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ORIGINAL PAPER

Quantification and localization of bacteria in plant tissues using quantitative real-time PCR and online emission fingerprinting

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Abstract In order to quantify and localize specific bacterial target genes in plant tissue, this project has generated relevant new insights in the combined application of quantitative real-time PCR in parallel with the in situ PCR + probe-hybridization and online emission fingerprinting using LSM 510 META. After designing an *Enterobacter radicincitans* species-specific probe, introduced bacterial cells were monitored in growing plant parts and their colonization behaviour was examined in relation to the native bacterial community. For this purpose, the plant growth-promoting rhizobacterial (PGPR) strain *Enterobacter radicincitans* was applied to *Brassica oleracea* plants in increasing inoculum concentrations 10^7, 10^8 and 10^9 cells per plant. Inoculation of 10^9 *E. radicincitans* cells per plant to *Brassica oleracea* leaves and roots resulted in significant increases of root, leaf and tuber growth. Total bacterial cell numbers were estimated using quantitative real-time PCR to be between 10^7 and 10^9 cells g^{-1} fresh leaf weight and about 10^8 cells g^{-1} fresh root weight of *Brassica oleracea* plants. Using quantitative real-time PCR, a significant colonization of *Brassica oleracea* leaves and roots with *E. radicincitans* cells was measured. Roots were colonized with a density of 10^7 cells g^{-1} fresh root weight up to at least 14 days after inoculation. That is equivalent to a proportion of *E. radicincitans* 16S rDNA-gene copy numbers compared to the total bacterial communities of about 10–16%. Online emission fingerprinting established that the introduced bacteria proliferated on and inside the root and that they colonized the intercellular spaces of the root cortex layer. Hence, *E. radicincitans* was able to successfully compete with the native bacterial population.

Keywords Bacterial competition · Confocal laser scanning microscopy · in situ PCR · Plant growth promotion · Rhizosphere bacteria

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Introduction

Many different bacteria are known to produce plant growth-promoting effects in a wide range of plant species. The presence of these bacteria can cause nitrogen fixation, phytohormone, vitamin and/or antibiotic production as well as a range of elicitorial effects (for a review, see Dobbelaere

et al. 2003). *Enterobacter radicincitans* sp. nov., isolated from the phyllosphere of winter wheat, is one of those plant growth promoting rhizobacteria (PGPR), which has shown to increase both growth and yield of different agricultural plants (Ruppel et al. 1994). In pure culture, the bacterial cells can fix atmospheric nitrogen (Ruppel and Merbach 1995), produce phytohormones (auxin-like compounds: indole-3-lactic acid, indole-3-acetic acid and cytokinine-like compounds: N^6-isopentyladenosine, N^6-isopentyladenine) (Scholz-Seidel and Ruppel 1992) solubilize calcium phosphate (Schilling et al. 1998) and inhibit the growth of phytopathogenic fungi (unpublished). These enzyme activities have been defined, at least under pure culture conditions. However, it is still unclear to what extent these activities occur in the rhizosphere and how these enzyme activities are regulated (Steenhoudt and Vanderleyden 2000). Furthermore, a better understanding of the mode of action in the plant–bacterial interaction can improve the success rate in applying biofertilizers in agricultural production systems.

In order to directly study the bacterial activity in vivo, it is helpful to detect, visualize and quantify the inoculated bacteria on or inside the plant tissue over the growth cycle of the plant and to measure their competitiveness against the native bacterial community. To date, traditional detection methods using introduced marker genes, such as resistance to an antibiotic, or chromogenic markers (e.g. xylE, lacYZ) that produce colour change in bacterial cells have been used (for a review see Pickup 1991). During the last decade immunofluorescence (fluorochrome-targeting specific antibodies) and fluorescence in situ hybridization techniques (FISH, rRNA-targeting bacterial species or genera-specific probes) combined with confocal laser scanning microscopy applications gave first images of bacterial localization on plant surfaces (Sørensen 2005). Likewise, gfp-labelling contributed further progress for detailed in situ localization of inoculants (Jansson 2003). Rothballer et al. (2003) showed a successful in situ identification of *Azospirillum brasilense* inoculants on and inside roots of wheat both by using gfp-labelled cells and by applying FISH-analysis using EUB338 probe. However, the advent of the quantitative real-time PCR technique, widely applied in medical approaches (Lyons et al. 2000; Yin et al. 2001), now offers the opportunity to detect and quantify target genes and thereby to assess bacterial levels in plant samples without the need to tag these target bacterial strains before plant inoculation. First quantifications of *Staphylococcus aureus* cells in cheese (Hein et al. 2001) and of *Desulfotomaculum* cells in soil (Stubner 2002) show the opportunity to quantify target genes using quantitative real-time PCR also in environmental samples. Additionally, this technique is very sensitive and it is possible to quantify specific targets down to 1 copy μl^{-1} extracted DNA (Boeckman et al. 2000a). In a former study a species-specific primer-probe set was developed to detect and quantify the *E. radicincitans* bacterial cells (Kämpfer et al. 2005).

In the present study, the *E. radicincitans* species-specific and a general bacterial-specific primer-probe sets were used to quantify the bacterial cell numbers by a real-time PCR approach. As it is normally impossible to completely recover 100% DNA from plant material, and as one sample can differ slightly from the next, a housekeeping gene, i.e. plant TEF-gene which encodes for a transcriptional enhancer factor (Wulf et al. 2003), was used as an internal control to allow more accurate quantification and the comparison of different experimental treatments. To directly observe *E. radicincitans* and total bacterial genes in root cuttings an in situ PCR + probe-hybridization technique was used to enrich the bacterial target genes in the root samples. Similar in situ PCR techniques were sucessfully applied to detect the amoA gene in biofilms (Hoshino et al. 2001) or to monitor *Ralstonia eutropha* KT1 in groundwater (Tani et al. 2002).

The aim of this study was to demonstrate the applicability of the quantitative real-time PCR technique in order to establish whether inoculated *E. radicincitans* cells colonize the plant and if so, to what extent compared to the native bacterial community. Simultaneously, the online emission fingerprint approach, using the laser scanning microscope 510 META (Zeiss, Germany), was tested to localize which plant parts

and tissues are colonized by *E. radicincitans* and by the native bacterial cells on and inside the root tissue.

Materials and methods

Greenhouse pot experiment

Brassica oleracea plants (*Brassica oleracea* L. convar. *caulorapa*, F1 Hybrid RZ Eder) were grown in a greenhouse under natural light conditions in quartz sand and watered to 60% of maximal water holding capacity with complex nutrient solution (Göhler and Drews 1986). Eight plants per replication (one plant per pot, size 12 cm in diameter, containing 1 kg sand) with four replicates, were grown for 6 weeks. When two leaves had emerged, plants were inoculated with *Enterobacter radicincitans* (DSM 16656) by pipetting 5 ml of the bacterial suspension onto the leaves. Some of the bacterial suspension also ran down to the root system, which resulted in root inoculation simultaneously.

Enterobacter radicincitans cells were grown in standard nutrient solution (MERCK) at 29°C in a rotary incubator at 100 rpm for 48 h. Plants were inoculated with 10^7, 10^8 and 10^9 *E. radicincitans* cells per plant to induce a wide range of introduced cell numbers. To remove all additional nutrients, cells were washed twice in sterile tap water by centrifugation at 8000*g* for 20 min before inoculation. Control plants were inoculated with 5 ml sterile tap water only. One day and 14 days after inoculation one plant per replicate was sampled to measure *E. radicincitans* and total bacterial cell numbers using quantitative real-time PCR. One day after *E. radicincitans* inoculation one plant per replicate of the 10^9 inoculation treatment was sampled to analyse the bacterial colonization behaviour using the in situ PCR + probe-hybridization and online emission fingerprint method. That time and treatment was chosen to ensure a dense bacterial population on the root to study the bacterial colonization behaviour at fresh root samples using online emission fingerprinting. Six weeks after inoculation all plants were sampled to measure root, leaf and emerging tuber dry matter (g dry matter per plant) to measure the plant growth promoting effect.

DNA extraction

DNA was extracted from 100 mg leaf and root material using the DNeasy Plant Mini Kit (QIAGEN). The amount of DNA was measured photometrically at 260 nm and the quality of extracted DNA was checked photometrically by the A_{260}/A_{280} ratio calculation to be above 1.9 and the A_{320} measurement was nearly 0.

Primer-probe design

The 16S rDNA of *E. radicincitans* bacterial cells (DNA isolated using UltraClean™ Microbial DNA Isolation Kit [MO BIO]) was amplified by PCR (polymerase chain reaction) using the primers 18f and 1492r (Table 1) and then sequenced (The Value read, MWG THE GENOMIC COMPANY). This sequence has been deposited in GenBank (Accession No. AY563134).

A *E. radicincitans* species-specific region for primer design was determined using the CLUSTALX (1.8) Graphical multiple alignment program (UK HGMP Resource centre) in conjunction with a multiple alignment of 17 different species of *Enterobacteriaceae* (*Yersinia enterocolitica* Z49829, *Rahnella aquatilis* X79940, *Edwardsiella ictaluri* AF310622, *Yersinia pestis* AJ232239, *Buchnera aphidicola* Y11972, *Escherischia coli* J01859, *Serratia proteamaculans* AJ288155, *Serratia* sp. AJ288154, *Erwinia* sp. AJ288156, *Erwinia herbicola* AF290417, *Erwinia amylovora* AF289542, *Xenorhabdus nematophila* AY286478, *Morganella morganii* AY043168, *Enterobacterium* EA61 AF283539, *Kluyvera ascorbata* AF310219, *Enterobacter intermedius* AF310217, *Edwardsiella tarda* AB050832). An unique region was determined to be between 740 bp and 820 bp and a *E. radicincitans* species-specific primer—TaqMan™ probe set was designed using the software Beacon Designer 3.0 (PREMIER Biosoft International, selected out of three different primer probe sets).

The specificity of primer and probe sequences was confirmed in a NCBI BLAST search in the

Table 1 List of primer and probe sequences

Target	Sequence 5′–3′	Source
16S rDNA		
18f (forward)	AGT CGA ACG GTA GCA CAG AGA	
1492r (reverse)	TAC GGY TAC CTT GTT ACG ACT T	Weisburg et al. (1991)
E. radicincitans		
519f (forward)	CAG CMG CCG CGG TAA NWC	Lane (1991)
E.radr (reverse)	CGT GGA CTA CCA GGG TAT CTA ATC	
E.rad TaqMan™ probe	[6-FAM] CTC CCC ACG CTT TCG CAC CTG AGC [BHQ-6-FAM]	
E.rad probe	[6-FAM] CTC CCC ACG CTT TCG CAC CTG AGC	
Bacteria		
519f (forward)	CAG CMG CCG CGG TAA NWC	Lane (1991)
907r (reverse)	CCG TCA ATT CMT TTR AGT T	Lane (1991)
27f (forward)	AGA GTT TGA TCV TGG CTC AG	Weisburg et al. (1991)
EUB338 probe	[Cy5]GCT GCC TCC CGT AGG AGT	Amann et al. (1990)
Plant TEF		
TEF f (forward)	ACT GTG CAG TAG TAC TTG GTG	Wulf et al. (2003)
TEF r (reverse)	AAG CTA GGA GGT ATT GAC AAG	Wulf et al. (2003)

GenBank + EMBL + DDBJ + PDB databases and checked within the PCR with the following bacterial species and strains: *Azospirillum* sp. A246, *Azospirillum irakense* DSM 11586, *Bacillus subtilis* DSM 15029, *Butyrivibrio fibrisolvens* DSM 3071, *Clostridium thermocellum* DSM 1237, *Dictyoglomus thermophilum* DSM 3960, *Herbaspirillum* spp. Accession No. AF364861, *Rhodobacter capsulatus* DSM 1710, *Rhodothermus marinus* DSM 4252, *Ruminococcus albus* DSM 20455, *Sporosarcina pasteurii* DSM 33, *Stigmatella aurantiaca* DSM 1035, *Vibrio proteolyticus* DSM 30189, CC322, K27, W7/15, CC307 (own bacterial isolates from roots and soil) and the archaeal strain *Methanococcus voltae* DSM 1537.

The *E. radicincitans* TaqMan™ probe was double labelled with 6-FAM (6-Carboxyfluorescein) and a black hole quencher at the 3′-end of the probe, for the real-time PCR approach (named E.rad TaqMan™ probe, Table 1). In order to use the same probe for the in situ PCR + probe-hybridization and microscopic investigations it was also labelled with 6-FAM (named as E.rad probe, Table 1).

Real-time PCR

16S rDNA-gene copy numbers of *E. radicincitans* cells, 16S rDNA-gene copy numbers of total bacterial cells and copy numbers of the plant TEF-gene, taken from the same plant part, were measured using two different real-time PCR procedures. *E. radicincitans* was enumerated using a TaqMan™ assay, while the 16S rDNA-gene bacterial and TEF-gene copy numbers were measured using the SybrGreen I approach.

E. radicincitans *quantification*

PCR conditions for the *E. radicincitans* species-specific primer/probe set were optimized. Reactivity and specificity of primers and *E. radicincitans* TaqMan™ probe were tested as described in the previous chapter. Final 25 µl PCR contained 300 nM 519f and *E. radicincitans* reverse primer (Table 1), 100 nM *E. radicincitans* TaqMan™ probe (Table 1), about 10 ng template DNA or 2.5 µl bacterial cell suspension and QuantiTect™ Probe PCR Kit (QIAGEN). The 16S rDNA-gene quantification conducted either using the extracted DNA or using the bacterial whole cell suspension gave comparable numbers (data not shown). The PCR was performed using the following PCR regime: Cycle 1, 95°C/15 min; cycle 2, 95°C/15 s, 53.2°C/30 s, 72°C/45 s (recording of fluorescence data), 50 repeats; cycle 3, 72°C/10 min and cycle 4, 4°C hold.

The TaqMan™ assay, described in detail by Boeckman et al. (2000a), was applied to quantify specific 16S rDNA-gene copy numbers of the *E. radicincitans* cells. The *E. radicincitans*-specific 6-FAM™ (6-Carboxyfluorescein) + BHQ™ (black

hole quencher™) labelled oligonucleotide probe allows for real-time monitoring of the PCR. The fluorescence of the reporter molecule, measured at 520 nm after an excitation at 490 nm using the iCycler™ detection system (BIO-RAD), increases as products accumulate with each successive round of amplification. At some point during the amplification, the accumulation of product results in a measurable change in the total fluorescence of the reaction mixture. The fluorescence rises appreciably above the background, i.e. at the threshold cycle. There is a linear relationship between the log of the starting amount of a template and the threshold cycle during real-time PCR. Therefore, given known starting amounts of the target nucleic acid, a calibration curve can be constructed by plotting the log of starting target amount versus the threshold cycle using the iCycler™ iQ optical system software 3.0a (BIO-RAD). This calibration curve can then be used to determine the starting amount of each unknown template based on its threshold cycle (Boeckman et al. 2000a).

The calibration curve for the *E. radicincitans*-specific 16S rDNA-gene was produced from the specific PCR product. The PCR product was cleaned using the MiniEluteTM PCR Purification Kit (QIAGEN) and the DNA concentration measured photometrically at an absorbance of 260 nm. Copy numbers were calculated using the known DNA concentration and the template length of 277 bp. Ten-fold dilution series within a range of 10^9 to 1 copy per µl were used. It was shown that one *E. radicincitans* cell contains two 16S rDNA-gene copies, measured in an in situ PCR with predefined *E. radicincitans* cell numbers.

Bacterial 16S rDNA-gene and TEF-gene quantification

The 16S rDNA-gene copy numbers of a wide range of bacterial cells were measured using the primers 519f and 907r specific for the bacterial domain (Primer sequences see Table 1, Lane 1991) and the real-time PCR approach with SybrGreen I as an intercalating dye (Boeckman et al. 2000b). Fluorescence signals were recorded at 520 nm after an excitation of 490 nm using the iCycler™ detection system (BIO-RAD). The final PCR mixture for both genes contained QuantiTect™ SYBR® Green master mix (QIAGEN), 300 nM of each forward and reverse primer and about 10 ng template DNA. The PCR protocol to quantify bacterial 16S rDNA-gene copy numbers was as follows: Cycle 1, 95°C/15 min; cycle 2, 95°C/15 s, 53°C/30 s, 72°C/42 s (record of fluorescence data), 50 repeats; cycle 3, 72°C/10 min; Cycle 4, melt curve 55°C increasing temperature every 10 s by 0.5°C (recording of fluorescence data), 85 repeats; and cycle 5, 4°C hold. The PCR protocol to quantify the TEF-gene copy numbers (primers TEF f and TEF r described in Table 1) was the same as for bacterial 16S rDNA-gene copy numbers with the exception of cycle 2 which was as follows: Cycle 2, 94°C/30 s, 56.5°C/30 s, 72°C/60 s (record of fluorescence data), 50 repeats. The data calculation was carried out using the iCycler™ iQ optical systems software 3.0a.

The quality of the SybrGreen I quantification method was further verified for each measurement to avoid the possibility of false positive signals induced by primer dimers or other non-specific PCR products. First, a melting profile was recorded after each run, which resulted in one melting peak of the first deviation with the specific melting temperature of the PCR product (83°C for TEF-gene and 87°C for bacterial 16S rDNA-gene). Second, PCR products were run on an agarose gel to confirm the presence of a single distinct band of expected size (155 bp for TEF-gene, 407 bp for bacterial 16S rDNA-gene). The calibration curves for TEF-gene and bacterial 16S rDNA-gene quantification were prepared as described for *E. radicincitans* 16S rDNA-gene quantification using specific PCR products.

In situ PCR + probe-hybridization

Root tissue slices of 1 mm thickness were cut by hand using a razor blade from fresh root tips (0.5 cm behind the root tip) of the treatment inoculated with 10^9 *E. radicincitans* cells per plant 1 day after the bacterial application. These segments or bacterial whole cell suspensions were used in two successive in situ PCRs before probe-hybridization and microscopic online emission fingerprint analysis. The first used primers to

amplify the *E. radicincitans*-specific region of the 16S rDNA and the second the bacterial domain-specific 16S rDNA region. The first PCR was carried out as for *E. radicincitans* quantification; however, *E. radicincitans* FAM labelled probe (E.rad probe) without black hole quencher (Table 1) was used and reactions were terminated after 20 repeats of cycle 2. The QuantiTect™ Probe PCR Kit (QIAGEN) included the enzymes to make bacterial and plant cells permeable to get access to the target bacterial DNA but keeping the overall structure of the cells in the in situ PCR. After the first PCR root segments were washed in water (molecular biology grade) 6 times and placed into the second PCR to amplify and hybridize bacterial DNA target copies using the 27f and 907r primers and the Cy5 labelled EUB338 probe (Table 1). A wide range of different bacterial genera and species produced fluorescent signals after hybridizing of the EUB338 probe as formerly documented by Amann et al. (1990), Daims et al. (2001) and Antón et al. (1999). The PCR protocol was the same as for the bacterial real-time PCR with the exception of using QuantiTect™ probe PCR Kit (QIAGEN) and an annealing temperature of 50°C. Again, the PCR was stopped after 20 repeats of cycle 2. Root segments were washed carefully about 6 times with water (molecular biology grade) to remove almost all adhering not hybridized fluorescence dyes. The *E. radicincitans* cells hybridized with both the specific FAM-labelled probe and the EUB338 Cy5-labelled probe since these probes have two different targets on the 16S rDNA. Therefore, the *E. radicincitans* strain has both specific regions, while all other bacteria do not have the *E. radicincitans*-specific sequence and can only hybridize with the bacterial Cy5 labelled probe. Then root segments were placed on a microscope slide, covered by water (molecular biology grade) and a coverslip, and observed under the laser scanning microscope 510 META.

Microscopy

After in situ PCR + probe-hybridization, bacteria and root segments were observed using the laser scanning microscope 510 META (Zeiss, Germany) and a Zeiss Plan-Apochromat 63×/1.4 oil objective. The online emission fingerprinting, which is based on the initial recording of the complete complex emission signals originated from the two differentially labelled samples and the root autofluorescence, was applied to separate fluorescence emissions in the root segments. Online emission fingerprinting was performed in three steps: (1) recording, (2) analysis and (3) separation of emission signals in multifluorescence imaging. For step (1), the fluorescence spectra of 6-FAM-labelled *E. radicincitans* cells, Cy5-labelled *Pseudomonas* cells (as a representative of the bacterial group) and the root autofluorescence spectrum were acquired separately as a lambda stack representing the complete spectral distribution of the fluorescence signals of the labelled cells and the root. In step (2), these spectra, characterizing the spectral emission properties for the three targets (*E. radicincitans* cells, bacterial cells and root cells) were selected as references and saved in the LSM 510 META software database. In step (3), these three reference spectra were tracked simultaneously on root samples hybridized in the in situ PCR. FAM and Cy5 fluorescence spectra were recorded on the root surface and in serial optical cuttings throughout the entire root (100 µm in diameter) using the online emission fingerprint mode within a Z-stack of 2.3 µm slices.

The EUB388 Cy5-labelled spectrum and the root autofluorescence spectrum were recorded after an excitation of 633 nm; the 6-FAM-labelled *E. radicincitans* spectrum was recorded after an excitation at 488 nm.

Statistics

Comparison of mean values of three or four replicates was performed using ANOVA analysis and Tukey's HSD test at a *P*-level of ≤5% (STATISTICA 6.0) (StatSoft 2001).

The 16S rRNA gene sequence for *E. radicincitans* is deposited at the GenBank, Accession No. AY563134. The bacterial strain *Enterobacter radicincitans* is deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen as DSM 16656^T and in the Collection Institut Pasteur as CIP 108468^T.

Results

Plant growth promotion

The inoculation of young *Brassica oleracea* plants with *Enterobacter radicincitans* (10^9 cells per plant) induced a significant increase in growth of all plant parts, i.e. leaves, roots and the emerging tubers as determined 6 weeks after inoculation (Table 2). Largest growth stimulating effects after *E. radicincitans* application were measured in the root system, where inoculated plants had 47% larger root dry matter production than non-inoculated control plants. Dry matter of leaves and emerging tubers increased by 35% and 37% over the control, respectively.

E. radicincitans quantification in *Brassica oleracea* tissues

The combined utilization of the *E. radicincitans* species-specific TaqMan™ assay and QuantiTect™ Probe PCR Kit (QIAGEN) allowed to quantify a target gene copy number in original isolated plant DNA samples without the need to dilute the samples. In our real-time PCR set up, pure DNA extracts did not inhibit the PCR. 10- and 100-fold diluted samples exactly quantified 10- and 100-fold lower copy numbers (data not shown).

The calibration curve, used to quantify target gene copy numbers in unknown samples, was generated from the specific PCR product of the *E. radicincitans* DNA and was adjusted within a range of nine 10-fold dilutions from 5.3×10^9 to 5.3 copies per µl DNA. Eight 10-fold dilutions were measurable down to 53 copies μl^{-1} DNA. This minimum copy number produced a fluorescence signal over the background at 44 PCR cycles.

Table 2 Root, leaf and tuber dry matter (dm) of *Brassica oleracea* plant, 6 weeks after *E. radicincitans* inoculation (10^9 cells per plant, greenhouse pot experiment)

Plant part	Control	*E. radicincitans*	% increase
Root	1.88 a	2.76 b	47
Leaves	1.80 a	2.44 b	36
Tuber	0.38 a	0.52 b	37

Tukey's HSD test at a *P*-level of ≤5%, different letters within a row indicate significant differences

Since DNA isolation from plant material can differ from one sample to the next a housekeeping gene was used as an internal reference. Using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen 2001) the increase (or decrease) of the target gene in the inoculated sample relative to the non-inoculated control sample and normalized to the abundance of the reference gene was calculated. One day after inoculation with 10^7 to 10^9 bacterial cells per plant, *E. radicincitans* 16S rDNA-gene copy numbers on the leaves were observed to be 2- to 2062-fold increased over the non-inoculated control. Up to 13-fold increases in the highest inoculum concentration treatment over the control was also seen in root tissues 1 day after inoculation (Table 3). Transferring these measured relative 16S rDNA-gene copy numbers to *E. radicincitans* bacterial cell numbers (two 16S rDNA-gene copies per cell and measured DNA concentration extracted per g plant material), a cell density of 3.9×10^4, 5×10^5 and 1.8×10^7 cells per gram leaf fresh weight was detected in the treatments inoculated with 10^7, 10^8 and 10^9 cells per plant, respectively.

To assess whether the introduced bacteria will survive or even proliferate on the plant and also to ascertain where these bacteria colonize, both roots and leaves were sampled again 14 days after inoculation. Results, shown in Table 3, clearly show a proliferation of introduced *E. radicincitans* on and inside the roots of all inoculated treatments 14 days after inoculation. The inoculated cells were analysed to have increased up to 10,000-fold over the non-inoculated control at this time, while the introduced bacteria did not survive in *Brassica oleracea* leaves (Table 3). The 10,000-fold increase on the roots corresponded to about 3×10^7 *E. radicincitans* cells per gram root fresh weight after 14 days.

Bacterial quantification using real-time PCR

To assess the level of competition between the introduced bacteria and the native bacterial community, the amount of total bacterial cells colonizing the *Brassica oleracea* roots and leaves were analysed from the same DNA samples used

Table 3 Relative quantification of *E. radicincitans* and of total bacteria normalized to the reference TEF-gene in roots and leaves 1 day and 14 days after *E. radicincitans* inoculation of 10^7, 10^8 and 10^9 cells per plant

Plant part	Treatment	Sampling time (d)	*E. radicincitans*	Bacteria
Leaves	10^7	1	2.07 ± 0.103	2.16 ± 0.11
	10^8	1	367.09 ± 18.3	4.47 ± 0.22
	10^9	1	2126427.15 ± 106321.36	0.15 ± 0.07
	10^7	14	0*	9.38 ± 0.47
	10^8	14	0*	4.47 ± 0.21
	10^9	14	0*	11.71 ± 0.58
Roots	10^7	1	1.30 ± 0.06	1.93 ± 0.09
	10^8	1	3.55 ± 0.17	0.96 ± 0.05
	10^9	1	12.99 ± 0.65	0.66 ± 0.03
	10^7	14	5556.65 ± 261.16	4.75 ± 0.24
	10^8	14	10960.30 ± 548.01	3.36 ± 0.17
	10^9	14	5556.65 ± 277.83	7.56 ± 0.38

Shown as *x*-fold increase of *E. radicincitans* and bacteria, respectively, in the inoculated treatment sample relative to the non-inoculated control sample and normalized to the reference gene (mean values of three replicates ± standard deviation)

*Below detection limit of 54 copy numbers per µl

for the *E. radicincitans* 16S rDNA-gene copy number and TEF-gene copy number quantification. The results show a minimal increase in the total bacterial colonization on leaves and roots of the inoculated treatments compared to the non-inoculated control (Table 3). These elevated total bacterial cell numbers in inoculated treatments over the non-inoculated control could be due to the bacterial cell numbers added with the *E. radicincitns* application.

The much larger increase of the *E. radicincitans* cells compared to the total bacterial cells in roots within the inoculated treatments indicate a successful colonization of the introduced bacteria and their competitiveness on the native bacterial community.

Localization of *E. radicincitans* and native bacteria in *Brassica oleracea* root segments

Localization was performed using the online emission fingerprinting at the LSM 510 META in conjunction with fluorescently labelled oligonucleotide probes. After cutting the root into 2.3 µm optical slices from the root surface throughout the cortex, endodermis to the vascular bundles, strongest emission signals originated from bacterial Cy5 labelled probes were recorded on the root surface and in the root cortex layer down to 14 µm below the root surface (blue signals in Fig. 1). This shows a bacterial colonization of the root surface and the root cortex. FAM-labelled signals originating from the introduced and native *E. radicincitans* cells, were detected in largest amounts within the root cortex and endodermis from 6.9 µm down to 23 µm below the root surface (green signals in Fig. 2). On the root surface, the *E. radicincitans* emission signals were lower than in the intercellular spaces of the root cortex and endodermis.

The inoculated *E. radicicncitans* cells successfully competed against the native bacterial community and colonized in nearly the same sites, mainly in the intercellular spaces of the root

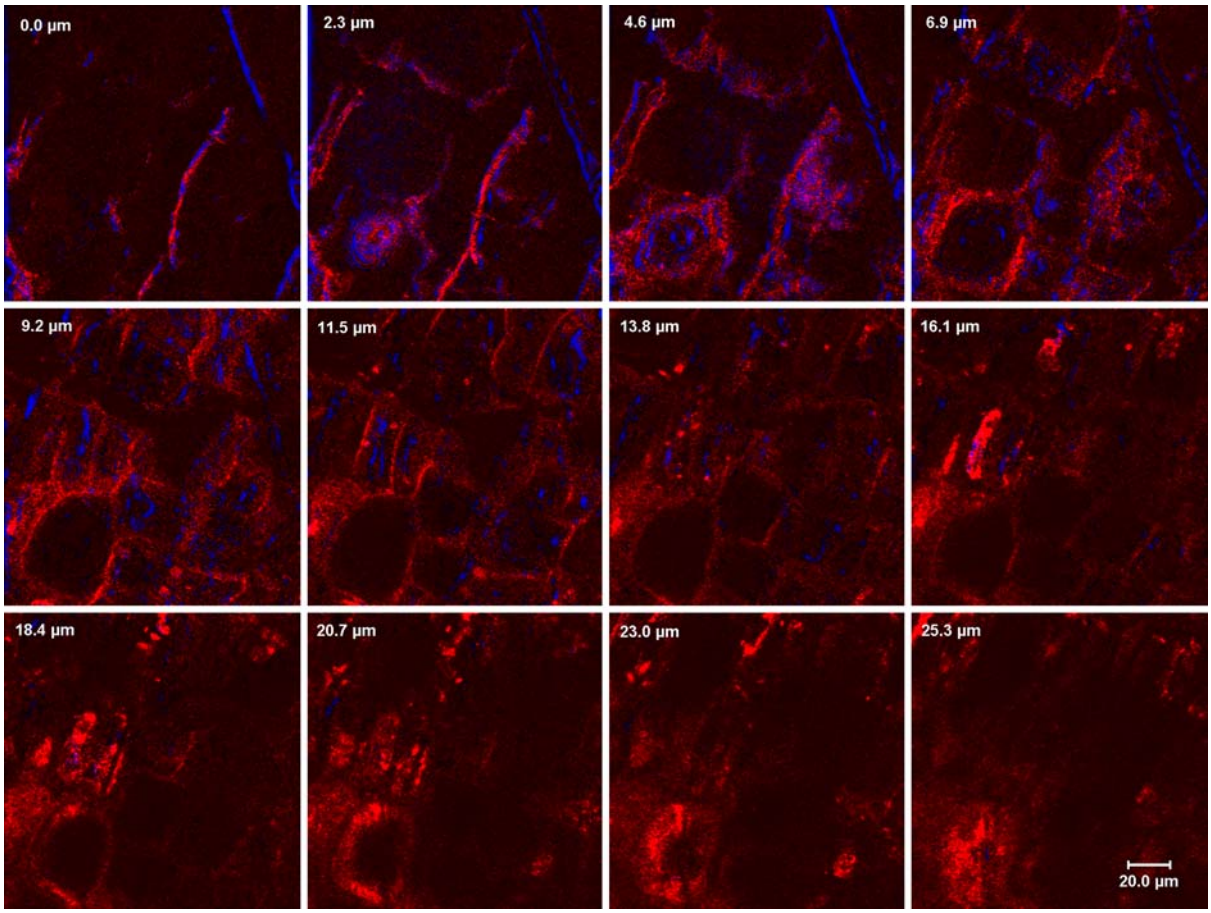

Fig. 1 Results of Cy5 labelled probe detection, specific for bacterial cells, within root tissues after cutting the *Brassica oleracea* root into 2.3 µm optical slices from the root surface throughout the cortex, endodermis to the vascular bundles till 25.3 µm in depth. Numbers indicate the depth of the optical cutting in µm and bars equals 20 µm

cortex cells (Fig. 3). In an optical cut at 13.7 µm below the root surface, divided into two recorded spectra, the root (Fig. 3A) and the Cy5 signal specific for the bacterial cells (Fig. 3B) are shown separately. The bacterial colonization of intercellular spaces are shown in Fig. 3C (marked by arrows). The next three pictures (Fig. 3D–F) show the same optical cut and spectra records for the root, the FAM labelled *E. radicincitans* (Fig. 3E) and the *E. radicincitans* colonization of intercellular spaces marked by arrows in Fig. 3F. In this root cortex layer some of the intercellular spaces were colonized predominantly by *E. radicincitans* cells (Fig. 3C, F).

Corroborating the real-time PCR data (Table 3), the emission fingerprinting (Fig. 3) also documented that the inoculated *E. radicincitans* cells represented a significant percentage of the total bacterial cells in the root of *Brassica oleracea* plants 1 day after bacterial inoculation of 10^9 cells per plant. *E. radicincitans* cells colonized nearly all visible intercellular spaces in the root cortex (Fig. 3F).

Discussion

Plant growth promotion

The PGPR strain *Enterobacter radicincitans*, used in this study, is known to improve plant growth and yield of various agricultural and vegetable plants such as winter wheat, corn or beans and to be quite stable in its plant growth-promoting

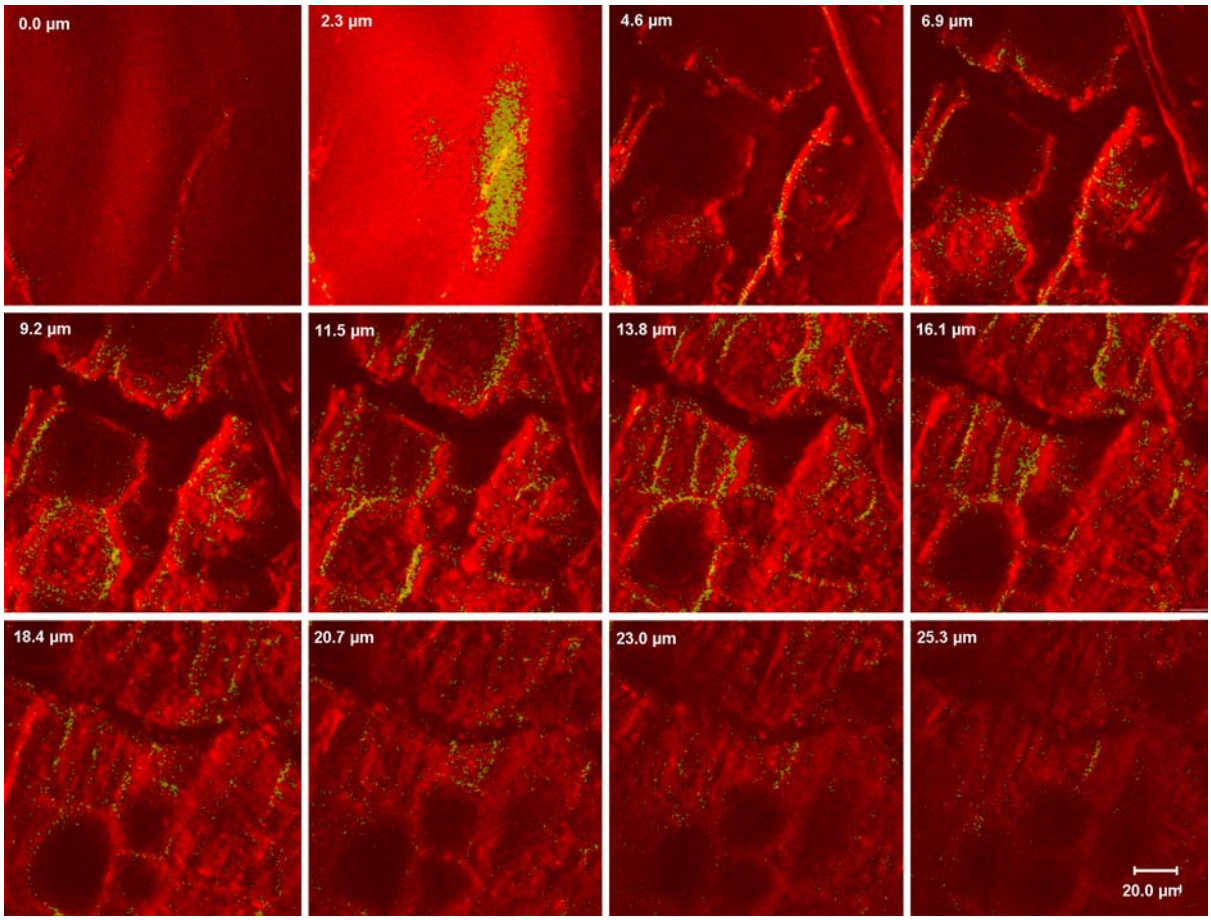

Fig. 2 Results of FAM labelled probe detection, specific for *E. radicincitans* cells, within root tissues after cutting the *Brassica oleracea* root into 2.3 µm optical slices from the root surface throughout the cortex, endodermis to the vascular bundles till 25.3 µm in depth. Numbers indicate the depth of the optical cutting in µm and bars equals 20 µm

effects (Höflich et al. 1992; Kämpfer et al. 2005; Ruppel et al. 1999). While the plant growth-promoting effects of various different bacterial strains have been well investigated in pure culture (see Dobbelaere et al. 2003 for a review), the mechanism by which the bacteria increase, e.g. the root surface area, plant growth or yield, is very poorly understood. It is still questionable, whether the plant growth promotion is caused by the inoculated strain itself, via repression of native harmful organisms or by an induced shift within the native microbial rhizosphere or phyllosphere population. First results showing that *Azospirillum brasilense* cells inoculated to wheat roots express the key gene for auxin biosynthesis using ipdC-gfp operon fusion constructs were shown by Rothballer et al. (2005). A localization of introduced *Azospirillum brasilense* Wa3 bacterial cells using strain-specific monoclonal antibodies and native bacterial species using rRNA-targeted oligonucleotides and fluorescence in situ hybridization (FISH) was reported by Aßmus et al. (1997). Lübeck et al. (2000) detected inoculated *Pseudomonas fluorescens* DR54 cells on sugar beet root surfaces using immunofluorescence technique and tested the FISH technique to monitor the native bacterial population. Both groups and also Franke et al. (2000) had difficulties to detect low bacterial cell densities using oligonucleotide probes in plant samples. This is probably due to the low ribosome content of some bacterial cells. Therefore, in our

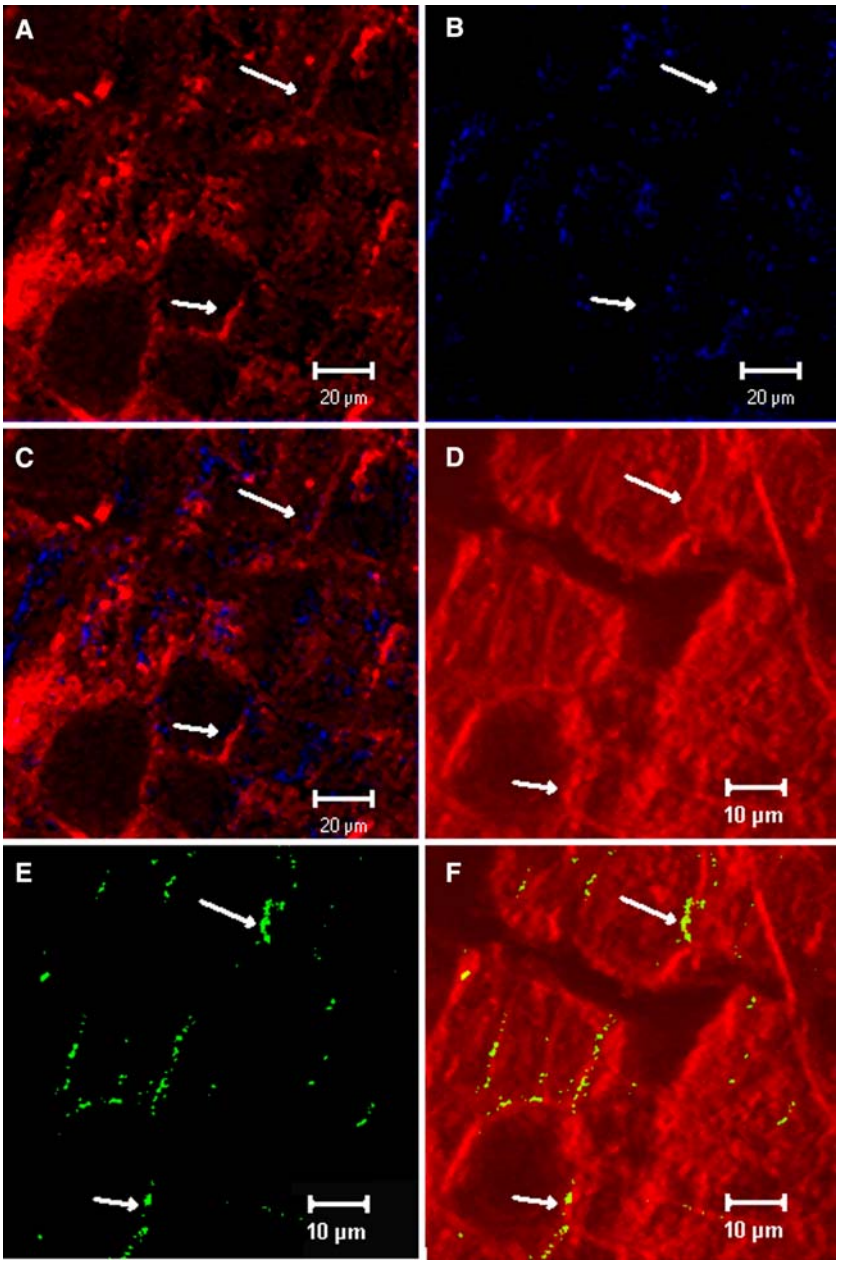

Fig. 3 Optical slice 13.7 µm below the root surface within the root cortex layer. Pictures acquired by online emission fingerprinting of the Cy5 labelled bacterial spectrum, the FAM labelled *E. radicincitans* spectrum and the root autofluorescence spectrum, documented after linear unmixing of Cy5 spectrum from root fluorescence (**A**, root signal; **B**, bacterial signals; **C**, both together) and after linear unmixing the FAM spectrum from root signals (**D**, root signal; **E**, *E. radicincitans* FAM labelled signals; **F**, both together). *E. radicincitans* colonization of intercellular spaces marked by arrows

experiment we inserted an in situ PCR step before probe-hybridization which increased the fluorescent signal to a detectable level. Additionally, the LSM 510 META system enables the exclusion of interfering autofluorescence from the plant tissue by searching for specific fluorescence spectra of labelled probes using the online emission fingerprinting. Besides excluding the strong autofluorescence signals, the advantage of emission fingerprinting is that it can separate even signals with strongly overlapping emission profiles as found in this study. Although, only 7 nm distance between both peak maxima were observed, the two spectra were easily detected and separated from each other in the root tip sample. Since the introduced PGPR strain and the native bacterial population were monitored in parallel using identical labelling methods, the competition of introduced cells for colonization spaces was clearly shown. The *E. radicincitans* strain colonized

intercellular spaces deeper in the root cortex and endodermis and they are more intense than the native bacterial community. For the first time we observed a replacement of native bacterial cells by introduced bacterial cells in intercellular spaces of the root cortex.

E. radicincitans strain and bacterial quantification

In this short term study, which was conducted to demonstrate the applicability of the new techniques, it was shown, that both quantitative real-time PCR techniques, i.e. a bacterial species-specific TaqMan™ probe and the SybrGreen I approach, were powerful methods to quantify *E. radicincitans* and a wide range of native bacteria in *Brassica oleracea* leaf and root samples. Using the housekeeping TEF-gene as an internal control allowed reliable quantification of target gene copy numbers for different treatments and plant parts. Thus, we were able to measure the changes in cell density of the introduced *E. radicincitans* cells in root and leaf samples.

The calibration curves of all three real-time PCR measurements were linear with between 10 and 10^9 target-gene copies μl^{-1} with correlation coefficients of 0.99 and 1.00. Hence, it was possible to measure the target-gene copy numbers within a wide range and no sample dilution was necessary to compare treatments with extremely large and low target-gene copy numbers. These results clearly demonstrate the possibility to enumerate target 16S rDNA-gene copy numbers of bacteria in plant tissues in original undiluted DNA samples.

The detection limit of *E. radicincitans* in plant material was estimated to be 2.7×10^4 16S rDNA-gene copy numbers g^{-1} plant fresh weight. Using the TaqMan™ probe for the *E. radicincitans* quantification has two advantages: (1) the specificity was improved and (2) the detection limit of 53 16S rDNA-gene copies μl^{-1} DNA or 2.7×10^4 16S rDNA-gene copies 1 g^{-1} plant material was 10-fold lower than the one achieved with the SybrGreen I approach. Hein et al. (2001) also obtained similar improvements using the TaqMan™ specific probes to quantify *Staphylococcus aureus* cells in cheese.

The advantage of quantitative real-time PCR is that it has been mostly automated and also offers the possibility to measure many different genes of interest in one sample by multiplexing or using parallel sets (Boeckman et al. 2000a, b). Currently, comparable results to quantify bacteria within environmental samples can only be achieved by time consuming and complex methods such as fluorescence in situ hybridization (FISH) combined with digital imaging analysis (Daims et al. 2001) and ELISA (enzyme linked immunosorbent assay) applications using target-specific antibodies (Ferguson et al. 2000). However, FISH with subsequently microscopic quantification were used only for sludge (Daims et al. 2001; Snaidr et al. 1997), soil (Chatzinotas et al. 1998), sediments (Liobet-Brossa et al. 1998) or ectomycorrhizospheres (Mogge et al. 2000) and have not yet shown to give quantitative results in plant analysis. Lübeck et al. (2000) firstly analysed images of FITC-labelled antibodies of *P. fluorescens* DR54 on sugar beet root surfaces and quantified these. They showed active, root-colonizing cells of *P. fluorescens* on root surfaces. However, the direct quantitative relationship between *P. fluorescens* and the native bacterial community could not be measured since two different methods were applied—immunofluorescence for the introduced *P. fluorescens* strain and FISH for the native bacterial population.

In this study, bacterial cell numbers measured using quantitative real-time PCR ranged between 10^7 and 10^9 cells g^{-1} fresh leaf weight of *Brassica oleracea* plants (based on the rough calculation of 7 operons of 16S rDNA present in bacterial cells and the known DNA amount isolated from 1 g fresh plant material) were 1–2 orders of magnitude larger than maximal epiphytic bacterial populations reported to be between 10^6 and 10^7 CFU g^{-1} fresh weight (Beattie and Lindow 1994; Hirano and Upper 1995). However, the latter numbers were achieved by cultivation methods, whereas ours are based on culture-independent procedures. Even if we calculate the bacterial cell numbers on the basis of 1 or 10 16S rDNA operon numbers which are known to occur in bacterial cells (The Ribosomal RNA Operon Copy Number Database, http://rrndb.cme.msu.edu), our measured bacterial cell numbers still are 1–2 orders

of magnitude larger than published epiphytic culturable populations. This comparison is in agreement with other studies that showed that only 1–10% of the total bacterial community are culturable (Ovreas and Torsvik 1998; Rosado et al. 1997; Torsvik et al. 1996).

Bacterial cell numbers of root samples (about 10^8 cells g^{-1} root fresh weight) are in the same order of magnitude as published by Stubner (2002) measured with the same culture-independent real-time PCR technique. Culturable bacterial cell numbers in the rhizosphere can range between 10^6 and 10^8 cells g^{-1} fresh root weight (Gardener and Weller 2001; Perebityk and Puchko 1989).

The inoculated *E. radicincitans* strain can successfully compete with the native bacterial community in plant tissues without inducing defence mechanisms of the plant. This phenomenon may constitute a part of its plant growth-promoting mechanisms. In earlier studies using electron microscopy, we demonstrated that inoculated wheat plants do not activate a defence mechanisms against the introduced *E. radicincitans* cells and that these bacteria were able to move and grow in the wheat endorhizosphere and phyllosphere (Ruppel et al. 1992). However, in these earlier studies no information about the native microflora was obtained. In general, only few results about the relationship between introduced bacterial cells and total bacterial numbers in plant tissues have been published. Stubner (2002) detected only 0.5–2% of the target Gram-positive sulphate reducers in rice root samples, but these were native organisms and not inoculated ones. Generally, experiments with direct inoculation of PGPR strains to plants exhibit population densities between 10^4 and 10^6 cells of the respective PGPR strain per g fresh root weight 2 weeks after inoculation (Burdman et al. 2000; James et al. 2002). However, the proportion of these bacteria to the native bacterial community was not determined.

Our initial results, obtained in quartz sand conditions, indicate high competitiveness and proliferation ability of the inoculated *E. radicincitans* strain on and inside *Brassica oleracea* roots. In roots, *E. radicincitans* represent 10–16% of the bacterial community 14 days after inoculation irrespective of inoculum density. That level was achieved independently of how much bacteria were inoculated. Such an active colonization ability offer an enormous advantage in biofertilizer applications if this competitiveness will be preserved in soil conditions. This has to be studied in further experiments.

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