Chapter 5: Real-time PCR detection of Holophagae (Acidobacteria) and Verrucomicrobia subdivision 1 groups in bulk and leek (Allium porrum) rhizosphere soils

Abstract

In the light of the poor culturability of Acidobacteria and Verrucomicrobia species, group-specific real-time (qPCR) systems were developed based on the 16S rRNA gene sequences from culturable representatives of both groups. The number of DNA targets from three different groups, i.e. Holophagae (Acidobacteria group 8) and Luteolibacter and Candidatus genus Rhizospheria (both from Verrucomicrobia subdivision 1), were determined in DNA extracts from different leek (Allium porrum) rhizosphere soil compartments and from bulk soil with the aim to determine the distribution of the three bacterial groups in the plant-soil ecosystem. The specificity of the designed primers was evaluated in three steps. First, in silico tests were performed which demonstrated that all designed primers 100% matched with database sequences of their respective groups, whereas lower matches with other non-target bacterial groups were found. Second, PCR amplification with the different primer sets was performed on genomic DNA extracts from target and from non-target bacteria. This test demonstrated specificity of the designed primers for the target groups, as single amplicons of expected sizes were found only for the target bacteria. Third, the qPCR systems were tested for specific amplifications from soil DNA extracts and 48 amplicons from each primer system were sequenced. All sequences were > 97% similar to database sequences of the respective target groups. Estimated cell numbers based on Holophagae-, Luteolibacter- and Candidatus genus Rhizospheria-specific qPCRs from leek rhizosphere compartments and bulk soils demonstrated higher preference for one or both rhizosphere compartments above bulk soil for all three bacterial groups.

* Authored by: Ulisses Nunes da Rocha, Jan Dirk van Elsas & Leo Simon van Overbeek
Published in: J Microbiol Methods (accepted)
Introduction

Acidobacteria and Verrucomicrobia are diverse bacterial phyla that are widely distributed and highly abundant in the soil environment (Hugenholtz et al., 1998; Barns et al., 1999; Lee & Cho, 2009). In spite of their high abundance and diversity, little information is available on their ecology, which is mainly due to the lack of culturable representatives in bacterial collections (Nunes da Rocha et al., 2009). Therefore, most of the ecological information about these taxa is based on the Acidobacteria and Verrucomicrobia 16S rRNA gene sequence distribution over different ecosystems. Analyses of 16S rRNA genes of Acidobacteria and Verrucomicrobia in samples from terrestrial habitats often revealed contradictory information about their preferred sites, especially in the distinction between bulk or rhizosphere soils (Chow et al., 2002; Sanguin et al., 2006; Zul et al., 2007; Kielak et al., 2008).

The phylogenetic diversity of Acidobacteria and Verrucomicrobia is high. In fact, both are deep-branching groups within the bacterial ‘tree of life’. Twenty six different phylogenetic groups have been defined so far for Acidobacteria (Barns et al., 2007) and seven subdivisions for Verrucomicrobia (Schlesner et al., 2006). Most studies on the numeric distribution of Acidobacteria and Verrucomicrobia in natural ecosystems have been performed with primers or probes that cover multiple groups or subdivisions within both phyla. Minority groups present among both phyla may have been overlooked and their behavior may be contrary to what is currently assumed to be true about the distribution of Verrucomicrobia and Acidobacteria in plant-soil ecosystems.

In spite of the fact that members of the Acidobacteria and Verrucomicrobia are considered to be ‘hard to culture’ (Jones et al., 2009; da Rocha et al., 2010), an increasing number of strains can currently be found in public databases. For instance, 209 16S rRNA gene sequences of culturable representatives of Acidobacteria next to 134 sequences of culturable Verrucomicrobia are currently available in the RDP Release 10 (update 19, March 31, 2010 - http://rdp.cme.msu.edu/index.jsp). The availability of culturable representatives of the two bacterial groups would allow the determination of metabolic, morphological and genetic parameters, which is impossible to accomplish with molecular techniques alone (Zengler, 2009). Further, such cultures would allow experimentation under controlled conditions mimicking the situation in the environment. The vast majority of these strains likely consists of slow growers, which require specific conditions that favor their growth in pure culture (Nunes da Rocha et al., 2009). Because of their recalcitrance to growth under laboratory conditions, combinations of culture-dependent and -independent approaches will further improve detection at finer resolution levels. This is important to allow distinction between
separate groups within both phyla, something required to gain understanding about the ecology of particular representatives within both groups.

Recently, two close related culturable representatives of the *Holophagae*, previously known as *Acidobacteria* group 8, were found in the leek rhizosphere at two independent occasions (Nunes da Rocha et al., 2010). Also, nine culturable members of three phylogenetically distinguishable groups of subdivision 1 of the *Verrucomicrobia* were isolated from leek and potato rhizospheres, sampled at different occasions (da Rocha et al., 2010; Nunes da Rocha et al., 2010). To the best of our knowledge, no information about their presence in soil compartments proximate to plants has been reported in literature (Nunes da Rocha et al., 2009). As these cultured *Holophagae* and *Verrucomicrobia* were recovered from soil adhering to roots (rhizosphere), it was hypothesized that both groups might show preferences for rhizosphere over bulk soil.

To test this hypothesis, bulk and rhizosphere soil samples taken from field-grown leek plants were analyzed for the presence of *Holophagae* and *Verrucomicrobia* subdivision 1 groups. Analyses were done with newly developed group-specific real-time PCR (qPCR) systems, based on the 16S rRNA gene sequences of culturable representatives of *Holophagae* and *Verrucomicrobia* subdivision 1. Three systems (one from the *Holophagae* and two from *Verrucomicrobia* subdivision 1) were evaluated and tested for their functionality in the detection of both groups in the plant-soil ecosystem.

**Material and Methods**

**Identity and growth of bacterial strains**

Isolation and identification of strains belonging to the *Holophagae* (2), *Luteolibacter* (3) and *candidatus Rhizospheria* (5) are described in da Rocha et al. (2010) and Nunes da Rocha et al. (2010) (Table 1). The taxonomic relationship, based on the 16S rRNA gene sequences of these strains and uncultured members of the *Acidobacteria* and *Verrucomicrobia* (SILVA database - Pruesse et al., 2007) is depicted in Fig. 1 of chapter 4 (page 111). R2A (Difco, France) was used for routine cultivation of all strains at 25°C.

**DNA extraction from pure cultures and soils**

For DNA extraction from all pure culture strains (Table 1), cells were scraped off from R2A agar plates with bacterial growth. The cells were then suspended in 500 µL of a sterile solution consisting of 0.85 % KCl in DNase/Rnase-free distilled water (Invitrogen, The Netherlands). DNA was extracted from the resulting cell suspensions
<table>
<thead>
<tr>
<th>Affiliation</th>
<th>Strain</th>
<th>Plant type</th>
<th>Year of isolation</th>
<th>Field/collection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acidobacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Verrucomicrobia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Luteolibacter</em></td>
<td>C20</td>
<td><em>Solanum tuberosum</em></td>
<td>2006</td>
<td>Droevendaal</td>
<td>da Rocha et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>ZNBB5</td>
<td><em>Solanum tuberosum</em></td>
<td>2006</td>
<td>Droevendaal</td>
<td>da Rocha et al. (2010)</td>
</tr>
<tr>
<td><strong>Verrucomicrobi um</strong></td>
<td>CNC16</td>
<td><em>Allium porrum</em></td>
<td>2007</td>
<td>Vredepeel</td>
<td>Nunes da Rocha et al. (2010)</td>
</tr>
</tbody>
</table>
using the MasterPure™ DNA purification kit (Epicentre Biotechnologies, WI, USA) following the instructions provided by the manufacturer. For extraction from soil, the PowerSoil Isolation Kit (MO BIO Laboratories, Inc., CA, USA) was used, following the instructions provided by the manufacturer.

**Design and testing of Holophagae-, Luteolibacter- and Candidatus genus Rhizospheria-specific primers**

Because the *Holophaga* (2), *Luteolibacter* (3) and *Candidatus* genus *Rhizospheria* (5) strains all formed separate clusters in the dendrogram (Fig 1), we decided to design three primer systems, one for each group. 16S rRNA gene sequences of these strains and of *Holophagae* (128 sequences), *Luteolibacter* (121 of *Luteolibacter* and 22 of *Prosthecobacter*) and *Candidatus* genus *Rhizospheria* (180 sequences) from the RDP Release 10, Update 19 (http://rdp.cme.msu.edu/) were separately aligned for each group using SINA Webaligner (http://www.arb-silva.de/aligner/).

The alignments were checked for similarities in conserved regions among the sequences within each of the three groups using ARB software (Ludwig et al., 2004). Then, primers were designed on the basis of the conserved regions and these were checked for *in silico* specificity using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). This recent software, last modified December 2009, enables the search for primer pairs that are specific for the intended PCR template and also allows to check for eventual occurrences of misprimed products and possible non-intended templates (http://www.ncbi.nlm.nih.gov/tools/primer-blast/primerinfo.html). The melting temperature of the primers was restricted to values between 59 and 61°C, to closely match the Taq polymerase optimal extension temperature of SYBR® Premix Ex Taq™ (TAKARA Bio Inc., Japan). The sequence, melting behavior and most relevant PCR amplification parameters of the designed primer systems are presented in Table 2.

*Luteolibacter* and *Prosthecobacter* are closely related genera of *Verrucomicrobia* subdivision 1, and only recently taxonomical distinction between both groups was made (Yoon et al., 2008). Although *in silico* analysis indicated *Luteolibacter*-specific amplification when using primers VS1Af and VS1Ar, sequencing of clones generated with this primer pair demonstrated that both *Luteolibacter* and *Prosthecobacter* members are actually amplified from soil DNA. The bacterial group distinguished by this specific qPCR system is therefore denoted as *Luteolibacter*.

The primers were further tested for their specificity using target (Table 1) and non-target DNA by PCR amplification. Non-target DNA from the following strains were included in the evaluation assays of the qPCR systems: *Agrobacterium tumefaciens* strain UBAPF2 (*Alphaproteobacteria*), *Burkholderia cepacia* strain LMG 1222T
<table>
<thead>
<tr>
<th>Phylum</th>
<th>Target group</th>
<th>Sense</th>
<th>Primer sequence (5' → 3')</th>
<th>Primer name</th>
<th>Tm(^a) (°C)</th>
<th>Amplicon length (bp(^b))</th>
<th>Dynamic range(^c)</th>
<th>Amplification efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidobacteria</td>
<td>Holophagae</td>
<td>Forward</td>
<td>TGGGATGTTGATGGTGAAAC</td>
<td>Acg8f</td>
<td>59.19</td>
<td>470</td>
<td>2.54 to 7.54</td>
<td>2.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>AGTCTCGGATGCAGTTCTTG</td>
<td>Acg8r</td>
<td>60.41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>Luteolibacter</td>
<td>Forward</td>
<td>CAGCTCGTGTCGTGAGATGT</td>
<td>VS1Af</td>
<td>60.04</td>
<td>199</td>
<td>2.26 to 8.26</td>
<td>1.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TCTCGGTCTCATTGTGCTG</td>
<td>VS1Ar</td>
<td>59.98</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candidatus genus</td>
<td></td>
<td>Forward</td>
<td>GCCCGACAGGGTTGATAGTA</td>
<td>VS1Bf</td>
<td>59.96</td>
<td>83</td>
<td>2.45 to 8.45</td>
<td>1.95</td>
</tr>
<tr>
<td>Rhizosphaeria</td>
<td></td>
<td>Reverse</td>
<td>CGCTTGGACCTTCGTATTA</td>
<td>VS1Br</td>
<td>60.06</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Tm, melting temperature  
\(^b\) bp, base pairs  
\(^c\) Theoretical dynamic range (expressed in, Log cell equivalents per gram of dry soil), which indicates the range of initial template concentrations in the soil DNA extracts over which reliable Ct values are obtained, assuming DNA extraction and qPCR reaction efficiencies of 100%  
\(^d\) The efficiency of the reaction was calculated by the following equation: \( Ae = 10^{(-1/slope)} \), where, \( Ae \), amplification efficiency, slope, slope calculated from standard curve
(Betaproteobacteria), Escherichia coli strain E1 (Gammaproteobacteria), Streptomyces griseus strain IPO 857 (Actinobacteria), Flavobacterium columnar strain 2003/035 (Bacteroidetes) and Bacillus subtilis strain Bs4 (Firmicutes); all derived from the strain collection available at Plant Research International (Wageningen, The Netherlands). Each 25-µl reaction mixture contained the following ingredients: 12.5 µl of SYBR Premix Ex Taq 2x (TAKARA Bio Inc., Japan), 0.5 µl of each primer (10 µM; Biolegio, NL), 0.5 µl of ROX Reference Dye II 50x (TAKARA Bio Inc., Japan), 6.0 µl H₂O and 5.0 µl template DNA (containing 1 ng of target or non-corresponding strains). All PCR reactions were run for one cycle at 50°C for 2 min; one cycle at 95°C for 10 s; 35 cycles at 95°C for 5 s and 60°C for 35 s. The number of cycles was limited to 35 to avoid occurrences of false positive signals (Sipos et al., 2007). Additionally, melting curves derived from each individual reaction were inspected in order to ascertain that the signals obtained originated from specific PCR reactions and not from primer dimer formation or any other artifact. Further, PCR amplicons made with all three primer systems with DNA from target and non-corresponding strains were checked in 1.5% agarose gels for presence of bands of expected sizes and absence of any secondary, or false-positive products.

As a final check on the specificity of the selected primer systems, amplification from soil DNA extracts (Vredepeel [V] soil) was performed. Thus, bulk soil DNA was added as template to PCR reaction mixtures (1 – 5 ng per reaction). Then, reactions were run under the same conditions as described before. Subsequently, PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, GE) and the resulting products were cloned into the pGEM-T Easy Vector (Promega, WI, USA) using the protocol provided by the manufacturer. Totals of 48 clones from each primer system were selected for sequencing. All clones were checked for the presence of inserts of the correct sizes before sequencing. The resulting sequences were aligned using MEGA 4 software (Kumar et al. 2008) and then individually compared with database sequences by BlastN-assisted searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Calibration of real time PCR (qPCR) systems.

The three qPCR systems, aimed to detect Holophagae, Luteolibacter and Candidatus genus Rhizospheria, were calibrated on cell lysates made from pure culture strains, one from each group. For each reaction (performed as described above), 5 µl of tenfold serial dilutions (ranging from approximately 10² to approximately 10¹⁰ cells per mL) per target strain per group-specific qPCR system was used. Holophagae strain CHC25, Luteolibacter strain CHC12 and Candidatus genus Rhizospheria strain CHC8 were used to yield DNA templates for the respective group-specific qPCR systems. Standard curves were made in separate for each series of serially-diluted suspensions and all
Detection of *Holophagae* and *Verrucomicrobia* subdivision 1 in soil
dilutions were made in triplicate. Group-specific qPCR systems were employed on
these series and the measured threshold cycle (Ct) values were plotted against the Log
cell number for each reaction. Line slopes and intercepts from resulting graphs were
calculated by regression analysis using GenStat 12th edition (VSN International Ltd.,
UK). The amplification efficiency (Ae) of the different primer systems was calculated
using the formula \( Ae = 10^{(-1/slope)} \), in which the slope represents the slope value
calculated by regression analysis.

**Leek plant growth and root and soil sampling procedures**

The site chosen for sampling was an agricultural field located at the experimental farm
‘De Vredepeel’, The Netherlands (51° 32’ 27.10” N and 5° 51’ 14.86” E). Here, leek
was grown in accordance with practices common for leek production, in particular
concerning soil tillage, crop rotation, chemical fertilization and chemical pest (thrips)
control. The V soil was characterized as sand, with pH 5.4 and 2.2% organic matter.
Roots with adhering soil of seven leek (*Allium porrum*) plants (cultivar Kenton,
Nunhems Zaden BV, The Netherlands) were collected, all at the same occasion, but at
different sites in the fields (minimum distance between plants was 5 m).

Three soil compartments were distinguished: bulk soil, outer rhizosphere and
inner rhizosphere. The soil free of roots in the neighborhood of the sampled plants (at
least at 1 m distance from each plant) was considered as bulk soil. The soil adhering to
leek roots was separated into two fractions: the outer rhizosphere was represented by the
soil adhering to the root surface after mild manual shaking. It was removed from the
roots by scratching with a spatula. Then, roots devoid of the outer rhizosphere were
shaken in a 1:10 ratio (v/v basis) in 0.1% sodium pyrophosphate solution. The resulting
soil suspension was considered to represent the inner rhizosphere. The inner rhizosphere
suspension was concentrated by centrifugation at 10,000 x \( g \) for 15 min followed by
resuspension of the resulting pellet in 1 ml of 0.85% NaCl solution.

**qPCR detection of Holophagae, Luteolibacter and Candidatus genus Rhizospheria in
different plant-soil compartments**

qPCR was conducted with the three selected primer systems using DNA from different
soil compartments (bulk, outer and inner rhizosphere soils) of the seven individual leek
plants. Three reaction mixtures per sample, each containing 5 ng of soil DNA , were
subjected to either one of the qPCRs for detection of *Holophagae*, *Luteolibacter* and
*Candidatus* genus *Rhizospheria*, as described above. One positive control (containing
DNA from a corresponding strain as a template) and seven negative controls, including
DNA from six non-corresponding strains (Table 1) and one with sterile demineralized
water only, were used in each run. Differences between the average Ct values per bacterial group in each soil compartment were statistically compared by one-way analysis of variance (ANOVA) and differences were considered to be significant at levels of $P \leq 0.05$.

Dynamic range here is considered as the range over which cell numbers can be reliably measured by qPCR. A theoretical dynamic range, using the extreme values of equivalent cell number determined by the calibration curves, was estimated for each primer system using the formula: $\text{Ev} = e\text{qc}_R \times V_{\text{PCR}} \times V_R^{-1} \times W_S^{-1}$; where, Ev is the estimated highest or lowest dynamic range value, $e\text{qc}_R$ is the equivalent cell number in the reaction mixture calculated from equations made by regression analysis, $V_{\text{PCR}}$ is the volume of DNA solution added to the qPCR reaction mixture (5 µl), $V_R$ is the volume in which the DNA pellet was resuspended (100 µl), $W_S$ is the precise amount of soil, calculated on dry weight basis, that was used for DNA extraction.

**Nucleotide sequence accession numbers**

Non-redundant sequences of the 144 partial 16S rRNA gene library clones constructed with the three primer systems (48 clones each) were deposited in the EMBL Nucleotide Sequence Database and are available under the accession numbers [FN796785](https://www.embl.de) to [FN796791](https://www.embl.de) and [FN796793](https://www.embl.de) to [FN796795](https://www.embl.de).

**Results**

**Specificity of the Holophagae, Luteolibacter and Candidatus genus Rhizospheria primer systems**

Three quantitative PCR primer systems aimed to specifically amplify 16S rRNA gene sequences of *Holophagae*, *Luteolibacter* and *Candidatus* genus *Rhizospheria* were designed (Table 2) and evaluated for quantification of members of these groups in soil. *In silico* comparisons of the sequences of individual primers with database sequences, using Primer-Blast, predicted that all primers (*Holophagae*, *Luteolibacter* and *Candidatus* genus *Rhizospheria*) would amplify 16S rRNA gene sequences that matched the sequences of their respective groups, yielding amplicons with expected sizes. Further, no matches with other bacterial groups were expected to be found.

PCR amplifications performed with genomic DNA of the two *Holophagae* strains, CHC25 and ORAC, using the primer system specific for the *Holophagae* resulted in the generation of single amplicons of the expected size of 470 bp, in the absence of any visible primer dimer or other products (data not shown). PCR amplification with this primer system, using genomic DNA from six non-corresponding
Detection of Holophagae and Verrucomicrobia subdivision 1 in soil

strains as well as all nine Verrucomicrobia strains (Table 1) resulted in the absence of any band under these amplification conditions. Using the primers designed for Luteolibacter in PCR reactions with genomic DNA of Luteolibacter strains CHC12, C20 and ONA9 showed the emergence of single amplicons of the expected size, of 199 bp in the absence of any primer dimer or other product after agarose gel electrophoresis. No PCR product was found from PCRs run with the same primers on genomic DNAs from six non-corresponding, five Candidatus genus Rhizospheria, two Holophagae and one Verrucomicrobium strains as templates (Table 1). Using the primers designed for the Candidatus genus Rhizospheria group with DNA from the five Candidatus genus Rhizospheria strains CHC8, IRVE, CR28, Z35 and ZNBB5 revealed the presence of single amplicons of the expected size of 83 bp in the absence of any primer dimer or other product in agarose gel. This band was absent when using genomic DNA from six non-corresponding strains and from all strains of Luteolibacter, Verrucomicrobium and Holophagae (Table 1) using the same PCR system and run under the same circumstances.

DNA sequence analyses of library clones constructed with the three primer systems (48 clones per system), using V soil DNA as template, always revealed the presence of insert sequences that matched with database sequences belonging to the expected groups (Table 3). DNA sequence analysis of the inserts of the clones made with Holophagae-specific primers resulted in the detection of two sequence groups, i.e. those denoted clone lib1_1_36 (encompassing 36 clones) and those denoted lib1_2_12 (encompassing 12 clones). Both groups were closest affiliated with Acidobacterium bacterium CHC25, albeit at different similarity levels, i.e. 97% for lib1_1_36 and 99% for lib1_2_12 (Table 3). Sequencing of the inserts of the 48 Luteolibacter library clones resulted in five different sequence groups; those denoted as lib2_3_16 (16 clones), lib2_5_15 (15) lib2_1_7 (7), lib2_4_8 (8) and lib2_2_2 (2). DNA sequences belonging to lib2_1_7 and lib2_2_2 showed closest matches with those of Prosthecobacter species (99% similarity) and those of lib2_3_16, lib2_5_15, lib2_4_7 groups matched closely with sequences of Luteolibacter species (> 98% similarity). Sequencing of the inserts of the 48 Candidatus genus Rhizospheria library clones resulted in three sequence groups, denoted as lib3_1_33 (33 clones), lib3_2_10 (10) and lib3_3_5 (5). All showed closest matches to different sequences belonging to the Candidatus genus Rhizospheria (100% similarity).

The primers designed within this study thus specifically targeted DNA sequences from Holophagae, Luteolibacter and Candidatus genus Rhizospheria species, both in pure culture as well as in complex soil DNA extracts.
**Table 2** Description of the group-specific *Holophagae* and *Verrucomicrobia* subdivision 1 qPCR primers.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Target group</th>
<th>Sense</th>
<th>Primer sequence (5’–3’)</th>
<th>Primer name</th>
<th>Tm(^{a})</th>
<th>Amplicon length (bp(^{b}))</th>
<th>Dynamic range(^{c})</th>
<th>Amplification efficiency(^{d})</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acidobacteria</em></td>
<td><em>Holophagae</em></td>
<td>Forward</td>
<td>TGGGATGTGTGATGGTGAAC</td>
<td>Acg8f</td>
<td>59.19</td>
<td>470</td>
<td>2.54 to 7.54</td>
<td>2.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>AGTCCTCGATGCAGTCTCTG</td>
<td>Acg8r</td>
<td>60.41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Verrucomicrobia</em></td>
<td><em>Luteolibacter</em></td>
<td>Forward</td>
<td>CAGCTCGTGTCGATGATGT</td>
<td>VS1Af</td>
<td>60.04</td>
<td>199</td>
<td>2.26 to 8.26</td>
<td>1.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TCTCGTCTCTCGGTTGCTG</td>
<td>VS1Ar</td>
<td>59.98</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Candidatus genus</em></td>
<td></td>
<td>Forward</td>
<td>GCCGACAGGGTGTAGTA</td>
<td>VS1Bf</td>
<td>59.96</td>
<td>83</td>
<td>2.45 to 8.45</td>
<td>1.95</td>
</tr>
<tr>
<td><em>Rhizospheria</em></td>
<td></td>
<td>Reverse</td>
<td>CGCTGCGGACCTTCATTATA</td>
<td>VS1Br</td>
<td>60.06</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Tm, melting temperature  
\(^{b}\) bp, base pairs  
\(^{c}\) Theoretical dynamic range (expressed in, Log cell equivalents per gram of dry soil), which indicates the range of initial template concentrations in the soil DNA extracts over which reliable Ct values are obtained, assuming DNA extraction and qPCR reaction efficiencies of 100%  
\(^{d}\) The efficiency of the reaction was calculated by the following equation: \(Ae = 10^{(10/k_Slope)}\); where, Ae, amplification efficiency, slope, slope calculated from standard curve
Detection of *Holophagae* and *Verrucomicrobia* subdivision 1 in soil

*Amplification efficiency and theoretical dynamic range of qPCR systems that detect the Holophagae, Luteolibacter and Candidatus genus Rhizospheria*

Standard curves of all three qPCR systems, constructed by plotting measured Ct values using cell lysates made from *Acidobacterium bacterium* strain CHC25 (*Holophagae*), *Verrucomicrobiaceae bacterium* strain CHC12 (*Luteolibacter*) and *Verrucomicrobiaceae bacterium* strain CHC8 (*Candidatus genus Rhizospheria*) against the respective log cell numbers revealed linear relationships between the two parameters for all three systems. For the *Holophagae* qPCR system, the slope of the curve, as calculated by regression analysis, was -3.296 ($R^2$ value 0.9963) and the amplification efficiency (Ae) value was 2.01. For the *Luteolibacter* qPCR system, the slope of the curve was -3.375 ($R^2$ value 0.9945) and the Ae value 1.98. For the *Candidatus genus Rhizospheria* qPCR system, the slope of the curve was -3.459 ($R^2$ value 0.9863) and the Ae value 1.95. The theoretical dynamic range, in Log equivalent cell number per gram of dry soil, of the different qPCR systems were 2.54 - 7.54 for the *Holophagae* qPCR system, 2.26 - 8.26 for the *Luteolibacter* qPCR system and 2.45 - 8.45 for the *Candidatus genus Rhizospheria* qPCR system.

*qPCR enumeration of Holophagae, Luteolibacter and Candidatus genus Rhizospheria in bulk soil and in the outer and inner rhizosphere of leek plants*

The distribution, in Log equivalent cell number per g of dry soil, of *Holophagae, Luteolibacter and Candidatus genus Rhizospheria* in three soil compartments, i.e. bulk soil and outer and inner rhizosphere of leek plants, is shown in Fig. 1. For the *Holophagae*, the highest numbers were found in the outer rhizosphere (6.25 - 6.41), followed by the bulk soil (6.08 - 6.20) and the inner rhizosphere (5.89 - 5.85). This stood in sharp contrast to the distribution of *Luteolibacter*, where the highest numbers were found in the inner rhizosphere (6.24 - 6.46), followed by the outer rhizosphere (5.91 - 6.21) and the bulk soil (4.11 - 4.39). For the *Candidatus genus Rhizospheria*, the highest numbers were found in both rhizosphere compartments: 5.33 - 5.39 in the outer rhizosphere, 5.25 - 5.35 in the inner rhizosphere and 4.31 - 4.48 in the bulk soil. Real-time PCR analysis of the natural V soil thus demonstrated higher numbers in log cell equivalents of *Holophagae, Luteolibacter and Candidatus genus Rhizospheria* in one or both leek rhizosphere compartments as compared to bulk soil.
Figure 1 Holophagae, Luteolibacter and Candidatus genus Rhizospheria cell estimates in bulk, outer rhizosphere and inner rhizosphere soils. Numbers were generated by Holophagae-, Luteolibacter- and Candidatus genus Rhizospheria-specific qPCRs. Averages followed by different letters significantly differ from each other, as determined by one-way ANOVA (P<0.05).

Discussion

Although representatives of the Acidobacteria and Verrucomicrobia are known for their recalcitrance to grow in pure culture, members of both taxa can be cultured from different environments. Indeed, several reports on successful isolation have appeared, being the work of da Rocha et al. (2010) and Nunes da Rocha et al. (2010), the first reports on recovery of Verrucomicrobia and Acidobacteria members from rhizosphere. Because of these findings, it was hypothesized that members of these groups might prefer the rhizosphere over bulk soil. To test this hypothesis, it was necessary to design a culture-independent quantitative method to determine cell equivalent numbers of the groups in different plant-soil compartments. This would allow an estimation of the cell numbers of the distinctive groups given that cultivation from soil still is cumbersome due to the specific requirements for growth and the long incubation times needed. Here, specific qPCR systems for each of the
three groups (Holophagae, Luteolibacter and Candidatus genus Rhizospheria) are presented. These were all designed on the basis of the 16S rRNA gene and evaluated for their specificity of detection in soil. The new qPCR systems will help us to explore the members of the three groups in the plant-soil ecosystem. To the best of our knowledge, the approach to develop such qPCR-based methods, validated on the basis of actual cell numbers, have never been developed before for the groups of Acidobacteria and Verrucomicrobia.

Two strains of the Holophagae (previously known as Acidobacteria group 8) were recovered at two different occasions from the leek rhizosphere (Nunes da Rocha et al., 2010). The Holophagae can be considered as a minority group in soil ecosystems, as they may comprise only up to 3.4% of total Acidobacteria sequences, as determined by pyrosequencing (Jones et al., 2009). More importantly, group 8 of Acidobacteria will not be included in Acidobacteria cell measurements in environmental samples when using primer Acd31F (Barns et al., 1999). Namely, it was recently reported that Holophagae-specific 16S rRNA sequences could not be amplified from soil with this primer (Kielak et al., 2009).

The Verrucomicrobia subdivision 1 strains, obtained from leek and potato rhizospheres at different occasions, were affiliated with two distinct groups in this subdivision, i.e. the Luteolibacter and the Candidatus genus Rhizospheria groups (da Rocha et al., 2010; Nunes da Rocha et al., 2010). Species belonging to Luteolibacter, i.e. Luteolibacter algae and Luteolibacter pohnpeiensis, were so far all only isolated from marine environments, namely from red algae and driftwood, respectively (Yoon et al., 2008). Another Verrucomicrobia subdivision 1 strain, initially identified only at the phylum level, also originated from seawater (Stingl et al., 2007). Later database comparisons revealed this strain to belong to the so called unclassified Verrucomicrobiaceae group of subdivision 1 of Verrucomicrobia and later the name of this group was coined to Candidatus genus Rhizospheria in Nunes da Rocha et al. (2010). The isolation of subdivision 1 Verrucomicrobia is therefore not new, but isolation from the plant-soil ecosystem is. It is still unknown whether the two groups are minority groups within the Verrucomicrobia. So far, only one report on the detection of Verrucomicrobia subdivision 1 was made in rhizospheres (Haichar et al., 2008), and hence we surmised that also this group may have been overlooked in the rhizosphere so far.

Quantitative PCR is currently a widely accepted approach in environmental microbiology. It is used to quantify bacterial gene or transcript numbers in environmental samples (Cardenas & Tiedje, 2008). Developing specific qPCR primers for detection in complex habitats like soil is a challenging endeavor because of the high bacterial diversity
in soil (van Elsas et al., 2008). Therefore, we opted for the validation of the designed qPCR primer systems in respect of their specificity in three steps, i.e. an *in silico* step followed by PCR on target and non-target DNA templates and by analysis of clone libraries generated with DNA from the same V soil. Further, specificity was improved by careful selection of melting temperature (around 60° C), annealing time (short, maximally 5 sec) and number of PCR cycles (relatively low, i.e. 35), in accordance with Edwards (2004) and Sipos et al. (2007). Our approach resulted in three sets of primers that were specific for the detection and quantification of the target *Holophagae*, *Luteolibacter/Prosthecobacter* and *Candidatus* genus *Rhizospheria* groups in soil. Microarray- and qPCR-assisted approaches for the detection and quantification of *Acidobacteria* and *Verrucomicrobia* in soil and thermal springs have been reported and all of these approaches have their merits and caveats (Hall et al., 2008; Kuramae et al., 2010; Liles et al., 2010). Our approach is different from these approaches because we validated the quantification systems using cells obtained from our cultured representatives of the three groups. This approach has not been done before with *Acidobacteria* and *Verrucomicrobia*. Our endeavor thus yielded a set of dedicated qPCR systems that quantify the cell numbers of three target groups of organisms in the plant-soil system.

The standard curves produced and the calculated theoretical dynamic ranges of all three qPCR systems revealed that cell numbers could be assessed over a broad spectrum (grossly, between Log 2 and Log 8 cell equivalents per g of dry soil) in soil. Quantification in soil was highly robust as demonstrated by the high $R^2$ values (over 0.980) for all three systems. The amplification efficiencies were all above 1.9, values that are acceptable for Sybr green detection in qPCR (Ruijter et al., 2009). The estimated limit of detection was between 100 and 500 cells per g of dry soil for all three qPCR systems, amounts that allow reliability in studies on the ecology of the three groups in the plant-soil ecosystem. An important further outcome of this study was that the three groups, *Holophagae*, *Luteolibacter* and *Candidatus* genus *Rhizospheria*, revealed to be more abundant in one or even both rhizosphere compartments than in the corresponding bulk soil. Moreover, the distinction between outer and inner rhizosphere made it possible to demonstrate that the numbers of both *Verrucomicrobia* groups became higher when coming into closer contact with the leek roots. For the *Holophagae*, this was different: although higher numbers were found in the outer rhizosphere than in bulk soil, the numbers were again lower in the inner rhizosphere. Supposedly, *Holophagae* cells do not thrive in spheres very proximate to roots or on the root surface, which may be the case for the *Verrucomicrobia* groups. One can only speculate about the driver of the preference for the outer rhizosphere in *Holophagae* cells. For instance, these cells may utilize some root-released compounds, but may be
Detection of *Holophagae* and *Verrucomicrobia* subdivision 1 in soil

Unable to compete with the root-associated bacteria typically occurring at root surfaces. We conclude that the members of the *Holophagae*, *Luteolibacter* and *Candidatus* genus *Rhizospheria* groups detected by our PCR systems appear to thrive in the rhizosphere. These groups may tentatively be considered ‘rhizosphere competent’ bacteria, as is the case for other well-known plant-associated bacteria like the fluorescent pseudomonads. Our data corroborate those of previous studies, such as those done in the rhizosphere of lodgepole pine (Chow et al., 2002) and different other plant communities (Zul et al., 2007). These studies, without specifying the subgroups, also demonstrated that *Acidobacteria* and *Verrucomicrobia* are numerically more abundant in the rhizosphere than in corresponding bulk soil. However, in this study we distinguish between bacterial groups at lower taxonomical resolution levels and demonstrated that particular groups of *Acidobacteria* and *Verrucomicrobia* may be considered as ‘rhizosphere competent’.

Acknowledgements

This research was part of the Ecogenomics program which is sponsored by the Dutch National Genomics Initiative and the basic research program on sustainable agriculture (KB4) sponsored by the Dutch ministry of agriculture, nature and food safety. We would like to thank Johnny Visser and field workers of ‘De Vredepeel’ experimental farm for their assistance in growth and sampling of leek plants.
Chapter 5

References


Detection of Holophagae and Verrucomicrobia subdivision 1 in soil


