Chapter 4: Isolation and partial characterization of *Holophaga*, *Luteolibacter*, unclassified *Verrucomicrobia* and *Verrucomicrobium* spp. from the leek (*Allium porrum*) rhizosphere*

Abstract

Strains affiliated with *Acidobacteria* (2) and *Verrucomicrobia* (5) were newly cultured from the leek rhizosphere. Phylogenetic analysis of these isolates plus four other verrucomicrobial strains - previously isolated from potato rhizosphere - were performed. The two acidobacterial strains isolated from leek were affiliated with the class *Holophagae* (former subgroup 8). All nine *Verrucomicrobia* belonged to subdivision 1 of this phylum, being that three of these resembled *Luteolibacter*, five unclassified *Verrucomicrobiaceae* and one *Verrucomicrobium*. Strains falling in the same group (*Holophaga*, *Luteolibacter* and unclassified *Verrucomicrobiaceae*) had >97% similarity on the basis of their 16S rRNA gene. They were therefore considered as the same species, but none of them was clonal (as determined by BOX-PCR). Also, a new name for the group of unclassified *Verrucomicrobiaceae* (*Candidatus* genus *Rhizospheria*) to be included in the family *Rubritaleaceae* (class *Verrucomicrobiae*, phylum *Verrucomicrobia*) was suggested. *Holophaga* isolates had similar phenotypic characteristics, indicating that they may occupy the same ecological niche. The phenotypic diversity within *Luteolibacter* and *Candidatus Rhizospheria* isolated from the rhizosphere of leek (this chapter) and those previously isolated from the potato rhizosphere (**chapter 3**) indicates that these isolates occupy different ecological niches in the soil-plant system.

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Submitted for publication

Introduction

Different reports have been published on the occurrence of *Acidobacteria* and *Verrucomicrobia* in the rhizosphere over the past decade (Sanguin et al., 2009; Kielak et al., 2008; Zul et al., 2007; Sanguin et al. 2006; Fillion et al., 2004; Gremion et al., 2003, Chow et al., 2002). *Acidobacteria* and *Verrucomicrobia* are bacterial phyla that are widely distributed over different ecosystems. Both represent phylogenetically very diverse groups. The phylum *Acidobacteria* is considered to be one of the most dominant bacterial groups present across soils (George et al., 2009), sediments (Ben Said et al., 2010), freshwater systems (Hardoim et al., 2009), lichen-associated bacterial communities (Hodkinson & Lutzoni, 2009), groundwater (Spain et al., 2007) and even in domestic toilets (Egert et al., 2010). The phylum *Verrucomicrobia* was also found to be among the dominant bacterial groups in communities present in soils and rhizospheres (Rosenberg et al., 2009), drinking water reservoirs (Lymperopoulou et al., 2010), human intestinal tract systems (Wang et al., 2005), contaminated groundwater (Herrmann et al., 2008), animal (gorilla) feces (Frey et al., 2006) and swine waste lagoons (Goh et al., 2009).

A total of twenty six different phylogenetic groups has been defined so far within the *Acidobacteria* (Barns et al., 2007), whereas seven subdivisions were found for the *Verrucomicrobia* (Schlesner et al., 2006). Information on the relative abundance of the individual groups within these phyla in different ecosystems is sparse. Therefore, the ecological niches of the different *Acidobacteria* and *Verrucomicrobia* are hardly characterized so far and so is their involvement in ecological processes. Moreover, contradictory information was provided on the occurrence of members of the two phyla in rhizosphere and bulk soils (Chow et al., 2002; Sanguin et al., 2006; Zul et al., 2007; Kielak et al., 2008). The general lack of knowledge is mainly due to the fact that many members of the two groups are recalcitrant to growth in pure culture (Nunes da Rocha et al., 2009). The availability of culturable strains would truly facilitate studies on their behavior in different ecosystems, including the putative involvement in key processes that can be predicted from biochemical, physiological, genetic and cell structural measurements in pure cultures (Zengler, 2009).

Although *Acidobacteria* and *Verrucomicrobia* are difficult to grow under confined conditions in the laboratory (Jones et al., 2009; da Rocha et al., 2010), procedures for their isolation, mostly from soil, have been described. In fact, successful isolations have been based on standard procedures with small modifications such as: (i) use of media low in nutrient availability (Janssen et al., 2002), (ii) increased levels of CO_2 in the surrounding atmosphere (Stevenson et al., 2004), (iii) selection of microcolonies (Ferrari et al., 2005), (iv) reduction of oxidative stress in the growth medium (Stevenson et al., 2004), (v) application of elongated incubation times (Janssen et al., 2002). Recently, an approach that included several of these modifications simultaneously was applied by us with the aim to improve bacterial isolation from the

potato rhizosphere. A total of four strains of subdivision 1 of the *Verrucomicrobia* (da Rocha et al., 2010) was successfully recovered upon plating of rhizosphere samples on agar media that allowed three- to ten-fold higher bacterial recoveries than on standard medium R2A. Questions about the prevalence and ecology of these strains in the rhizosphere still remain open. Also, the potential to obtain other hitherto uncultured *Verrucomicrobia* and *Acidobacteria* awaits further work. For our understanding of the ecology of *Verrucomicrobia* subdivision 1 strains, it is interesting to assess the culturability of organisms from this group from a rhizosphere different from that of the dicotyledonous potato and whether these are distinct from those from potato.

In this chapter, we hypothesized that novel *Acidobacteria* and *Verrucomicrobia* can be obtained by isolation from the rhizosphere of the monocot leek (*Allium porrum*). We thus searched for organisms of both groups defined on the basis of 16S rRNA gene sequences. Following isolation, selected strains from both phyla were characterized using a suite of biochemical, physiological, genetic and cell structural approaches. The final goal was to compare strains of the same group from different rhizospheres in order to infer their potential ecological roles in the plant-soil environment.

Material and Methods

Site description and leek rhizosphere soil collection

The site chosen for sampling in this study is an agricultural field located at the experimental farm 'De Vredepeel', The Netherlands (51^{o} 32' 27.10" N and 5^{o} 51' 14.86" E) where leek was grown. The soil was characterized as a sand (pH 5.4 and 2.2% of organic matter). Leek (*Allium porrum*) cultivar Kenton (Nunhems Zaden BV, The Netherlands) plants were collected by the end of January 2007 (*experiment* 1) and the beginning of February 2008 (*experiment 2*).

At both sampling occasions, seven leek plants, including the entire root system and the soil adhering to the roots, were randomly collected from the field with minimal distances of 5 m between the plants. The plants were transported to the lab and processed within 4 hours after collection (da Rocha et al., 2010). Following process, rhizosphere soil suspensions, made from the soil adhering to roots were prepared as previously described (da Rocha et al., 2010) and used for dilution plating, cell counting and total DNA extraction.

Bacterial recovery from the leek rhizosphere on agar media low in available carbon

Bacterial cells in the rhizosphere were enumerated by direct microscopy, as described by Bloem et al. (1995). Preparation of agar media for isolation, i.e. oligotrophic agar medium (OLI) amended or not with catalase (CAT) or leek rhizosphere extract (LEX) was performed as described by da Rocha et al. (2010). R2A (Difco, France) was used as

the reference medium. The following modifications to the incubation conditions used before (da Rocha et al., 2010) were applied, in addition to the previously used ones, for CAT and LEX plates: these plates were also incubated in an atmosphere of 5% CO_2 (elevated CO_2) and 16% O_2 (further denoted as CAT H and LEX H, respectively). Different plates were used in *experiment 1 and 2*, as follows: *Experiment 1* - R2A, OLI, CAT, LEX, CAT H and LEX H; *Experiment 2* - CAT H plates were used.

Significance of differences between cell and CFU numbers on different agar media were calculated by analysis of variance (ANOVA) using GenStat 12th edition (VSN International Ltd., UK). Differences were considered to be significant at levels of $P = 0.05$ and lower.

After 15 days of incubation, 360 (60 from each agar medium/incubation condition) colonies next to 225 (45 each from OLI, CAT and LEX incubated at normal and elevated CO_2) microcolonies (mCFU, colonies between 80-250 µm and only visible under 66 x magnification) from *experiment 1* and 150 colonies from *experiment 2* were randomly picked from plates that had received the two highest dilutions of the rhizosphere soil suspensions. Material from all colonies grew when streaked to purity on their respective agar media under the adequate CO_2 incubation conditions after 5 days of incubation for colonies and up to 150 days for mCFUs. Upon colony formation, material from single separate colonies was again transferred to fresh agar media of the same composition and incubated under the respective CO_2 conditions to allow the formation of new colonies.

All pure isolates were taken up and stored in stock solutions containing oligotrophic broth (OLI medium with omission of agar) supplemented with glycerol (final concentration 20%, w/w) at -80^{o}C.

Screening for representatives of Acidobacteria and Verrucomicrobia via 16S rRNA gene-assisted identification

Genomic DNA was extracted from all (in total 735) isolates using the MasterPure DNA purification kit (Epicentre, WI, USA) following instruction of the manufacturer. PCR amplification using primers 27F (Lane et al., 1985) and 1492R (Rochelle et al., 1992) was then performed on all DNA extracts, followed by single-strand sequencing of all amplicons using primer 1492R. Almost-complete 16S rRNA genes (> 1200 bp) were then sequenced for those isolates that showed closest matches with *Acidobacteria* and *Verrucomicrobia* (SILVA database, release 102 NR - Pruesse et al., 2007) as described in da Rocha et al. (2010). These larger sequences were again compared to those of the SILVA database using ARB software (Ludwig et al. 2004). Strains that showed closest matches with 16S rRNA gene sequences of *Acidobacteria* and *Verrucomicrobia* were selected for further analyses. These were supplemented with four strains from the potato rhizosphere identified as *Verrucomicrobia* subdivision 1 isolates (da Rocha et al. 2010).

Strains from leek and potato rhizospheres were different at the genome level as evidenced using BOX-PCR

BOX-PCR genomic profiling (Rademakers *et al*., 1998) of the 11 selected strains was performed to further distinguish these. DNA extraction was performed using the MasterPure DNA purification kit (Epicentre, WI, USA) following instruction of the manufacturer. Aliquots (1 µL) containing 1 ng of DNA were used as the templates for PCR reactions. After PCR amplification, 10 µL of each mixture was loaded onto 1.5 % agarose gel (20 cm in size), and gels were run at 24 V for 16h, 4^{o}C. Each gel contained three lanes, located at different places, loaded with 5 µL of 1-kb ladder (Invitrogen, Carlsbad, Ca) for normalization of the fingerprints. After staining with ethidium bromide, gel images were digitized using a digital camera and the digitized fingerprints were used for analysis with Gelcompar II software (Applied Maths, Belgium).

Morphological and physiological characterization of selected strains from leek and potato rhizospheres

Morphological and physiological parameters of selected isolates on individual cell and colony levels were determined. Suspensions with grown cells of all stains were Gram-stained. Then, the morphologies (cell morphological type, length and width) of at least 100 individual cells (at 1,000 x magnification) were determined according to Doetsch (1981). Further, cell motility was determined (Smibert & Krieg, 1981), assessing at least 50 cells per suspension, using the same magnification level. Also, colony size and morphology on the same colonies were described on at least 10 colonies per isolate (Doetsch, 1981).

To test for growth in liquid medium, suspensions were made of cells from single colonies on OLI agar. Optical densities of the resulting suspensions (OD600) were set at 0.05 before introduction into liquid OLI, 0.1 strength trypticase soy broth (1/10 TSB, BD, France), R2A, King's B (Roitman et al., 1990) and Luria-Bertani (LB) media (Sambrook et al., 1989). Cultures were incubated at 28^{o} C, with shaking, and the increase of OD600 values was measured. To test for growth on solid media, 5 µL cell suspensions in OLI were spread over the surfaces of R2A, 1/10 TSA, King's B and LB agar media (purified agar, 12.5 g L^{-1} used). Plates were incubated at 28^{o} C in the dark and monitored daily for colony growth to up to 3 months after inoculation.

Growth rates at colony level were determined on R2A in accordance with Wimpenny & Lewis (1977). *Escherichia coli* K12 was used as a reference strain for growth rate measurements at colony level.

Colony growth at different pH was tested on OLI agar with modified pH. The pH buffer composition described in Costilow (1981) was used, i.e. pH 4.0 was set with 0.1 M acetate buffer, pH 5.0 - 0.1M acetate buffer, pH 6.0 - 0.1M citrate-phosphate buffer, pH 7.0 - 0.1M phosphate buffer and pH 8.0 - Tris-Cl buffer. To test for growth

on organic acids and amino acids, we used OLI agar without glucose and casein hydrolysate supplemented with 54 mg L^{-1} of either oxalic acid, DL-(-)-malic acid, succinic acid, citric acid, L-glutamine or DL-alanine (Sigma-Aldrich Company Ltd., UK). Following inoculation, plates were incubated for up to 2 months at 25^{o}C and colony growth was determined regularly. As controls, OLI (da Rocha et al., 2010) without any carbon source was also inoculated and these plates were incubated and examined similarly. Growth on cellulose and cellulolytic activity measurements near the colonies were performed on OLI agar, with the glucose and casein hydrolysate substituted by 54 mg L^{-1} of cellulose (Sigmacell®, Sigma Chemical Co., USA). Colony formation and eventual formation of haloes surrounding individual colonies were recorded over time as in Smibert & Krieg (1981).

The presence of putative laccase genes in the genomes of the selected isolates was determined on genomic DNA extracts as templates using the PCR primers and conditions described by Ausec & Mandic-Mulec (2010).

Nucleotide sequence accession numbers

DNA sequences of the almost-complete 16S rRNA genes of the *Acidobacteria* and *Verrucomicrobia* strains recovered in this study were deposited in the EMBL Nucleotide Sequence Database under accession numbers **FN554388** to **FN554392** and **FN689719** to **FN689720**. DNA sequences of putative laccase gene were deposited in the EMBL Nucleotide Sequence Database under accession numbers **HM453207** to **HM453211**

Results

Bacterial numbers in the leek rhizospheres

Experiment 1. Seven healthy mature leek plants sampled from the V field soil revealed the presence of between Log 9.71 and 9.97 DTAF-stainable cells per g of dry soil (Table 1). The total Log colony numbers (including macro- and microcolonies) per g of dry soil from the same samples were in the range 7.87 - 8.61 on R2A; 8.91 - 9.39 on OLI; 9.10 - 9.47 on CAT; 9.16 - 9.48 on CAT H; 9.13 - 9.49 on LEX; and 9.12 - 9.39 on LEX H. This yielded colony recovery percentages (expressed as fraction of the number of DTAF-stainable cells) of 1.3 -4.4 on R2A, 15.9-26.1 on OLI, 24.6-31.9 on CAT, 25.5-32.3 on CAT H, 23.3-33 on LEX and 23.4-28.8 on LEX H. The culturability levels were clearly raised on OLI, CAT, CAT H, LEX and LEX H as compared to R2A. In fact, the differences between the CFU numbers on R2A versus those on OLI, CAT, CAT H, LEX and LEX H , irrespective of CO_2 level, were significant ($P<0.05$), whereas the CFU numbers on the latter media were statistically similar. We decided to focus on

Table 1. Total (DTAF-stainable) bacteria and CFU (normal plus microcolonies) in 7 different leek plants sampled in two different occasions.

Plant	*Experiment 1*							*Experiment 2*	
	DTAF stainable cells	R2A	OLI[a]	CAT	CAT H	LEX	LEX H	DTAF stainable cells	CAT H
	9.71	7.87 (1.5)[b]	8.91 (15.9)	9.10 (24.6)	9.16 (28.6)	9.13 (26.3)	9.12 (25.9)	9.65	9.11 (28.8)
	9.77	7.87 (1.3)	9.01 (17.6)	9.18 (25.8)	9.18 (26.1)	9.18 (25.9)	9.15 (24.3)	9.62	9.03 (25.7)
۲	9.80	8.09 (2.0)	9.02 (16.7)	9.22 (26.3)	9.20 (25.5)	9.22 (26.3)	9.19 (24.4)	9.67	9.07 (25.1)
	9.83	8.27 (2.7)	9.08 (17.6)	9.25 (26.1)	9.31 (30.0)	9.25 (26.1)	9.21 (23.7)	9.72	9.09 (23.4)
۴	9.90	8.38 (3.0)	9.16 (18.4)	9.29 (24.9)	9.36 (29.0)	9.28 (24.1)	9.23 (21.7)	9.78	9.17 (24.5)
	9.96	8.55 (3.9)	9.20 (17.5)	9.43 (29.6)	9.40 (27.5)	9.33 (23.3)	9.29 (21.5)	9.59	8.96 (23.4)
۶	9.97	8.61 (4.4)	9.39 (26.1)	9.47 (31.9)	9.48 (32.3)	9.49 (33.0)	9.39 (26.2)	9.74	9.18 (27.5)
Avarage[c]	9.85	8.24 (2.7)^{A}	9.11 (18.6)^{B}	9.28 (27.0)^{D,E}	9.30 (28.4)^{E}	9.27 (26.4)^{C,D,E}	9.23 (24.0)^{C}	9.68	9.09 (25.5)
Standard deviation	0.10	0.30 (1.2)	0.16 (3.4)	0.14 (2.7)	0.12 (2.3)	0.12 (3.1)	0.09 (1.8)	0.07	0.08 (2.0)

[a] OLI, CAT, LEX: low carbon availability agar medium, respectively non-amended or amended with catalase or leek rhizosphere extract; H for medium incubated at 5% CO_2 and 16% O_2 concentration

[b] Ratio (%) between CFU and DTAF stainable cells

[c] Significantly different, where A > B > C. Statistical analysis was made with the ratio (%) of CFU g^{-1} dry $soil^{-1}$ number to percentage and DTAF stainable cells (arcsine transformation) and calculated with one way ANOVA ($p < 0.05$)

CAT H for further isolations in experiment 2, as this medium yielded highest fractional colony recoveries.

Experiment 2. The DTAF-stainable cell numbers in seven leek rhizosphere samples were between Log 9.59 and 9.78 cells per g dry soil and CFU numbers on CAT H were between Log 8.96 and 9.18 CFU per g of dry soil, resulting in recovery percentages of between 23.4 and 28.8. CFU recovery on CAT H was again significantly higher than that on R2A in the same experiment (1.8-3.1%; not shown).

Selection of isolates belonging to the Acidobacteria and Verrucomicrobia

A total of 735 isolates (585 from *experiment 1* and 150 from *experiment 2*) were screened for the presence of isolates belonging to the *Acidobacteria* or *Verrucomicrobia* by partial sequencing of their 16S rRNA genes (amplicons approximately 350 bp in size). Among the isolates of *experiment 1*, a total of five in 585 (0.9%) presumptively fell in the *Acidobacteria*/*Verrucomicrobia*. Specifically, one isolate (CHC25) from CAT H showed >90% similarity (sim) to *Geothrix fermentans* (*Acidobacteria*), and four to *Rubritalea marina* (*Verrucomicrobia* subdivision 1). Of the latter, one (ONA9) was obtained from OLI, one (CNC16) from CAT, and two (CHC8 and CHC12) from CAT H (Table 2). In *experiment 2*, two of 150 (1.2%) isolates fell in the target phyla, being one (ORAC) from CAT H. This organism showed >90% similarity to *Geothrix fermentans* (*Acidobacteria*). The other one (IRVE), obtained from CAT H, affiliated to *Rubritaea marina* (*Verrucomicrobia* subdivision 1) (Table 2).

All isolates were isolated from media and conditions that allowed higher colony recoveries and even so appeared at a prevalence of roughly 1%. This strongly indicated that increased culturability on specific media is required to efficiently obtain representatives of these phyla from the leek rhizosphere. There was no preference for growth on any agar medium or CO_2 level in particular.

The strains obtained from the leek rhizosphere were pooled with four strains isolated from the potato rhizosphere, belonging to *Verrucomicrobia* subdivision 1 (da Rocha et al. 2010). On the basis of their almost-complete 16S rRNA genes, the new leek rhizosphere strains were subjected to further phylogenetic analyses. Strains CHC25 and ORAC, which were tentatively found to affiliate with *Acidobacteria* in the first run, again affiliated with this phylum, showing closest matches with *Geothrix fermentans* (accession number U41563, 94.2% sim), class *Holophagae* (previously known as *Acidobacteria* group 8). However, the highest similarity (97.5%) of both was with an uncultured bacterium (accession number GU169059) recovered from “synthetic river water with humic substances” (Fig 1A). The five strains isolated from leek rhizosphere

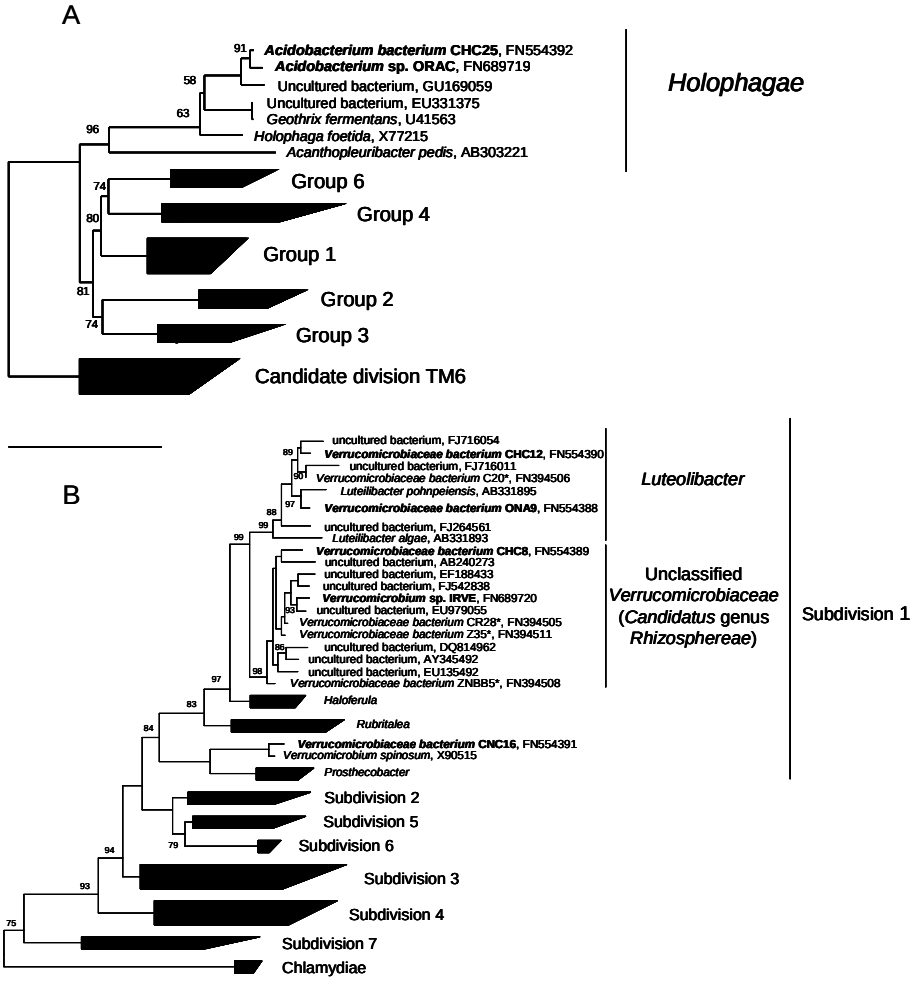

Figure 1 Taxonomic affiliation of *Verrucomicrobia* (A) and *Acidobacteria* (B) isolates obtained from the leek and/or potato rhizosphere. Distances between partial 16S rRNA gene sequences over 1100 bp in length were calculated using ARB software, the topology was reconstructed using ARB neighbour joining. A cluster made of 16S rRNA gene sequences of 10 different species from the *Chlamydiae* (A) or Candidate division TM6 (B) were used as out groups. Isolates followed by an asterisk (*) were recovered by Nunes da Rocha et al. (2010); and isolates in bold were recovered in this study. Bar at the bottom indicate 10% divergence among sequences and bootstrap values (calculated from 1000 iterations, %) are presented near each junction in the tree.

Table 2. Bacterial isolates used in this study.

Affiliation	Isolate/isolate	Nearest match[a]	Plant type	Year of isolation	Field/collection	Reference
Holophagae	CHC25	uncultured bacterium EU937971 (94.7%)	*Allium porrum*	2007	Vredepeel	This study
	ORAC	uncultured bacterium EU937971 (94.1%)	*Allium porrum*	2008	Vredepeel	This study
Luteolibacter	C20	uncultured bacterium FJ715972 (98.6%)	*Solanum tuberosum*	2006	Droevendaal	Nunes da Rocha et al. (2010)
	CHC12	uncultured bacterium FJ716054 (97.6%)	*Allium porrum*	2007	Vredepeel	This study
	ONA9	*Luteolibacter pohnpeiensis* AB331895 (98.0%)	*Allium porrum*	2007	Vredepeel	This study
Unclassified *Verrucomicrobiaceae* (*Canditatus* genus *Rhizospheria*)	CR28	uncultured *Verrucomicrobia* bacterium EU979055 (98.2%)	*Solanum tuberosum*	2006	Droevendaal	Nunes da Rocha et al. (2010)
	Z35	uncultured *Verrucomicrobia* bacterium EU979055 (98.8%)	*Solanum tuberosum*	2006	Droevendaal	Nunes da Rocha et al. (2010)
	ZNBB5	uncultured bacterium EU135492 (97.9%)	*Solanum tuberosum*	2006	Droevendaal	Nunes da Rocha et al. (2010)
	CHC8	uncultured bacterium DQ815271 (97.3%)	*Allium porrum*	2007	Vredepeel	This study
	IRVE	uncultured *Verrucomicrobia* bacterium EU979055 (98.5%)	*Allium porrum*	2008	Vredepeel	This study
Verrucomicrobium	CNC16	uncultured bacterium FJ230907 (98.7%)	*Allium porrum*	2007	Vredepeel	This study

[a] In accordance with RDP using SIMO analysis tools (http://simo.marsci.uga.edu/public_db/analysis_login.asp)

that fell in the *Verrucomicrobia* were shown to affiliate with different groups within this phylum. Strains CHC12, C20 and ONA9 showed nearest matches with *Luteolibacter pohnpeiensis* (accession number AB331895, 97.1 and 98.0% sim), genus *Luteolibacter*, subdivision 1 *Verrucomicrobia* (Fig. 1B). Strains CHC8 and IRVE grouped in a separate cluster denoted in the RDP classification as 'unclassified *Verrucomicrobiaceae*' of subdivision 1 (Fig. 1B). Finally, strain CNC16 showed the closest match with *Verrucomicrobium spinosum* (accession number X90515, 98.6% sim), genus *Verrucomicrobium*, subdivision 1 (Fig. 1B). Of the four potato rhizosphere strains, one, i.e. C20, grouped with *Luteolibacter pohnpeiensis* (accession number AB331895, 97.3% sim), whereas the remaining strains, i.e., CR28, Z235 and ZNBB5, clustered with unclassified *Verrucomicrobiaceae* (Fig. 1B).

Reports on the internal matches between the novel strains on the basis of their almost-complete 16S rRNA gene sequences, *Holophaga* CHC25 and ORAC were 98.9% similar, *Luteolibacter* CHC12, C20 and ONA9 97.4 - 99.0%, whereas unclassified *Verrucomicrobiaceae* CHC8, IRVE, CR28, Z35 and ZNBB5 were 97.6 - 99.1% similar (Table 3). In contrast, the BOX-PCR profiles (see Appendix Fig. A1) never showed similarities exceeding 91% Pearson correlation between strains (the cut-off limit set to 100 % similarity) (see Appendix Fig. A1), indicating that none of the isolates was identical to any other one.

Mophological and physiological characterization of the novel strains

The 11 novel *Holophagae*, *Luteolibacter* and unclassified *Verrucomicrobiaceae* strains were characterized at cell and colony levels (Table 4). In all cases, strains turned out to be Gram-negative, motile and able to grow on OLI agar, and even R2A media under atmospheric conditions. They were, therefore, considered to be aerobic and heterotrophic bacteria. *Luteolibacter* CHC12 and ONA9, unclassified *Verrucomicrobiaceae* CHC8 and *Verrucomicrobium* CNC16 were able to grow on 1/10 TSA, whereas none of the isolates grew on King's B or LB agar. Unclassified *Verrucomicrobiaceae* CHC8 and *Verrucomicrobium* CNC16 were the only strains that grew in liquid 1/10 TSB. *Holophagae* CHC25 and ORAC formed small rough colonies (1.0 mm in diameter), whereas those of *Verrucomicrobia* were larger (between 1.4 - 4.8 mm in diameter), isolate CHC8 being the largest for the unclassified *Verrucomicrobiaceae*. The colonies of all *Verrucomicrobia* isolates have a smooth shiny surface.

Table 3. Similarity[a] (%) of the 16S rDNA gene of the different *Holophagae* and *Verrucomicrobia* subdivision 1 isolates

Taxa	*Holophagae*		*Luteolibacter*			Unclassified *Verrucomicrobiaceae* (*Canditatus* genus *Rhizospheria*)					*Verrucomicrobium*
Isolate	CHC25	ORAC	CHC12	C20	ONA9	CHC8	IRVE	CR28	Z35	ZNBB5	CNC16
CHC25		98.9	71.1	73.9	70.8	70.5	71.2	72.6	73.4	73.8	71.1
ORAC			70.5	74.5	70.5	70.5	70.3	73.0	73.5	74.2	70.0
CHC12				99.0	97.4	93.3	93.0	93.9	93.8	94.4	83.7
C20					97.8	94.4	93.5	94.0	94.0	94.5	84.9
ONA9						93.4	93.5	94.4	94.3	94.6	83.8
CHC8							97.1	97.6	97.5	97.4	84.0
IRVE								99.1	99.0	97.6	82.9
CR28									99.0	97.6	84.3
Z35										98.2	84.7
ZNBB5											80.9
CNC16											

[a] Similarity levels were determined using ARB software (Ludwig et al., 2004)

Holophaga foetida CHC25 and ORAC only grew on OLI agar set at pH 4.0 and 5.0 and not on those with higher pH. *Luteolibacter* CHC12 and ONA9 grew on OLI agar with pH 6.0, 7.0 and 8.0 and not on those with lower pH. C20 only grew on OLI with pH 7.0 and 8.0. Unclassified *Verrucomicrobiaceae* strain CHC8 only grew on OLI agar at pH 5.0, 6.0, 7.0 and 8.0, CR28 only at pH 6.0 - 8.0 and IRVE, Z35 and ZNBB5 only at pH 7.0 and 8.0. *Verrucomicrobium* CNC16 grew on OLI agar at pH 8.0 and not at lower pH levels. The *Holophagae* strains thus grew under more acidic circumstances, whereas subdivision 1 *Verrucomicrobia* preferred more neutral to basic circumstances.

All strains were then examined for growth on oxalic acid, malic acid, succinic acid and citric acid, as well as the amino acids glutamine and alanine, as sole carbon sources. After 60 days of incubation, *Holophaga* CHC25 and ORAC grew on OLI agar containing either malic acid, succinic acid, citric acid, glutamine or alanine, but not on that containing oxalic acid. *Luteolibacter* CHC12, C20 and ONA9 differed from each other in carbon source utilization. C20 grew on all six OLI agar media, ONA9 only on the ones with glutamine and alanine and CHC12 did not form colonies at all on any of the six agar media. The five unclassified *Verrucomicrobiaceae* also showed different carbon source utilization patterns; CHC8 grew on all six carbon sources, IRVE only on oxalic acid, malic acid, glutamine and alanine, CR28 on succinic acid, citric acid and glutamine, Z35 on citric acid, glutamine and alanine and ZNBB5 did not form colonies with organic acids but grew on glutamine and alanine. *Verrucomicrobium* CNC16 only grew on OLI agar with glutamine and alanine.

The estimated growth rates of *Holophaga* strains CHC25 and ORAC were 12.3h and 13.8h, respectively, per cell division. The subdivision 1 *Verrucomicrobia* strains varied in estimated doubling times, i.e. between 9.6 and 68.8 h per cell division. The *Luteolibacter* CHC12, C20 and ONA9 had growth rates between 9.6h and 65.5h per cell division, the unclassified *Verrucomicrobiaceae* CHC8, IRVE, CR28, Z35 and ZNBB5 between 15.6h and 68.8h and *Verrucomicrobium* CNC16 14.6h. All strains grew significantly slower than the reference strain *E. coli* K12 (37 min) under the same conditions.

Growth on OLI agar with cellulose, cellulose hydrolysis activity and presence of putative laccase genes were measured. Only unclassified *Verrucomicrobiaceae* CHC8 showed growth and halo formation on OLI agar containing cellulose as sole carbon source. Putative laccase genes, using laccase gene-specific PCR amplification, were found to be present in five subdivision 1 *Verrucomicrobia*, ie. *Luteolibacter* CHC12 and ONA9, unclassified *Verrucomicrobiaceae* IRVE and CR28 and *Verrucomicrobium* CNC16. Sequence analyses of amplified fragments from all five isolates by BLAST-assisted searches revealed that all five isolates contained a putative 3-domain laccase gene (see Appendix Table 1).

Overall, the two *Holophagae* strains strongly resembled each other, whereas the nine subdivision 1 *Verrucomicrobia* substantially differed from each other.

Discussion

The major outcome of this chapter is the isolation of novel *Acidobacteria* and *Verrucomicrobia* strains from leek. Remarkably, all 11 strains belonged to single subgroups within each phylum, i.e. to the *Holophagae* (formerly known as group 8 *Acidobacteria*) and to the subdivision 1 *Verrucomicrobia*. The isolation of subdivision-1 *Verrucomicrobia* from leek was in line with previous isolations from potato (da Rocha et al., 2010). This would indicate that subdivision-1 *Verrucomicrobia* are typical rhizosphere bacteria that abound in different plant species. However, the nine strains that we analyzed strongly differed from each other in phenotypic and genotypic terms, being classified in three distinct clades within the subdivision. Differences in their preferred niches in the rhizosphere are likely on the basis of the data; this aspect will be further investigated in a follow-up study. The close resemblance of the two leek *Holophaga foetida* strains indicates this monophyletic group within the *Acidobacteria* may particularly associate with leek. However, it is not possible to draw firm conclusions on the basis of the low number (two) of strains from this group obtained in the current study.

All novel strains were isolated under conditions that allowed significantly raised recovery rates from rhizosphere soil. Bacterial counts rising up to 33% of the total bacterial cell fractions were reached, corroborating what we previously achieved for the potato rhizosphere using the same media (da Rocha et al., 2010). Improved bacterial culturability as a result of incubation under conditions better-tuned to the natural environment has been achieved before with bulk soil (Janssen et al., 2002; Sait et al., 2002; Schoenborn et al., 2004; Davis et al., 2005) and freshwater samples (Bruns et al., 2003). In these studies, key factors contributing to improved cultivation were: i) reduced nutrient availability, ii) prolonged incubation times and iii) reduction of oxidative stress by the addition of protective agents. Recoveries from natural environments can thus be increased by simple modifications of already existing protocols, yielding access to hitherto uncultured bacterial groups, as demonstrated in the current study as well as in others (Janssen et al., 2002).

Strikingly, two *Holophagae* were obtained from the leek rhizosphere. To the best of our knowledge, this is the first report on isolation of an *Acidobacterium* species from the plant rhizosphere. The class *Holophagae* represents a small group within the *Acidobacteria.* Estimated population sizes may range from 0 and 3.4 % of the total *Acidobacteria* community present in (bulk) soils (Jones et al., 2009). Due to the fact that

Table 4. Morphological, phenotypical and physiological characteristics of *Acidobacteria* and subdivision 1 *Verrucomicrobia* isolates from leek and potato rhizospheres

Characteristic	*Acidobacteria Holophagae*		*Verrucomicrobia* subdivision 1								
			Luteolibacter			Unclassified *Verrucomicrobiaceae* (*Canditatus* genus *Rhizospheria*)					*Verrucomicrobium*
	CHC25	ORAC	CHC12	C20	ONA9	CHC8	IRVE	CR28	Z35	ZNBB5	CNC16
Gram staining[a]	neg.	neg.	neg	neg	neg	neg. pos.	neg	neg	neg. pos.	neg	neg
Cell morphology	Filamentous short chains	Filamentous short chains	Single rod	Single rod	Staphylo cocci	Single fusiform	Staphylo rod	Single rod	Single fusiform	Single rod	Staphylo rod
Cell motility[b]	+	+	+	+	+	+	+	+	+	+	+
Growth[c]											
Liquid Media[d]	-	-	-	-	-	+	-	-	-	-	+
Agar Media[e]											
OLI	+	+	+	+	+	+	+	+	+	+	+
R2A	+	+	+	+	+	+	+	+	+	+	+
1/10 TSA	-	-	+	-	+	+	-	-	-	-	+
King's B	-	-	-	-	-	-	-	-	-	-	-
LBA	-	-	-	-	-	-	-	-	-	-	-
pH 4.0	+	+	-	-	-	-	-	-	-	-	-
5.0	+	+	-	-	-	+	-	-	-	-	-
6.0	-	-	+	-	+	+	-	+	-	-	-
7.0	-	-	+	+	+	+	+	+	+	+	-
8.0	-	-	+	+	+	+	+	+	+	+	+
OAs[f] Oxalic acid	-	-	-	+	-	+	+	-	-	-	-
Malic acid	+	+	-	+	-	+	+	-	-	-	-
Succinic acid	+	+	-	+	-	+	-	+	-	-	-
Citric acid	+	+	-	+	-	+	-	+	+	-	-
AAs[g] Glutamine	+	+	-	+	+	+	+	+	+	+	+
Alanine	+	+	-	+	+	+	+	-	+	+	+
Colony morphology											

Size (diameter, mm)	1.0	1.0	2.3	1.9	1.7	4.8	1.8	2.0	1.8	1.4	1.9
Form	punctiform	punctiform	circular	circular	circular	slightly spindle	circular	circular	circular	circular	circular
Surface	rough	rough	shine	shine	shine	shine	shine	shine	shine	shine	shine
Texture	dry	dry	viscous	viscous	viscous	mucoid	viscous	viscous	viscous	viscous	viscous
Color	yellow / brownish	yellow / brownish	white / pink	opaque yellow	bright yellow	bright yellow	bright yellow	bright yellow	bright yellow	bright yellow	white
Elevation	flat	flat	flat	convex	convex	convex	convex	convex	convex	convex	flat
Margin	entire	entire	entire	curled	curled	curled	curled	curled	curled	curled	entire
Colony doubling time (g)[h]	12.3h	13.8h	32.7h	65.5h	9.6h	18.2h	68.8h	68.8h	33.8h	15.6h	14.6h
Cellulase activity	absent	absent	absent	absent	absent	present	absent	absent	absent	absent	absent
Laccase gene	absent	absent	present	absent	present	absent	present	present	absent	absent	present

[a] neg., cell stained Gram negative; pos., cells stained Gram positive; neg. pos., cells of young culture were Gram negative and old cultures (more than 30 days old) stained Gram positive

[b] Determined by light microscopy – bacterial cells were considered to be motile when movements arrested upon administration of 0.1% sodium azide

[c] +, positive growth; -; negative growth

[d] Liquid media used – Low carbon availability medium (OLI), R2B (R2A without addition of agar), 1/10 TSB (trypticase soy broth), King's B and LB. Positive growth was only detected on 1/10 TSB

[e] OLI, low carbon availability medium; TSA (trypticase soy agar broth)

[f] OAs, organic acids. Growth in organic acids and amino acids was measured changing Glucose for the respective organic or amino acid

[h] AAs, amino acids

[g] Doubling time estimated on the bases of colony size during exponential growth phase on R2A; h, hours

[i] Pink colour was developed after 14 days incubation

other *Acidobacteria,* i.e. those of groups 1, 2, 3, 4 and 6, are present in much higher numbers in soils, *Holophagae* may have been overlooked in the past. Also, members of this group may prefer sites proximate to plant roots. Other *Acidobacteria*, especially group 6 ones, have been detected before in the rhizosphere of different plants via culture-independent approaches (Schmalenberger & Tebbe, 2003; Sharma et al., 2005; Wang et al., 2007; Hao et al., 2008). However, no culturable representatives from the group-6 *Acidobacteria* have ever been recovered from the rhizosphere.

Many uncertainties still exist about the roles of the cultured fastidious bacteria in the rhizosphere. The *Holophagae,* strains CHC25 and ORAC, were motile, indicating the possibility of chemotaxis, adherence to substrates, biofilm formation and even swarming (Young, 2007). The filamentous short chains formed by CHC25 and ORAC cells may be an indication that these bacteria are adapted to resist bacterivory (Young, 2007). Also, strain CHC25 and ORAC cells could not grow in liquid media, a phenomenon observed for other *Acidobacteria* isolates (Valáková et al., 2009). Within the group of *Holophagae*, only three cultures have been described to date, i.e.*Holophaga foetida* (accession number X77215), *Geothrix fermentans* (accession number U41563) and *Acanthopleuribacter pedis* (accession number AB303221). Both *Geothrix fermentans* (Coates et al., 1999) and *Holophaga foetida* (Liesack et al., 1994) are strictly anaerobic bacteria isolated from hydrocarbon-contaminated areas, whereas *Acanthopleuribacter pedis* is a strictly aerobic bacterium isolated from a beach chiton (Fukunaga et al., 2008). This demonstrates that the physiology within this group may be diverse. Recently, *Holophagae* were detected in clone libraries made from the endophytic bacterial community in rice (*Oryza sativa* L.) roots (accession number DQ340903) (Sun et al., 2008), as well as from the rhizosphere of *Phragmites* (accession number AB240249). We hypothesize that particular members of *Holophagae* live in association with plants, either in the rhizosphere or even as endophytes. It may well be that the ones that live in association with plants, or eukaryotes in general, actually have an aerobic and heterotrophic lifestyle.

A high diversity among the subdivision-1 *Verrucomicrobia* was further observed in the current study and in that of da Rocha et al. (2010). Although 16S rRNA gene sequences from this phylum may be more abundant in rhizosphere than in bulk soils (Chow et al. 2002; Zul et al. 2007), no information about their ecological roles in the rhizosphere is available. All unclassified *Verrucomicrobiaceae* were able to grow on organic acids and/or amino acids. These compounds are common in the rhizospheres of different plant species and our novel strains will likely be able to grow in the proximity of roots of plant species that exude such compounds (Jones, 1998; Baudoin et al., 2003). Cells of CHC8 showed cellulase activity, indicating that they may be able to grow on plant material in soil and

even colonize living roots (Mostajeran et al., 2007) and/or the interior tissues of plants (Bischoff et al., 2009). Five of the nine *Verrucomicrobia* subdivision 1 isolates yielded evidence for the presence of laccase genes, which is interesting as these enzymes may specifically oxidize phenolic and non-phenolic lignin-related compounds (Kunamneni et al., 2008).

Subdivision-1 *Verrucomicrobia* have been isolated before from other environments, e.g. from soil, freshwater (Schlesner, 1987; Hedlund et al., 1996) and marine environments (Hedlund et al., 1996; Scheuermayer et al., 2006; Yoon et al., 2008). The strains found here fell in three groups, *Luteolibacter*, unclassified *Verrucomicrobiaceae* and *Verrucomicrobium* (*Verrucomicrobia* subdivision 1). This is the first report on cultivation of members of this "unclassified *Verrucomicrobiaceae"* group, which is a tight hitherto-unnamed, group in the verrucomicrobia. We propose to coin this group "*Candidatus* genus *Rhizospheria*". It is naturally included in the family *Rubritaleaceae* (class *Verrucomicrobiae*, phylum *Verrucomicrobia*). The name of the proposed group is based on the repeated isolation of the cultured representatives of this group from the leek rhizosphere.

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. We also thank An Vos, Meint Veninga and Jaap Bloem for their help with total bacterial counts.

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