observed on the root surface (data not shown). In some cases, *L. monocytogenes* established a strong colonization of the root surface, but again, no endophytic behaviour of the bacteria was observed.

The colonization behaviour of *E. coli* HB101 was similar to that of the three *Listeria* species. The bacteria were found on the root hairs but no colonization of the root surface or endophytic bacteria were observed (data not shown).

Herbaspirillum sp. N3 showed a typical endophytic colonization behaviour of barley roots with rapid colonization of cells in the root central cylinder (data not shown). In this case, more single cells of *Herbaspirillum* sp. N3 than microcolonies were found distributed in the inner cortex and central cylinder of the roots.

The colonization behaviour of strains used in this study is summarized in Table 2.

Specific PCR detection of the inoculated strains in different parts of the plant

To confirm the viable cell count data and microscopic FISH experiments and to investigate possible systemic spreading of *Salmonella* through the vascular system towards the shoot we used a PCR-based approach to detect bacterial DNA in and on the roots, as well as in the shoot. For detection of the two *Salmonella enterica* strains we used the primer pair S139/S141.

Using DNA isolated from roots and shoots of plants inoculated with the *Salmonella* strains and the PCR system specific for *Salmonella* DNA, *S. enterica* serovar *typhimurium* LT2 could be detected not only in barley roots after 4 weeks (Fig. 3a, lanes 3–6) but also in the shoot after 4 weeks (Fig. 3a, lane 7). Identical results were achieved with strain S1 as the inoculant (data not shown).

Results of PCR performed with DNA extracted from plants inoculated with *L. ivanovii*, *L. monocytogenes* sv. 4b or *L. innocua* and the species-specific primer sets (Table 2)

are summarized in Fig. 3. Figure 3(b) shows results for *L. innocua* 127, which was taken as representative for all three *Listeria* strains. In contrast to *Salmonella*, no evidence for colonization of the shoots by *Listeria* spp. after 4 weeks could be found (Fig. 3b, lanes 3, 5, 7, 9). However, detection via PCR in the roots was possible (Fig. 3b, lanes 2, 4, 6, 8).

Discussion

In a monoxenic hydroponic model system it could be demonstrated that the food-borne pathogenic bacteria *Salmonella enterica* serovar *typhimurium* and *Listeria* spp. colonize barley plants differently. This was demonstrated not only by applying the plate count technique with and without surface sterilization, but also by *in situ* localization of the bacteria using the FISH technique with specific oligonucleotide probes in combination with CLSM as well as by specific PCR assays performed with DNA from different parts of the plant.

Applying the microscopic *in situ* labelling approach with FISH and CSLM, localization of the inoculated bacteria on or within barley roots was much clearer. Bacteria located at the root surface, which are protected by their localization in crevices or when embedded in the mucigel layer of the rhizoplane, may survive surface sterilization and mimic true endophytic colonization. Application of microscopic *in situ* detection techniques enabled us to demonstrate endophytic colonization of barley roots by *S. enterica*. By producing three-dimensional images from a *z*-stack of optical sections an exact determination of the position of colonizing cells in the tissue down to a depth of 40 μm was possible (orthogonal images). Because of the low accessibility of deeper root tissues for the probes it was necessary to prepare radial slices not exceeding a thickness of about 100 μm. Cells that were transferred during the cutting process from their original position to a location within the tissue were probably washed away during subsequent fixation, dehydration, hybridization and washing (Rothballer *et al.*, 2003). The complete absence of *Listeria* cells inside the roots supports this conclusion. By contrast, both *Salmonella* strains were repeatedly found in the intercellular space of the root cortex of barley plants.

Systemic spreading in the whole plant could be found using the PCR-based detection system only for the *Salmonella* strains used in this study. They were detectable by PCR and CFU counts in the stem and leaves of root-inoculated barley plants. This systemic spreading could have occurred through water transport in the vascular system, but could also have been actively supported by the *Salmonella* cells in a hitherto unknown way (Dong *et al.*, 2003). A similar invasion process for colonizing plants is also known for plant pathogens. Vasse *et al.* (1995) characterized a three-phase process in which roots of hydroponically grown tomato plants become infected with *Ralstonia solanacearum*. Colonization of the root surface, followed by infection of the vascular parenchyma and invasion of the xylem, was described. Penetration into the xylem leads to systemic colonization of the plants with the pathogen. Endophytic colonization by nitrogen-fixing bacteria such as *Gluconoacetobacter diazotrophicus*, *Herbaspirillum seropedicae* or *Azoarcus* sp. in different crop plants, such as sugar cane, rice or grasses (Boddey *et al.*, 1995; Reinhold-Hurek & Hurek, 1998a, b) has also been described. These bacteria colonize lateral root junctions in high numbers, making these junctions a likely site of plant entry. These bacteria also enter the xylem and may infect the stem cortex through the xylem (James & Olivares, 1998; James *et al.*, 2002).

Despite the widely held view that the inner tissues of plants are usually free of bacteria, Guo *et al.* (2002) found high numbers of *Salmonellae* in some hypocotyls, cotyledons, stems and leaves of hydroponically grown tomato plants via viable cell count determination. Through inoculation studies with different types of vegetables, Jablasone *et al.* (2005) showed internalization of *Salmonella typhimurium* into lettuce and radish. Similar to our study, Jablasone *et al.* found that *L. monocytogenes* did not internalize within seedlings, but did persist on the surface of plants throughout the cultivation period. Invasion of *Medicago truncatula* and *M. sativa* by enteric bacteria was demonstrated by Dong *et al.* (2003), where extensive colonization of lateral root cracks by enteric bacteria, similar to the colonization by nitrogen-fixing endophytes, leads to plant entry. Infection of root hairs is unlikely, as observed by rhizobia with legumes, although there is a higher level of nutrients than in the rhizosphere. However, Cooley *et al.* (2003) pointed out that colonization of *Arabidopsis thaliana* by *S. enterica* takes place at many locations. *Salmonella enterica* is concentrated initially at the root tips and the branch points of lateral roots, but at later incubation times the apoplast of roots (intercellular) were often found to be colonized. *Salmonella typhimurium* appears not to need wounded tissue or root cracks to invade the root. This seems to be a new mode of entry as plant pathogens often invade through breaks of the epidermis upon development of lateral roots (Gough *et al.*, 1997).

Gandhi *et al.* (2001) showed that a *Salmonella stanley* strain could reside within the interior tissues of alfalfa shoots only to a depth of 18 μm from the surface. By contrast, by using immunofluorescence microscopy and scanning electron microscopy, Itoh *et al.* (1998) showed invasion of *E. coli* O157:H7 in radish tissue on shoots grown from contaminated seeds. The bacteria were found in and on xylem elements of the hypocotyls, supporting a conclusion that *E. coli* O157:H7 can move through the vascular system. In another recent study, Solomon *et al.* (2002) also concluded that *E. coli* O157:H7 was moving within the

plant, presumably via the vasculature system, when the bacteria were shown to be present within surface-sterilized lettuce leaves after root inoculation.

There is evidence that invasion into the roots by human pathogens could lead to systemic spreading and contamination of seeds and fruits of plants. Guo *et al.* (2001) inoculated different *Salmonella* strains into stems of tomato plants by injection at flowering and early stages of fruit development, and observed their survival through fruit ripening.

It is reasonable to propose that interactions occur frequently between human-pathogenic bacteria and plants because enteric bacteria are able to survive outside their host organisms, e.g. in soils. *Salmonella enterica* has been shown to persist for extended periods in soil and on plant surfaces (Baloda *et al.*, 2001). Furthermore, enteric bacteria frequently come into contact with plants once they are shed from their animal host, and survival on plants would help ensure that they are again ingested. Seemingly, there is a benefit to survival of enteric bacteria in the plant environment long enough and in sufficient numbers to ensure infection of a new host. By contrast, it is quite possible that colonization of plant roots and human tissue may require similar traits, which make the rhizosphere a natural source of bacteria having the potential to invade human tissues (Berg *et al.*, 2005).

Although these results may be of interest with regard to public health concern, it is important to note that this, and many other, study was carried out in monoxenical hydroponic systems. Such controlled environments do not imitate the multiple biotic and abiotic environmental influences that may stimulate or suppress infection. Factors that influence internalization of bacteria in plant tissues may also be different in hydroponically grown plants and in plants grown in soil. The macrostructures of roots may differ substantially in the two systems, thus potentially affecting the behaviour of endophytic bacteria. However, axenical growing plants offer the unique ability to demonstrate the potential of colonization by a particular bacterium and to test the response of the plant towards bacterial colonization. Cooley *et al.* (2003) showed differences in the survival of different *Salmonella* strains with *Arabidopsis thaliana* growing in three different soils (autoclaved, unautoclaved and amended with the strain of interest). In soil, cell counts of pathogenic bacteria colonizing the root were reduced compared with the hydroponically grown plants. *Enterobacter asburiae* mainly affected the persistence of *S. enterica* in a hydroponic system in the form of reduced survival. It is not only competition with other microbes that affects survival and fitness of human pathogenic bacteria in the rhizosphere; bacterial and host genotypes also influence the infection by genetic determinants. Dong *et al.* (2003) demonstrated enhanced colonization by *S. enterica* of an *Medicago trunculata* mutant, which is deficient in root nodulation by *Sinorhizobium meliloti* and/or mycorrhizal infection by *Glomus*.

Although the gnotobiotic method described does not mimic the complexity of the natural environment, it does mimic commercial shoot production and hydroponic farming. It is an important tool towards discovery of bacterial and host genes involved in the interactions between microbes and plants. Some of these genes may also be important in crop plants grown in nature and may therefore provide clues to developing new methods to minimize contamination of products with human pathogens.

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