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Indigenous Populations of Three Closely Related *Lysobacter* spp. in Agricultural Soils Using Real-Time PCR

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Abstract Previous research had shown that three closely related species of *Lysobacter*, i.e., *Lysobacter antibioticus*, *Lysobacter capsici*, and *Lysobacter gummosus*, were present in different *Rhizoctonia*-suppressive soils. However, the population dynamics of these three *Lysobacter* spp. in different habitats remains unknown. Therefore, a specific primer–probe combination was designed for the combined quantification of these three *Lysobacter* spp. using TaqMan. Strains of the three target species were efficiently detected with TaqMan, whereas related non-target strains of *Lysobacter enzymogenes* and *Xanthomonas campestris* were not or only weakly amplified. Indigenous *Lysobacter* populations were analyzed in soils of 10 organic farms in the Netherlands during three subsequent years with TaqMan. These soils differed in soil characteristics and crop rotation. Additionally, *Lysobacter* populations in rhizosphere and bulk soil of different crops on one of these farms were studied. In acid sandy soils low *Lysobacter* populations were present, whereas pH neutral clay soils contained high populations (respectively, <4.0–5.87 and 6.22–6.95 log gene copy numbers g^{-1} soil). Clay content, pH and C/N ratio, but not organic matter content in soil, correlated with higher *Lysobacter* populations. Unexpectedly, different crops did not significantly influence population size of the three *Lysobacter* spp. and their populations were barely higher in rhizosphere than in bulk soil.

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Introduction

The genus *Lysobacter* is known for its strong lytic activities as well as production of secondary metabolites with antibiotic activity [26]. Several *Lysobacter* isolates have been described as potential biocontrol agent of a variety of plant pathogenic fungi, oomycetes, and bacteria [7, 9, 10, 14, 25]. The increased interest in this genus was followed by the isolation and description of several new species. Most recently, the presence of *Lysobacter* spp. in soil was correlated with disease suppression of *Rhizoctonia solani* [23, 24]. These suppressive soils contained members of three closely related species of *Lysobacter*, i.e., *Lysobacter antibioticus*, *Lysobacter capsici*, and *Lysobacter gummosus*. However, details about occurrence and population dynamics of *Lysobacter* spp. remain unknown.

A suitable selective medium is not available for *Lysobacter* spp. In early studies, *Lysobacter* spp. were determined as plaque-forming units (PFU) using media with cyanobacteria. Abundance in natural habitats was relatively low (500 PFU cm^{-3} soil, 400 PFU ml^{-1} freshwater) [26]. An extensive search for antagonistic bacteria, using an agar medium together with a fungus, showed the presence of antagonistic *Lysobacter* species in several soils [24]. Population densities of introduced *Lysobacter enzymogenes* strains were determined on plant roots in biocontrol experiments with plate counts and immunofluorescent colony staining [4, 13] and more recently by real-time PCR detection [19]. In several other studies, reviewed by Hayward et al., only one or few *Lysobacter* isolates were found in various niches (soil, rhizosphere, compost water, sludge, salamander skin) of different continents (Europe,

Asia, and North America) [7], showing the general appearance of *Lysobacter* but not giving any quantitative information.

Quantitative information about the indigenous *Lysobacter* spp. in soil is scarce and factors influencing the population density are unknown. Now that *Lysobacter* spp. are gaining ecological importance, as potential biocontrol agents [7] as well as being involved in natural disease suppression [24], several key questions about the presence and abundance of *Lysobacter* populations arise. Is their population size influenced by soil factors, such as soil type, pH, texture, or organic matter? What is the influence of crop species, rotation, or agricultural measures? Have *Lysobacter* spp. preferred niches in soil, i.e., is their population size enhanced in rhizosphere soil in the vicinity of plant roots? A specific detection method to quantify *Lysobacter* populations in different habitats is required to answer such ecological key questions.

Currently, quantitative molecular detection techniques are increasingly applied to specifically quantify bacterial or fungal strains, species, genera, or larger taxonomic entities [15, 16, 19, 20, 27]. Meanwhile, recent improvements in extraction methods for DNA or RNA and the sufficient removal of inhibitory compounds with easy-to-use commercial kits has also made these techniques suitable for application on complex substrates such as soil.

This paper describes the design and application of a specific primer–probe combination for quantifying the three closely related species *L. antibioticus*, *L. capsici*, and *L. gummosus* simultaneously in one TaqMan system. The detection method was applied to assess the *Lysobacter* populations in soils of arable fields at different locations within the Netherlands during three subsequent years. These soils differed in soil characteristics and crop rotation. To study the influence of different crops on *Lysobacter* communities, rhizosphere and bulk soil, samples were taken from different crops on one farm where *Lysobacter* was known to be present. These samples were subjected to DNA and RNA analyses to compare total and active community sizes.

Methods

Cultivation of and DNA Extraction from *Lysobacter* Strains

Lysobacter strains were derived from the culture collection of Plant Research International (Table 1). Four type strains were obtained from DSMZ (Braunschweig, Germany) and two strains of *L. enzymogenes* were kindly supplied by D.Y. Kobayashi (Rutgers University, New Bunswick, NJ, USA) and G. Yuen (University of Nebraska, Lincoln, NE, USA). Genetic affiliation of the three target species is shown in Fig. 1. The strains, stored at −80°C in 10% glycerol, were cultured on nutrient poor agar medium R2A (BD Laboratories, Franklin Lakes, NJ, USA). The inoculated R2A plates were incubated 48 h at 25°C. Single colonies were picked and resuspended in 5 ml 1/10 strength Trypticase Soy Broth (TSB, BD Laboratories) and incubated 2 days at 25°C under rotation at 200 rpm. Genomic DNA was isolated from the cultures using the MasterPure™ Complete DNA Purification Kit (Epicentre Biotechnologies Madison, WI, USA) according to the manufacturer's instructions. DNA was further purified using Wizard DNA clean-up (Promega, Madison, WI, USA) because of excess polysaccharides. To confirm product integrity and to estimate yield, DNA preparations were visualized by using standard 1.5% (*w*/*v*) agarose 0.5× TBE gel electrophoresis with ethidium bromide staining.

Design of Primers and Probe for Real-Time PCR Detection of *L. gummosus*, *L. antibioticus*, and *L. capsici*

Beacon designer 5.0 software (Premier Biosoft, Palo Alto, CA, USA) was used to design the primers and probe for the simultaneous detection of *L. gummosus*, *L. antibioticus*, and *L. capsici* based on their 16S rRNA sequences. *L. enzymogenus*, a closely related species, was used as non-target. Sequences of four strains of different species derived from the culture collection of Plant Research International, as well as sequences of the corresponding type strains were used for this purpose (Fig. 2). To increase hybridization specificity and enhance the probe discrimination between single nucleotide polymorphisms, a Locked Nucleic Acid (LNA™) dual label probe was developed (P_Lyso_guanx1-5′ TGACATCCACGGAACTTTC 3′, underlined letter is a LNA base), labeled at 5′ end with the fluorescent dye 6-FAM and at 3′ end with the quencher BHQ1. Following the probe design, a selective reverse primer (Rv_Lyso_guanxl-5′ TGCAGCACCTGTCTCAC 3′) and a non-selective forward primer (Fw_Lyso_guanxl-5′ CAACGCGAA GAACCTTACC 3′) were designed using FastPCR V3.7.43 (University of Helsinki, Finland). Figure 2 depicts the primer and the probe sequences developed to detect *L. antibioticus*, *L. capsici*, and *L. gummosus* for real-time PCR. The probe and the primers used in this study were synthesized by Biolegio (Nijmegen, the Netherlands).

Specificity of the primer–probe combination was tested in silico with Visual OMP (DNA software, Ann Arbor, MI, USA) by comparing the theoretical 89-bp product of the probe and primer combination with sequences retrieved from public databases. In addition, specificity of this primer–probe combination was tested in duplicate with the TaqMan assay on DNA of in total 29 target and six non-target strains (Table 1).

Table 1 Isolates used in this study

Species	Isolate nr.	EMBL Accession	Origin	Reference
Lysobacter antibioticus	3.2.10	AM941209	Bulk soil; Pietersbierum, NL	[24]
L. antibioticus	1.4.11	AM941210	Bulk soil; Strijen, NL	[24]
L. antibioticus	4.1.2	FR822759	Bulk soil; Marknesse, NL	[24]
L. antibioticus	4A.2.4	FR822760	Bulk soil; Marknesse, NL	[24]
L. antibioticus	9.4.19	AM941219	Bulk soil; Strijen, NL	[24]
L. antibioticus	11.2.4	AM941208	Bulk soil; Marknesse, NL	[24]
L. antibioticus	12.3.1	AM941206	Bulk soil; Engwierum, NL	[24]
L. antibioticus	12.4.1	AM941218	Bulk soil; Marknesse, NL	[24]
L. antibioticus	66	FN398325	Bulk soil; Zwaagdijk, NL	[23]
L. antibioticus	76	FR822755	Bulk soil; Zwaagdijk, NL	[23]
L. antibioticus	156	FN398326	Potato; Marknesse, NL	[31]
L. antibioticus	DSM 2044^T	AB019582	Culture collection	
L. capsici	1.3.3	FN357195	Bulk soil; Strijen, NL	[22]
L. capsici	6.2.3	FN357196	Bulk soil; Hoensbroek, NL	[22]
L. capsici	10.4.5	FN357197	Bulk soil; Marknesse, NL	[22]
L. capsici	55	FN357198	Bulk soil; Zwaagdijk, NL	[22]
L. capsici	177	FN398327	Potato; Marknesse, NL	[31]
L. capsici	YC5194^T (DSM 19286^T)	EF488749	Culture collection	
L. gummosus	3.2.11	FN600120	Bulk soil; Pietersbierum, NL	[24]
L. gummosus	1A.1.1	FR822756	Bulk soil; Engwierum, NL	[24]
L. gummosus	1A.1.10	FR822757	Bulk soil; Engwierum, NL	[24]
L. gummosus	1A.1.13	FR822758	Bulk soil; Engwierum, NL	[24]
L. gummosus	2.4.7	AM941212	Bulk soil; IJzendijke, NL	[24]
L. gummosus	3.3.1	AM941214	Bulk soil; Pietersbierum, NL	[24]
L. gummosus	5.1.5	FR822761	Bulk soil; Hensbroek, NL	[24]
L. gummosus	10.1.1	FR822762	Bulk soil; IJzendijke, NL	[24]
L. gummosus	11.3.5	AM941213	Bulk soil; Marknesse, NL	[24]
L. gummosus	13.3.12	AM941220	Bulk soil; Pietersbierum, NL	[24]
L. gummosus	DSM 9680^T	AB161361	Culture collection	
L. enzymogenes	3.1T8	AM930380	Cucumber rhizosphere; NL	[5]
L. enzymogenes	1.1.4	FN398321	Bulk soil; Bakel, NL	[19]
L. enzymogenes	C3	AY074793	Turfgrass leaf	[29]
L. enzymogenes	N4.7	U89956	Agricultural soil	[29]
L. enzymogenes	DSM 2034^T	AJ298291	Culture collection	
Xanthomonas campestris pv. *campestris*	ATCC 33913^T	AE012505	Culture collection	

Real-Time PCR Procedure

TaqMan assays to detect *L. antibioticus*, *L. capsici*, and *L. gummosus* were performed in 96-well plates with 3-µl samples in a total volume of 25 µl. Reaction mixtures contained 12.5 µl 2× Premix Ex Taq and 0.5 µl ROX reference Dye (both Takara Bio Inc, Otsu, Japan), 600 nM forward and reverse primer, and 100 nM FAM-labeled probe. Amplification reactions were performed on a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Amplification was carried out 15 s at 95°C and 60 s at 60°C for 40 cycles. Data analysis was carried out with Sequence Detection System software (Applied Biosystems).

Size of the PCR products of several samples was checked on a 2% Agarose gel (Tebu-Bio, Le Perray en Yvelines, France).

Quantification of *Lysobacter* spp. and Total Bacterial Populations

Standard curves for quantification of *Lysobacter* spp. were made from genomic DNA of the target strains *L. antibioticus* 3.2.10, *L. capsici* 1.3.3, and *L. gummosus* 3.2.11 described

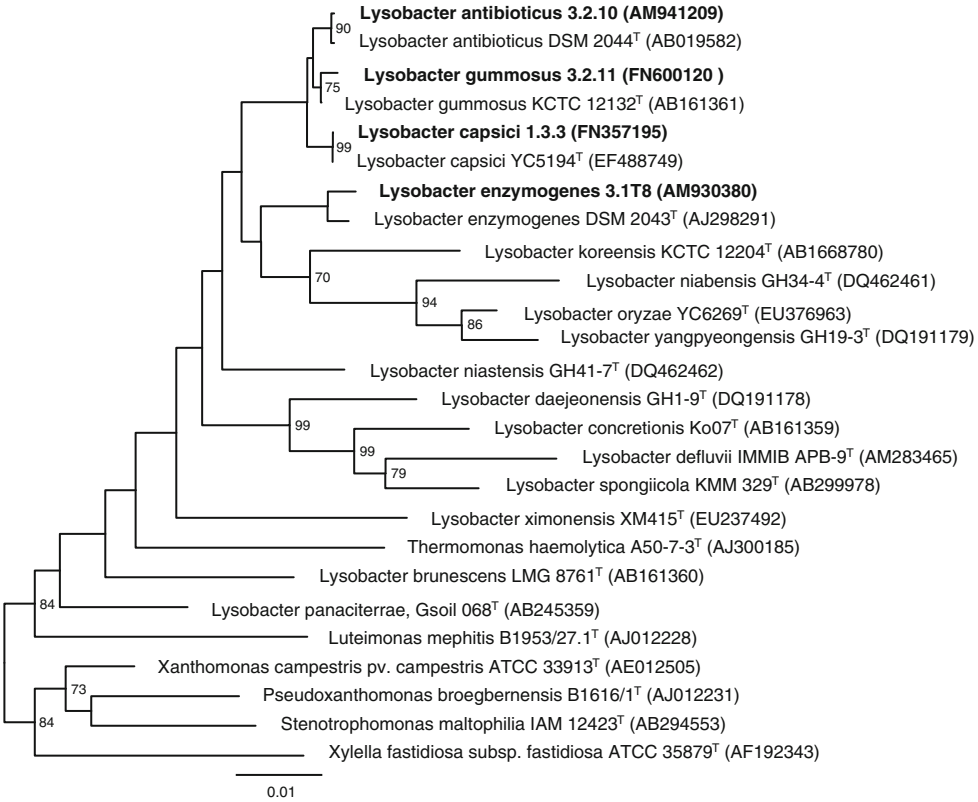

Figure 1 Phylogenetic tree constructed using 16S rRNA gene sequences available in GenBank database employing the neighbor-joining method. The three *Lysobacter* target strains 1.3.3, 3.2.10, and 3.2.11 and the non-target strain 3.1T8 are presented in *bold*; type strains of *Lysobacter* and related other species are presented with their strain number and the accession number in *parentheses*. *Numbers* at the nodes indicate percentage bootstrap values, >70%, from 1000 resamplings of the data. *Scale bar* presents sequence difference

in Table 1. Purified DNA (described before) was quantified using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and the number of gene copies per microliter was calculated. Using tenfold increment, the standards concentrations were adjusted from 10^7 to 10^1 gene copies per microliter for each species. Real-time PCR was performed as described in the previous paragraph. The amplification efficiency (E) was

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                                                                  Fw_Lyso_guanxl •
La AM941209     AATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGA      898
La AB019582     ............................................................      964
Lc FN357195     ............................................................      941
Lc EF488749     ............................................................      955
Lg FN600120     ............................................................      932
Lg AB161361     ............................................................      958
Le AM930380     ...........................................................M...      963
Le AJ298291     ...........................................................C...      958

                                          P_Lyso_guanxl
La AM941209     ACCTTACCTGGTCTTGACATCCACGGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAAC      958
La AB019582     ...........................................................-..      1023
Lc FN357195     ..........C.....................T......A.....................      1001
Lc EF488749     ..........C.....................T......A.....................      1015
Lg FN600120     ..........C.....................................................      992
Lg AB161361     ..........C.....................................................      1018
Le AM930380     ..........C........GTCGA........................................      1023
Le AJ298291     ..........C........GTCGA........................................      1018

                ← Rv_Lyso_guanxl
La AM941209     CGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC      1018
La AB019582     ............................................................      1083
Lc FN357195     ............................................................      1061
Lc EF488749     ............................................................      1075
Lg FN600120     ............................................................      1052
Lg AB161361     ............................................................      1078
Le AM930380     TCGA.C......................................................      1083
Le AJ298291     TCGA.C......................................................      1078
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Figure 2 Alignment of 16S rRNA fragment of target species *L. antibioticus* (La), *L. capsici* (Lc), *L.gummosus* (Lg) and a related non-target species *L. enzymogenes* (Le). For each species the sequence of one isolate from the PRI collection as well as from the corresponding type strain was used. Location of primers (*dark grey box*) and TaqMan probe (*light grey box*) are marked

calculated by the formula: $E = 10^{(-1/\text{slope})} - 1$. Additionally, the standard curve from the target isolate *L. gummosus* was compared with a dilution series of DNA extracted from cells of the same isolate, which had been quantified by plate counting on R2A.

TaqMan reactions were performed on 3 µl DNA preparations from soil samples which were diluted 10 or 50 times to avoid inhibition. A standard curve was included in every TaqMan run. Measurements of negative (undetermined) samples were repeated. Data analysis was carried out with the Applied Biosystems software; the baseline was set automatically. From the obtained cycle threshold (C_t) values from the DNA dilution series and the DNA extracts of soil samples, the gene copy numbers of the target organism were calculated per gram of soil sample.

Inhibition of TaqMan reactions with DNA preparations of soil samples (10 or 50 times diluted) was tested in separate TaqMan runs with the *gfp*-coding gene assay developed as internal control by Klerks et al. [12].

The total bacterial population in soil samples was quantified with real-time PCR (SYBRGreen) following the method of Fierer et al. [3]. Reactions were performed on 3 µl DNA preparations from soil samples which were diluted 100 times.

Recovery of Inoculated *L. gummosus* Cells from Soil by Plate Counting and TaqMan

L. gummosus was cultured in TSB during 2 days at 25°C. Inoculum (1×10^9 washed cells) or water (control) was added to soil portions (2 g) of a clay (farm A) and a sandy soil (farm F) (three replicates per soil type). Part of the treated soil was frozen (−20°C), part was analyzed directly for colony-forming units. For plate counts, soil was suspended by shaking 1 g soil in a 250 ml Erlenmeyer flasks with 10 g gravel (2–4 mm) and 99 ml 0.1% NaPP (tetra-sodium diphosphate) at 460 rpm for 10 min. Tenfold dilution series were made in 1/4-strength Ringers solution (one tablet Ringers (Oxoid) in 500 ml distilled water, autoclaved at 121°C) and plated in triplicate on R2A plates. Total bacteria and *Lysobacter* colonies (distinguished by its colony morphology) were enumerated after 2 days incubation at 25°C. DNA was extracted from frozen soil as described by Postma et al. [24] using a Mo Bio Ultra Clean soil DNA isolation kit (Mo Bio Laboratories, BIOzymTC, Landgraaf, the Netherlands) and analyzed by TaqMan as described in the previous paragraph.

Soil Samples of Different Locations

Arable fields of 10 organic farms at different locations within the Netherlands, having different soil characteristics and crop rotations, were sampled in August of 2004, 2005, and 2006 [24]. Sandy and marine clay soils were sampled in different regions of the Netherlands, in three and five different provinces, respectively (Fig. 3). These farms were part of a network with organic farmers [28]. From each field, four independent soil samples of 1.5 l were taken at 3–20 cm depth with minimally 10 m distance between each sampling site. In total, 144 soil samples (31 fields each with four replicates) were subjected to DNA extraction with the Mo Bio Ultra Clean soil DNA isolation kit and stored at −20°C until use.

Soil Samples of Different Crops from One Farm

Rhizosphere and bulk soil samples were taken from different crops on farm A (Engwierum, the Netherlands) in June 2008. Fields with a grass–clover mixture, red beet, onion, potato, and wheat were sampled in four replicates per field. Individual plants with adhering soil were harvested. Rhizosphere soil was defined as the soil that remained attached to roots after removal from field and gentle shaking. Remaining soil was defined as bulk soil. Subsequently, the rhizosphere soil was carefully removed from the roots with forceps and a spatula. These samples were subjected to DNA extraction.

September 2008, additional rhizosphere and bulk soil samples were taken on farm A in order to analyze DNA as well as RNA of the microbial community. Sampled crops were the grass–clover mixture, red beet, and pumpkin (onion, wheat and potato were already harvested). All soil samples were frozen immediately following sampling at

Figure 3 Location of sampled farms in different provinces of the Netherlands

field location with dry ice, transported to the lab, and stored at −80°C until DNA and RNA extraction.

Extraction of DNA and RNA from Soil Samples

Soil genomic DNA was isolated from 0.5 g (wet weight) rhizosphere or bulk soil using the Mo Bio Ultra Clean soil DNA isolation kit. The RNA isolation of soil samples was, from sampling until cDNA synthesis, performed under RNAse-free conditions. The working area and materials reserved for RNA handling were treated with RNAse decontamination solution (RNase*Zap*®, Ambion). Total RNA was extracted from 0.5 g (wet weight) using the RNeasy Mini Kit (Qiagen) according to the manufacturer's specifications using 900 μl instead of 450 μl Buffer RTL. Total RNA was resuspended in 50 μl of RNAse-free water. Total RNA was quantified using the NanoDrop ND-1000 Spectrophotometer (Bio-Rad Laboratories Inc., the Netherlands).

RNA samples were reverse-transcribed using Moloney Murine Leukemia Virus reverse transcriptase with low RNAse H activity (200 μ/μl, RevertAid™ First Strand cDNA Synthesis Kit, M-MuLVRT, Fermentas) using random hexaminer primers (0.2 μg/μl) according to the manufacture's protocol (RevertAid™ First Strand cDNA Synthesis Kit, Fermentas). The reaction mixture (20 μl) contained 5 μl of template RNA.

Populations of *L. antibioticus*, *L. capsici*, and *L. gummosus* in the soil samples were quantified in the DNA and cDNA extracts from rhizosphere and bulk soil samples using TaqMan as described previously.

Statistical Analysis

Statistical analyses were performed with GenStat Release 12.1 (PC/Windows XP) (Rothamsted Experimental Station, Harpenden, UK). Differences among populations of *Lysobacter* spp. in different soil samples were assessed using analysis of variance (ANOVA). Gene copy numbers of *Lysobacter* spp. were analyzed after logarithmic transformation. Negative TaqMan reactions were left out of the statistical analysis. After ANOVA, least significant differences were calculated at a significance level of $P=0.05$. Multiple regression analyses were performed on mean values per field with generalized linear models to analyze the influence of environmental factors on the *Lysobacter* population size.

Results

Specificity of the Primer–Probe Combination

All target strains without any exception reacted perfectly with the primer–probe combination; C_t values were in all cases between 12 and 18. The non-target of another genus (i.e., *Xanthomonas*) had a similar C_t value (36) as the water control. The strains of the closely related *L. enzymogenes* species reacted to some extent: C_t values were between 30 and 35. A 17-fold higher cycle threshold for this related species implies that only very high population numbers of this related non-target will interact with measurements of the target strains (17 cycles correspond with 2.5 10^5). Besides, *L. enzymogenes* was never isolated from one of the soils used in this study [24]

Quantification of *Lysobacter* spp.

Separate standard curves of the three target *Lysobacter* strains were evaluated in repeated TaqMan runs. The prepared standard curves were within the range of −3.1 to −3.6, corresponding with a PCR efficiency between 80% and 110%, and a recommended r^2 value of above 0.98 [1, 27]. Thus, all target strains showed appropriate reactions with real-time PCR. The standard curve prepared with *L. gummosus* showing an efficiency of 98%; slope, −3.38; and r^2, 1.00 was used throughout the study as standard.

Taking sample size and dilution steps into account, lowest measured gene copy numbers of *Lysobacter* spp. in soil samples with TaqMan were about 10^4; thus, 4 log g^{-1} soil is regarded as minimum detection limit.

Comparing the standard curve of *L. gummosus* with a dilution series of DNA template from freshly cultured cells of the same isolate, showed that the copy numbers were about 1.5–2 times higher than the cell numbers (data not presented). Regression analyses between both dilution series resulted in a significant fit ($F<0.001$): log (number of gene copies) = 0.07+1.05 log(CFU), accounting for 99.9% of the variance.

Presence of the 89-bp PCR products was confirmed for several different soil samples on agarose gel (results not shown).

Recovery of Inoculated *L. gummosus* Cells from Soil by Plate Counting and TaqMan

High numbers of *L. gummosus* cells were added to the soils to be able to enumerate the introduced bacteria among the indigenous populations. Recovered CFU in both soils were not statistically different with the inoculum. With TaqMan, 8.00 and 8.57 log *Lysobacter* cells g^{-1} soil were detected in sand and clay soil, respectively, compared to 8.65 log *Lysobacter* cells which had been introduced (Table 2). Thus, sandy soil showed a lower recovery than marine clay (23% compared to 86%). TaqMan values in the non-inoculated soils were comparable with the indigenous *Lysobacter* populations presented in Table 3.

Table 2 Recovery of *L. gummosus* cells after their introduction in two soil types with plate counts and TaqMan

Soil type	Inoculum (10 log *Lysobacter*/g soil)	Plate counts (10log *Lysobacter*/g soil)	Recovery (%)[b]	TaqMan (10log *Lysobacter*/g soil)	Recovery (%) [b]
Marine clay (farm A)	–	<3.00		6.36	
Marine clay (farm A)	8.65	8.80	152	8.57	86
Sandy soil (farm F)	–	<3.00		5.88	
Sandy soil (farm F)	8.65	8.69	113	8.00	23
LSD[a]		0.25		0.10	

[a] Least significant differences (LSD) at $P=0.05$; three replicates per treatment

[b] Recovery (%) is expressed as 100×numbers detected in soil just after inoculation/numbers in the inoculum

Table 3 Soil characteristics for bulk soil (3–20 cm depth) collected at arable fields of 10 organic farms in 2004, 2005, and 2006 at different locations within the Netherlands, ordered from low to high *Lysobacter* spp. populations (gene numbers expressed as log g^{-1} soil)

Year	Farm code[a]	Crop	Soil type[b]	Clay particles (<2 μm) (%)[b]	pH-KCl[b]	Organic matter (%)[b]	C/N quotient[b]	*Lysobacter* gene copies (log g^{-1} soil)[d]	Total bacteria gene copies (log g^{-1} soil)	*Lysobacter* percentage of total bacteria
2004	C	Grass–clover	Sandy soil	2.0	5.2	10.2	26.1	<4.00 (4/3)	8.27	–
2005	C	Wheat	Sandy soil	1.5	5.1	10.8	18.4	4.71 (2/1)	8.87	0.01
2005	F	Grass–clover	Sandy soil	2.0	4.9	3.8	16.7	5.57	9.12	0.03
2004	F	Triticale, grass–clover	Sandy soil	2.0	4.7	2.8	18.9	5.69 (2/0)	9.11	0.04
2006	J	Pumpkin	Sandy soil	2.5	4.9	1.7	23.2	5.71	8.40	0.21
2006	F	Wheat, winter radish	Sandy soil	1.5	5.0	2.6	19.0	5.75 (1/0)	8.43	0.21
2004	J	Pumpkin	Sandy soil	1.8	5.4	1.9	21.3	5.76 (3/1)	8.84	0.08
2005	J	Alfalfa	Sandy soil	1.8	5.4	2.4	18.1	5.87 (2/1)	8.05	0.67
2006	A	Red beet	Marine clay	12.3	7.4	1.9	12.7	6.22	9.11	0.13
2005	A	Carrot	Marine clay	12.0	7.5	2.3	9.2	6.25	8.21	1.09
2006	Aa	Grass-clover	Marine clay	24.3	7.4	2.7	10.1	6.27	8.86	0.26
2006	I	Pea	Marine clay	28.0	7.4	2.5	11.9	6.37	8.94	0.27
2005	I	Sugar beet	Marine clay	27.3	7.6	2.7	10.1	6.37	8.61	0.58
2006	D	Potato	Marine clay	13.8	7.4	3.0	13.3	6.44	9.30	0.14
2005	B	Carrot	Marine clay	13.5	7.3	2.5	9.7	6.44 (1/0)	8.54	0.80
2006	Ga	Grass-clover	Marine clay	15.5	7.4	4.0	12.7	6.47	9.34	0.13
2006	Ea	Grass-clover	Marine clay	23.5	7.2	8.9	11.9	6.48	9.18	0.20
2005	G	Wheat	Marine clay	14.0	5.9	12.0	11.7	6.51 (1/0)	8.30	1.60
2006	E	Cabbage	Marine clay	26.3	7.2	7.5	11.1	6.51	9.01	0.32
2006	Da	Grass-clover	Marine clay	14.3	7.6	2.9	13.6	6.51	9.13	0.24
2005	D	Broccoli	Marine clay	13.3	7.5	3.5	12.6	6.54	8.56	0.95
2005	H	Celery	Marine clay	20.3	7.5	3.5	9.8	6.57 (1/0)	8.88	0.49
2004	A	Wheat	Marine clay	11.5	7.4	1.7	13.6	6.57 (1/0)	8.72	0.70
2004	B	Cauliflower	Marine clay	13.8	7.3	1.6	12.4	6.58 (1/0)	8.72	0.71
2005	E	Wheat	Marine clay	25.8	7.2	4.1	10.6	6.63	8.53	1.25
2004	I	Grass-clover	Marine clay	29.3	7.4	1.9	15.7	6.64 (1/0)	8.33	2.03
2006	G	Parsnip, pumpkin	Marine clay	16.3	7.0	9.4	12.7	6.65	9.59	0.11
2004	E	Shallot, winter radish	Marine clay	27.5	7.2	6.8	11.6	6.67	8.83	0.69
2004	D	Onion	Marine clay	11.8	7.5	2.9	18.4	6.79	8.69	1.26
2004	G	Brussels sprouts	Marine clay	19.0	6.8	10.3	13.4	6.95 (1/0)	9.54	0.26
2004	H	Cabbage	Marine clay	20.5	7.4	2.9	12.2	6.95 (1/0)	8.67	1.90
LSD[c]				2.4	0.4	1.5	2.3	0.51	ns	

[a] "a" is a field from the same farm but with grass–clover

[b] Data are compiled from previous research [24]

[c] Least significant differences (LSD) at $P=0.05$; four replicates per treatment; *ns* no significant differences

[d] Between brackets: number of undetectable samples/number of samples with inhibition; 10^4 is estimated to be the detection limit

Effect of Soil Characteristics on *Lysobacter spp*. Populations

Populations of the species *L. antibioticus*, *L. capsici*, and *L. gummosus* were quantified using the *Lysobacter*-specific TaqMan in bulk soil samples collected in 2004, 2005, and 2006 in arable fields of 10 organic farms (Table 3). Sandy soils from farms C, F, and J had the lowest values of *Lysobacter* spp.; data ranged from undetectable (<4.0) up to 5.87 log gene copies per gram of soil. In clay soils, numbers of *Lysobacter* were in all cases higher than in sandy soils ranging from 6.22 to 6.95 log gene copies per gram of soil. This difference in *Lysobacter* populations between clay and sand (32- and 12.5-fold between minimum and maximum values of clay and sand, respectively) is larger than the difference in the recovery of the inoculated cells in both soils (fourfold). Moreover, the part of the total bacteria (real-time, SYBRGreen) that is detected to be *Lysobacter* was in general much higher in clay than in sandy soil (Table 3). Mean percentage of *Lysobacter* in sand and clay were 0.18% and 0.70%, respectively.

Several of the sandy soil samples (tenfold diluted) had negative TaqMan reactions. Part of them (six out of 14) showed inhibition of the TaqMan reaction, as tested with the *gfp*-coding gene method. Of the clay samples, only few (eight out of 92) samples showed a negative reaction, even though their replicates showed high *Lysobacter* numbers (Table 3, see data between brackets). None of these samples showed inhibition of the TaqMan reaction using the *gfp*-coding gene method. Thus, the negative reactions in clay soils were not due to failure of the method, but due to low numbers present in the sample. The variation between replicates resulted in an LSD value of 0.51 (negative samples were excluded from ANOVA).

The influence of the different environmental factors on the *Lysobacter* populations was analyzed using multiple regression analyses. Most important factors contributing to the density of the combined *L. antibioticus*, *L. capsici*, and *L. gummosus* populations were soil type and the sampled field ($F<0.001$). No significant differences in *Lysobacter* densities were recorded across the years of sampling ($F=0.500$) and the crop grown in the field ($F=0.898$).

Physical, chemical, and biological soil parameters measured from the same soil samples in a previous study [24] were used to analyze the influence of these factors on the *Lysobacter* populations. Multiple regression analyses between these soil characteristics and *Lysobacter* populations assessed using TaqMan, showed that these populations strongly correlated with the pH, percent clay particles, and C/N quotient in the soil (all $F<0.001$). Sandy soils, with low *Lysobacter* populations, were low in percent clay, low in pH, and high in C/N quotient (Table 3). Organic matter content in the soils did not correlate with *Lysobacter* populations ($F=0.236$). Also soil suppressiveness against *R. solani* (data presented in previous study [24]) did not correlate with the *Lysobacter* populations measured with TaqMan ($F=0.484$).

Effect of Specific Crops on *Lysobacter spp*. Populations

Different crops were sampled from fields of one selected farm (A) with a marine clay soil in June and September 2008. None of these clay samples showed inhibition of the TaqMan reaction. Populations of the species *L. antibioticus*, *L. capsici*, and *L. gummosus* in bulk soil ranged from 6.77 to 7.29 log gene copies per gram of soil in June (Table 4). Only onion bulk soil had a higher value of 7.74 log gene copies per gram. Rhizosphere populations ranged from 7.17 to 7.78 log gene copies per gram of soil in June. On average, populations were higher in the rhizosphere soil compared to bulk soil; 7.58 and 7.26 log gene copies per gram, respectively, soil for all crops (Table 4).

Bulk soil and rhizosphere soil samples taken in September 2008 were all between 6.60 and 7.19 log gene copies per gram of soil, with no significant difference between rhizosphere and bulk soil (Table 5).

In June, the part of the total bacterial population that was detected to be *Lysobacter* was 0.13% and 0.21% on average for the different crops in the bulk and rhizosphere soil, respectively. In September, the % *Lysobacter* was 0.23% and 0.25%, respectively. It seems that the fluctuation of the percentage of *Lysobacter* is bigger among plant species than between rhizosphere and bulk soil.

Table 4 *Lysobacter* populations (gene copies expressed as log g^{-1} soil and% *Lysobacter* between brackets) in rhizosphere and bulk soil of different crops sampled June 2008 on farm A

Crop	Bulk soil	Rhizosphere soil
Grass[a]	7.29 (0.14)[c]	7.17 (0.10)
Clover[a]	–	7.56 (0.16)
Onion	7.74 (0.40)	7.66 (0.35)
Potato	6.77 (0.04)	7.78 (0.26)
Red beet	7.24 (0.14)	7.57 (0.19)
Wheat	6.99 (0.07)	7.64 (0.28)
Mean of crops	7.26 (0.13)	7.58 (0.21)
LSD[b]	0.20	

[a] Grass and clover were grown together as a mixtures in one field

[b] Least significant differences (LSD) at $P=0.05$; crop as replicate, each with four subsamples

[c] Percent *Lysobacter*=100×mean number of *Lysobacter*/mean number of total bacteria

Table 5 DNA and RNA-based detection of *Lysobacter* populations (resp. gene or transcript copies expressed as log g^{-1} soil and% *Lysobacter* between brackets) in rhizosphere (Rhiz) and bulk soil of different crops sampled in September 2008 on farm A

Crop	DNA-based analyses		RNA-based analyses	
	Bulk	Rhiz	Bulk	Rhiz
Grass[a]	7.14 (0.24)[c]	7.19 (0.79)	5.08 (0.03)	4.46 (0.16)
Clover[a]	–	7.02 (0.33)	–	5.89 (0.62)
Red beet	7.01 (0.15)	6.60 (0.24)	6.01 (0.21)	7.78 (0.70)
Pumpkin	6.76 (0.25)	6.88 (0.06)	4.91 (0.06)	6.54 (2.15)
Mean of crops	7.00 (0.23)	6.92 (0.25)	5.24 (0.09)	6.17 (0.63)
LSD[b]	0.24		0.84	

[a] Grass and clover were grown together as a mixtures in one field

[b] Least significant differences (LSD) at $P=0.05$; crop as replicate, each with four subsamples

[c] Percent *Lysobacter*=100×mean number of *Lysobacter*/mean number of total bacteria

Active Part of *Lysobacter spp*. Populations

RNA was extracted from soil samples of different crops in September 2008 and compared with DNA-based analyses of the *L. antibioticus*, *L. capsici*, and *L. gummosus* community. None of these samples showed inhibition of the TaqMan reaction, as tested with the *gfp*-coding gene method. RNA-based analyses were on average 5.24 log transcript copies per gram in bulk soil (Table 5). In rhizosphere, large variations were present between the crops: ranging from 4.46 to 7.78 log transcript copies per gram for grass and pumpkin, respectively. The average for all crops in rhizosphere was 6.17. Consistent lower values were observed in RNA (cDNA)-based versus DNA-based *Lysobacter* TaqMan measurements, except for red beet rhizosphere where the RNA values were higher than those of DNA measurements. Roughly, the activity, i.e., RNA/DNA ratio, of the *Lysobacter* population was 1–10%.

The active part of *Lysobacter* within the active total bacterial population was 0.09% and 0.63% on average for the different crops in the bulk and rhizosphere soil, respectively.

Discussion

A specific primer–probe combination was successfully designed to simultaneously quantify three closely related *Lysobacter* spp. using TaqMan. These species, i.e., *L. antibioticus*, *L. capsici*, and *L. gummosus*, form one cluster within the *Lysobacter* genus (Fig. 1). They were all three isolated from several *Rhizoctonia*-suppressive soils in the Netherlands and could strongly inhibit mycelium growth of this fungal plant pathogen [24]. Specificity of this detection system was demonstrated by in silico evaluation of the theoretical 89-bp product of the primer–probe combination, as well as by running TaqMan with strains of the three target species and several strains of the closely related *L. enzymogenes*, which had a much higher (17-fold) cycle threshold, and a nontarget *Xanthomonas* which did not show any positive reaction. These four tested species are the main species recovered from soil up to now. Although we had never isolated *L. enzymogenes* from one of the used soils in the current experiment. Other *Lysobacter* species have not been detected in soil regularly; they are either water-borne (e.g., *Lysobacter brunescens*) or described on the basis of one or few isolates.

For quantification of *Lysobacter* in the soil samples, a standard curve with genomic DNA of *L. gummosus* strain 3.2.11 was used. Some assumptions (e.g., size of the *Lysobacter* genome) had to be made for calculating the gene copy numbers in the soil samples. As a consequence, the absolute values are an estimation, but the measured values can be compared with each other to study the influence of several factors in soil on the population dynamics of *Lysobacter*. Gene copy numbers were 1.5–2 times higher than the values obtained by cell estimations via CFU counts. Recovery of freshly grown *Lysobacter* cells immediately after their introduction indicated that the detection efficiency of TaqMan in sandy soil and clay soil were approximately 23% and 86%, whereas recovery by plate counts was >100%. The reason for these differences in efficiencies is not clear, but a cell division during the extraction procedure to detect CFU can be an explanation for the high recovery with plate counts.

The influence of soil type on the population size of *Lysobacter* was remarkable. The three sandy soils contained low *Lysobacter* populations in all 3 years of sampling, ranging from undetectable ($<10^4$) to 5.87 log gene copies per gram bulk soil. In clay soils, the numbers were always higher: 6.22–6.95 log gene copies per gram bulk soil. Even if the difference in recovery between sandy and clay soil as

was found with inoculated *Lysobacter* cells is taken into account, clay soils seem to contain larger *Lysobacter* populations than sandy soils. Several soil characteristics significantly correlated with the size of the *Lysobacter* populations, i.e., more *Lysobacter* gene copies were detected in soils with high pH, high percentage clay, and a lower C/N ratio. However, organic matter content in soil appeared to have no influence on the *Lysobacter* population size. In our previous study, antagonistic *Lysobacter* spp. had only been isolated from clay soils and not from sandy soils with low pH [24]. In other studies, *Lysobacter* spp. was isolated from agricultural soils and sand dunes in Scotland where the pH was not below 6 [26]. This strong effect of soil type on the detected *Lysobacter* populations is in line with other studies on microbial community composition, where soil type had a larger effect than land use on community composition [2, 6]. Another study showed that the bacterial community was largely driven by the pH and soil texture [17].

It was hypothesized that also the presence and type of crop would influence the *Lysobacter* population, since in a previous study we isolated about five times more *Lysobacter* isolates from fields with a grass–clover crop than from fields of the same farm with another crop [24]. However, TaqMan analyzes of the same samples (farm A, D, E, and G in 2006) resulted in similar population sizes of *Lysobacter* spp. in grass–clover and other crops. Also, new samples taken from fields with different crops, all from farm A, did not show higher *Lysobacter* populations when grass and clover were grown. Moreover, the vicinity of plant roots often did not enhance *Lysobacter* populations. *Lysobacter* populations were higher in rhizosphere than in the bulk soil in only a few cases. For this reason, we speculate that *Lysobacter* is not a genuine rhizosphere bacterium, such as fluorescent *Pseudomonas* spp. which can occur in much higher populations in the rhizosphere compared to bulk soil [18, 30]. *Lysobacter* spp. is known for their capacity to degrade various biomacromolecules; they proliferate on chitin and decompose nematodes, bacteria, yeasts, fungi, etc. [26]. Some isolates of the three target *Lysobacter* spp. do not proliferate on citric and malic acid and on glucose and maltose [21, 22], which are important components of plant exudates [11]. Consequently, *Lysobacter* spp. is probably not stimulated directly by plant exudates as is the case for *Pseudomonas* spp.

The number of *Lysobacter* gene copies in clay bulk soil samples was on average 7.0 log gene copies per gram soil, whereas the antagonistic *Lysobacter* isolates had been estimated previously to be 5.5 log CFU g^{-1} soil (approximately 0.3% of culturable bacteria) [24]. Similarly, Huijsdens et al. [8] demonstrated that quantification by real-time PCR yielded approximately 100-fold higher numbers of *Escherichia coli* in the human gut than quantification by plate counts. Higher numbers of gene copies than antagonistic isolates can be expected if: (1) not all cells are antagonistic, (2) not all DNA originates from culturable bacterial cells, (3) more than one gene copy is present per bacterium, and (4) bacteria occur clustered in the soil environment. In contrast to soil with freshly inoculated target cells, the indigenous *Lysobacter* population might contain poorly culturable cells, or even dead intact DNA. Another crucial point would be the presence of *Lysobacter* cells which are not antagonistic against *Rhizoctonia*. This would also explain the lack of correlation between *Lysobacter* gene copy numbers and *Rhizoctonia* disease suppression.

In addition to the size of the population, bacterial activity will influence beneficial aspects such as disease suppression. For this reason the activity of the *Lysobacter* population was assessed by comparing DNA and RNA quantities of *Lysobacter* spp. Although the percentage of RNA/DNA was roughly 1–10%, it did not support the enhanced suppressiveness in grass–clover crops; i.e., the activity of *Lysobacter* was not higher in grass or clover than in other crops.

One not-yet-discussed result is the large variation which sometimes occurred between replicate samples of 0.5 g soil which had been taken from larger soil samples. In several fields, high populations of *Lysobacter* were present in three replicates, where the fourth replicate had undetectable populations. This was not due to inhibition in the TaqMan reaction, which had been checked by the *gfp* assay. Also during isolation of antagonistic *Lysobacter* isolates [24] large variations in isolate numbers between replicates were found. Probably, cells of *Lysobacter* appear in clusters in the soil, which makes research on the ecology of *Lysobacter* more complicated.

This paper describes the first attempt to quantify *Lysobacter* spp. in different soils and in the vicinity of different crops. Conclusions which can be drawn from these first experiments about the habitat of *L. antibioticus*, *L. capsici*, and *L. gummosus* are: (1) sandy soil with a low pH is unfavorable, (2) soil factors stimulating *Lysobacter* populations are clay content, pH, and C/N ratio, but not organic matter content, (3) equal and sometimes higher populations are present in rhizosphere compared to bulk soil, (4) the effect of the crop is unclear, since antagonistic isolates were previously found to be higher in grass–clover crops, but total gene copy numbers assessed with TaqMan were not influenced by these crops.

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