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SOIL MICROBIOLOGY

Isolation and Partial Characterization of Bacterial Strains on Low Organic Carbon Medium from Soils Fertilized with Different Organic Amendments

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Abstract A total of 720 bacterial strains were isolated from soils with four different organic amendment regimes on a low organic carbon (low-C) agar medium (10 μg C ml^{-1}) traditionally used for isolation of oligotrophs. Organic amendments in combination with field history resulted in differences in dissolved organic carbon contents in these soils. There were negative correlations between total and dissolved organic carbon content and the number of isolates on low-C agar medium, whereas these correlations were absent for bacterial strains isolated from the same soil on high-C agar medium (1,000 μg C ml^{-1}). Repeated transfers (up to ten times) of the isolates from low-C agar medium to fresh low- and high-C agar media were done to test for exclusive growth under oligotrophic conditions. The number of isolates exclusively growing under oligotrophic conditions dropped after each subsequent transfer from 241 after the first to 98 after the third transfer step. Identification on the basis of partial 16S rRNA gene sequences revealed that most of the 241 isolates (as well as the subset of 98 isolates) belong to widespread genera such as *Streptomyces*, *Rhizobium*, *Bradyrhizobium*, and *Mesorhizobium*, and the taxonomic composition of dominant genera changed from the first transfer step to the third. A selected subset of 17 isolates were further identified and characterized for exclusive growth on low-C agar medium. Two isolates continued to grow only on low-C agar medium up to the tenth transfer step and matched most closely with *Rhizobium sullae* and an uncultured bacterium on the basis of the almost full-length 16S rRNA gene. It was concluded that the vast majority of strains which are isolated on low-C agar media belong to the trophic group of microorganisms adapted to a "broad range" of carbon concentrations, including well-known and widespread bacterial genera. Oligotrophy is a physiological, not a taxonomic property, and can only be identified by cultural means so far. We showed that true oligotrophs that are unable to grow on high carbon media are rare and belong to genera that also contain broad-range and copiotrophic strains.

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Introduction

The impact of soil management on trophic groups of bacteria in soil is poorly understood. Several studies have been conducted to understand the relationship between microbial community structure and soil characteristics after different soil treatments or cropping regimes [9, 45]. Others focused on the impact of organic amendments on bacterial communities [4] or on the relationship between soil

nutrients (especially carbon and nitrogen) and microbial populations [7]. However, only a limited number of studies are available on the effects of organic substrates or inorganic nutrients on oligotrophic populations in soils [17, 48]. We define "oligotrophs" as bacteria able to grow at (extremely) low and not at high nutrient availabilities, in accordance with definitions proposed before [31, 34].

Oligotrophic bacteria are widely distributed and have been isolated from different environments [8, 11, 19, 34, 38]. This trophic group is taxonomically diverse and includes Gram-positive as well as Gram-negative species [15, 46]. Although oligotrophic bacteria have not been studied as frequently as copiotrophic or eutrophic strains, experts in this field contend that oligotrophs constitute the majority of bacteria in natural environments [29]. Oligotrophs are distinct from other bacterial groups because of their trophic properties, i.e., their ability to exploit ecological niches that are low in substrate concentrations and energy flows [34]. Oligotrophs are *K* strategists (although not all *K* strategists are oligotrophs), while copiotrophs are often *r* strategists [43]. *K* strategists grow slowly and consistently, both at low and high nutrient availabilities, whereas *r* strategists grow faster and respond more abruptly to (high) amounts of easily available nutrients and may die or become dormant when their surroundings are deprived of readily accessible nutrients. Oligotrophic bacteria are commonly called "true" or "obligate" oligotrophs when they possess narrow reaction ranges in terms of extremely low Km values, energy maintenance coefficients, and low respiratory rates [34]. There is no clear consensus on the definition of oligotrophic bacteria, but they share their common preference for (extremely) low nutrient availabilities [34].

These bacteria are of great interest because they may play an important role in the decomposition of organic matter and nutrient dynamics, as they can bring the glucose concentrations below the threshold level for catabolite repression of hydrolytic enzymes, thereby contributing to the activity of eutrophic bacteria [34, 37]. The limited amount of knowledge about this group of bacteria restricts further exploration on their role in important soil processes, like mineralization and plant growth support. Main constraining factors are: (1) the lack of (known) functional commonalities among oligotrophs that distinguish them from copiotrophs, (2) the intrinsic difficulties to cultivate them with respect to nutrient availability and composition of their growth media [31], and (3) their taxonomic heterogeneity.

Standardized techniques for detection of oligotrophs in natural environments are not available, irrespective whether they are based on culture-dependent or independent approaches. Only traditional carbon-limited culture media can be used for isolation of oligotrophs from the environment. Common low-carbon media used for isolation of these bacteria are 10^4-fold diluted broth [12, 37, 43] or 100-fold diluted S medium originally containing 1,000 μg C ml^{-1} [39]. The obtained isolates are sometimes tested for the absence of growth on high-C agar medium, to distinguish (true) oligotrophs from copiotrophs [10, 29, 33, 44]. Molecular markers in oligotrophic bacteria suitable for detection have been proposed [20], but not yet developed. Oligotrophic bacteria commonly belong to particular bacterial groups, like the *Alphaproteobacteria* and *Gammaproteobacteria* [5, 10, 33, 47]. Knowledge about characteristics of oligotrophic bacteria is important for development of detection tools, based on phylogenetic (e.g., 16S rRNA-gene-based) or functional (physiological) markers that can quantify oligotrophic populations in natural environments like soils.

Based on our previous research [17], we hypothesized that the oligotrophic bacterial fraction will be lower in soils with relatively higher amounts of available carbon and that repetitive growth on low-C agar medium will result in the selection of true oligotrophs.

The aims of this study were to:

1. Isolate bacteria on low organic carbon media from soils differing in organic matter management
2. Investigate the relation between the population densities of these bacteria and the carbon contents in the soils
3. Test the growth rates of selected isolates on high- and low-C agar media and determine the proportion of true oligotrophs among the isolates growing on low-C agar media
4. Tentatively identify the isolates on the basis of the nearest matches of their 16S rRNA sequences with 16S rRNA genes available in databases

Materials and Methods

Site Description and Sample Collection

Two fields (denoted as fields 1 and 6) located at the organic experimental farm Droevendaal, The Netherlands (coordinates, W 5.66 and N 51.99), were selected for sampling. These fields differed in the history of agricultural management practices and crop rotation during at least 3 years before sampling (in 2005). Field 1 was previously used for conventional arable crop production (potatoes in 2001), whereas field 6 was a pasture with organic management (i.e., without chemical fertilizers or pesticides). In the last 3 years before sampling, both fields were managed organically and planted with field crops rotated with a grass-clover ley. In both fields, small plots were established in 2002 with

different organic amendments plowed under to a depth of 20 cm. There were four treatments: (1) 72 m^3 ha^{-1} slurry (liquid cattle manure; S), (2) 12 ton ha^{-1} plant-derived carbon amendments (green waste compost; C), (3) 43 m^3 ha^{-1} liquid and 27 ton ha^{-1} solid cattle manure (dung; SD), and (4) 37 m^3 ha^{-1} slurry, 27 ton ha^{-1} dung, and 11 ton ha^{-1} plant-derived amendments (CSD). The treatments were applied in duplicate plots in each field. Fields were 36× 28m in size including paths, and plot sizes were 8×9 m. From each plot, duplicate soil samples (10 kg, including roots) were collected in September 2005 from the top layer (0–20 cm). The samples were stored for not longer than 1 week at 15°C for chemical and microbiological analyses

Soil Chemical Analyses

Subsamples (12 g) of the large soil samples were dried at 40°C for 24 h and ground. Total carbon was measured by the Dumas method [24] followed by detection by a CHN1110 element analyzer (CE Instruments, Milan, Italy). Total nitrogen was determined by the Kjeldahl method [3]. Nitrate content was determined with an Autoanalyzer II (Technicon Instrument Corporation, Tarrytown, NY, USA) after addition of 0.01 M $CaCl_2$. Phosphorus and potassium contents were measured according to Novozamsky protocols [25]. The pH and dissolved carbon were determined according to the protocol of Houba [16].

Dilution Plating on Organic Low-C and High-C Agar Media

For bacterial isolation, 0.5 g subsamples were suspended in 5 ml sterile tap water, sonicated (47 kHz) for 1 min and vortexed for 10 s, and the suspensions were tenfold serially diluted. Fifty microliters of each suspension was pipetted onto high-C agar medium (0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KNO_3, 1.3 g $KH_2PO_4 \cdot 3H_2O$, 0.06 g $Ca(NO_3)_2 \cdot 4H_2O$, 2.5 g glucose, 0.2 g enzymatic casein hydrolysate, 15 g technical agar [Oxoid nr.3], 1 l demineralized water; and 100 mg l^{-1} filter [0.22 μm pore size]-sterilized cycloheximide (100 mg l^{-1}) was added to the medium after autoclaving at 121°C for 20 min). The low-C agar medium [35] had the same composition as high-C agar medium, except that enzymatic casein hydrolysate and glucose were 100-fold lower (2 and 25 mg, respectively) and that Noble Agar (Difco Labs, Detroit) was used. All plates were incubated at 25°C for 2 days (high-C agar medium) or for 15 days (low-C agar medium) before colony counting. Log-transformed colony-forming units (CFUs) were calculated per gram of dry soil.

Selection for Oligotrophic Isolates

A total of 45 colonies per sample (16 samples) from the highest diluted suspensions (10^{-4} dilution) on low-C agar media were aseptically transferred to fresh low- and high-C agar media (first transfer). Upon incubation, those colonies from low-C agar medium that did not form visible colonies on high-C agar medium but did on low-C medium were selected and subsequently transferred to fresh low-C and high-C agar media (second transfer). Upon incubation, again, those colonies that exclusively grew on low-C agar medium were further selected (third transfer). All isolates from the first and third transfer steps were streaked onto fresh low-C agar medium to be used for taxonomic identification. A selected subset (17) of the colonies obtained after the third transfer were further tested for growth on low- and high-C agar media up to the tenth transfer step.

Colony Growth Measurements

Colony growth of the selected subset of 17 isolates was followed on low- and high-C agar media during 14 days at the same incubation temperature. Colonies were inspected at ×50 magnification using a StemiSV11 binocular (ZEISS, Germany) attached to an AxioCam MRc camera (ZEISS, Germany), and images of individual colonies were digitized daily. Diameters from ten digitized images per isolate were measured using Axio software (ZEISS, Germany) for calculation of colony growth rate.

Molecular Identification of Oligotrophic Isolates

Bacterial cells from pure colonies were suspended in liquid low-carbon medium (same composition as low-C agar medium, except that agar was omitted). DNA extracts were made from these cell suspensions using the PUREGENE Genomic DNA Isolation Kit (Gentra systems, USA) according to the protocol for cultured cells provided by the manufacturer. Partial 16S rRNA gene fragments were amplified from these extracts by polymerase chain reaction (PCR) using bacterial primers 27F [18] and 1492R [32]. Fifty-microliter PCR reaction mixtures were prepared containing 1 μl of DNA extract (5 – 50 ng), 200 μM of each deoxyribonucleoside triphosphate, 0.2 μM of each primer, 1× SuperTaq buffer (HT Biotechnology LTD, Cambridge, UK), and 5 U SuperTaq Polymerase (HT Biotechnology LTD). Amplification was performed in a PTC-100 thermal cycler (MJ Research, Inc., Tilburg, The Netherlands) using a program of 94°C for 4 min followed by 35 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 1 min, followed by a final extension step at 72°C for 5 min.

PCR fragments of approximately 1,400 bp were purified using the Wizard DNA Cleanup kit (Promega, USA) for sequencing. For that purpose, purified PCR products were added to reaction mixtures containing 5 μl of sequencing mixture, 1 μl of DETT Dye (DYEnamic ET Terminator

Cycle Sequencing Kit, Healthcare, GE), 3 μl of dilution buffer, and 1 μl (0.5 μM) of primer 1492R. Linear amplifications were performed for 25 cycles at 94°C, 20 s; 50°C, 15 s; and 60°C, 60 s. The amplified products, approximating 600 bp in size, were sequenced in an ABI prism automatic sequencer by making use of the services of Greenomics (Plant Research International, Wageningen, The Netherlands). For sequencing of larger fragments of 16S rRNA genes, PCR amplicons were first PCR-amplified with the following primers: 27F, R530 [23] and 968R [13] and 1492R. Then contiguous fragments of approximately 1,400 bp in size were assembled. Sequences from all fragments were compared with Ribosomal Database Project (RDP) sequences using the RDP analysis tool Sequence Match (http://rdp.cme.msu.edu/) and Basic Local Alignment Search Tool at default settings [1, 6]. All 16S rRNA gene sequences were deposited in the EMBL database and are available under accession numbers AM709973 through AM710213.

Statistical Analysis

The experiment was designed according to a randomized split-plot scheme, consisting of two fields, divided into two blocks having four plots each with one treatment per plot including two sampling points per plot. Duplicate values of the logarithm of the original CFUs on low- or high-C agar medium per gram of dry soil from the same plot were averaged and so were the duplicate values of each chemical variable. The Shannon diversity values were calculated on the basis of phylogenetic differences in low-C for each treatment. For calculation, the following equation was used: $H = -P_i \log P_i$, where $P_i = n_i/N$ and n_i is the number of isolates per genus on low-C agar medium, and N is the total number of isolates on this medium. Pairwise comparisons of treatments and fields with respect to log CFU, chemical, and Shannon diversity values were made by two-tailed t tests using SAS statistical analysis software (SAS Institute, Cary, NC, USA). Quantitative relationships between the fractions of total and dissolvable organic carbon and log-transformed CFUs were determined using linear regression analysis in SAS statistical analysis software. Differences were considered to be significant at levels of $P \leq 0.05$.

Results

Soil Chemical and Microbiological Measurements

Chemical parameters measured in soils from the 16 plots differed per treatment and field (Table 1). The pH values among the eight soil samples ranged between 4.48 and 4.95, and they were significantly lower in field 1 than in field 6. Per treatment, the pH was lowest in C-treated soils of field 1 and in SD-treated soils of field 6.

Total organic carbon in the differently treated soils ranged between 10.03 and 18.16 g kg^{-1} soil, and the dissolvable organic carbon content ranged between 65.47 and 101.67 mg kg^{-1}. Per treatment, values for total carbon and dissolved organic carbon were significantly lower in field 1 than in field 6, and the lowest values in total C were for S-treated soils in both fields.

Values for total nitrogen ranged between 1.15 and 1.68 g kg^{-1} and those of nitrate between 0.16 and 0.26 g kg^{-1}. Values for total nitrogen were highest in SD- and CSD-treated soils of field 1 and in S-treated soils of field 6, whereas nitrate was approximately the same for all treatments.

Values for total C/N ratio ranged between 7.65 and 11.98, and the C/N ratio was lower in field 1 than in field 6 soils. Values were lowest in CSD-treated soils of field 1 and in S-treated soils of field 6.

Log-transformed CFUs on low-C agar medium were between 6.45 and 6.73 g^{-1} dry soil, and these numbers were one to two orders higher than log CFUs on high-C agar medium, which ranged from 4.62 to 5.11 g^{-1} dry soil. The average log CFUs on low-C agar medium were about the same in both fields (6.63 in field 1 and 6.61 in field 6, both gram of dry soil) and so were the average log CFUs on high-C agar medium (5.05 in field 1 and 4.97 in field 6). Per treatment, no significant differences were found between CFUs of both trophic groups.

Negative linear correlations were present between log CFUs on low-C agar medium and total carbon (Ctot) and dissolvable organic carbon (DOC) fractions in the 16 soil samples (Table 2), indicating that the carbon contents in these soils have a negative effect on the number of CFUs recovered on low-C agar medium. No correlations were found between the number of CFUs on high-C agar medium and the concentrations of total or dissolvable carbon in the soils.

Bacteria Isolated on Low-C Agar Medium and Selection of Oligotrophic Isolates

In total, 32 Petri plates with 10^{-4} diluted suspensions were obtained for the low-C agar medium and the same number of plates for the high-C agar medium. On average, 22 colonies grew on each low-C plate after 15 days of incubation (longer incubation until 21 days did not lead to significantly more CFU but led to overlapping of colonies [35]). A total of 717 colonies (three colonies did not grow further after transfer) were obtained on low-C agar plates with 10^4 diluted suspensions from all soil samples. These isolates must be considered as the culturable fraction that could grow at a "broad range" of carbon concentrations,

Table 1 Mean values of soil chemical and microbiological parameters measured in differently treated plots from fields 1 and 6 at the Droevendaal experimental farm of Wageningen UR, the Netherlands

Soil parameter	Field 1[a]				Field 6			
	S	C	SD	CSD	S	C	SD	CSD
pH	4.88a	4.74b	4.95a	4.89a	4.57	4.5	4.48	4.58
Total C (g kg^{-1})	10.03	11.12	11.27	10.06	15.25	18.16	16.84	17.94
DOC (mg kg^{-1})	65.47b	68.16b	74.13a	75.03a	91.40	98.66	101.67	89.68
Total N (g kg^{-1})	1.15b	1.24ab	1.42a	1.32a	1.68a	1.58b	1.51b	1.53ab
N-NO_3 (g kg^{-1})	0.18	0.23	0.24	0.26	0.22	0.19	0.17	0.16
C/N ratio	8.76a	8.98a	7.96b	7.65b	9.11b	11.52a	11.19a	11.98a
Log CFU on low-C agar media[b]	6.73	6.61	6.69	6.50	6.55	6.50	6.45	6.47
Log CFU on high-C agar media[b]	5.00	5.09	5.11	4.99	4.80	4.78	4.75	4.62

[a] S, C, SD, and CSD denote soils treated with, respectively, slurry, compost, slurry and dung, and compost, slurry plus dung. Values with different letters indicate a significant difference ($P \leq 0.05$), where a > b

[b] Expressed in log CFU per gram dry soil

including oligotrophic conditions. All isolates were tested for exclusive growth on low-C agar medium in the absence of growth on high-C agar medium. After the first transfer, 241 isolates were found to grow exclusively on low-C agar medium, and this number decreased after the second (124) and third (98) transfer steps (Fig. 1). There was thus a tendency for leveling off towards a number of oligotrophic isolates that exclusively grew on low-C agar medium (Fig. 2).

Preliminary Identification of Oligotrophic Isolates

All 241 isolates obtained on low-C agar medium after the first and the subset of 98 isolates obtained on the same medium after the third transfer step were identified by comparison of their 16S rRNA gene sequences with those present in the RDP database. Oligotrophs selected after the first transfer showed best matches with bacterial sequences from 32 different genera (Fig. 3). The genera occurring at the highest frequencies among this pool of isolates were: *Streptomyces* (30.7%), *Mesorhizobium* (11.2%), *Bradyrhizobium* (10.4%), *Rhizobium* (7.1%), and *Nocardia* (7.1%). The isolates selected after the third transfer step belonged to 11 genera, and the most frequent occurring ones were: *Mesorhizobium* (27.6%), *Bradyrhizobium* (25.5%), and *Rhizobium* (17.3%). The clearest difference between both sets of isolates was the number of isolates affiliated with *Streptomyces* species, which was absent among the isolates after the third transfer step. Most remarkable is the high abundance of isolates affiliated with *Alphaproteobacteria* (73.5% after the third transfer step), indicating that most of the oligotrophs in these soils belonged to this bacterial class.

The Effect of Soil Treatment on CFUs on Low-C Agar Medium

After the first transfer step, the total number of isolates on low-C agar medium were highest in S-treated soil from field 1 (42) and lowest in the SD-treated soil from field 6 (19; Table 3). Taking the data for both fields together, the highest number of isolates was found in S-treated soils (80) and the lowest in the CSD-treated soils (48) and so were the isolates in the *Alphaproteobacteria* (respectively, 31 and 8), *Bradyrhizobium* (respectively, 11 and 2), *Mesorhizobium* (respectively, 10 and 2), and *Rhizobium* (respectively, 8 and 1). However, no clear effect of soil treatment was found for the Shannon diversity values for low-C isolates, which were highest in SD-treated soils from field 1 and lowest in CSD-treated soils from field 6. After the third transfer step, the numbers of oligotrophs per treatment in fields 1 and 6 were, respectively, 19 and 18 for S-treated, 14 and 12 for SD-treated, 15 and 8 for C-treated, and 8 and 4 for CSD-treated soils; the highest numbers were again found in S-treated soils and the lowest in the CSD-treated ones. This

Table 2 Equations describing the linear relationships between the number of CFU on low-C agar media and total (Ctot) and dissolved organic carbon (DOC) in the differently treated soils

Dependent variable	model	*p* value	R^2
LogCFU on low-C agar media	6.851 (±0.118)−0.021 (±0.008)×Ctot	0.04	0.51
LogCFU on low-C agar media	7.049 (±0.156)−0.006 (±0.002)×DOC	0.02	0.62

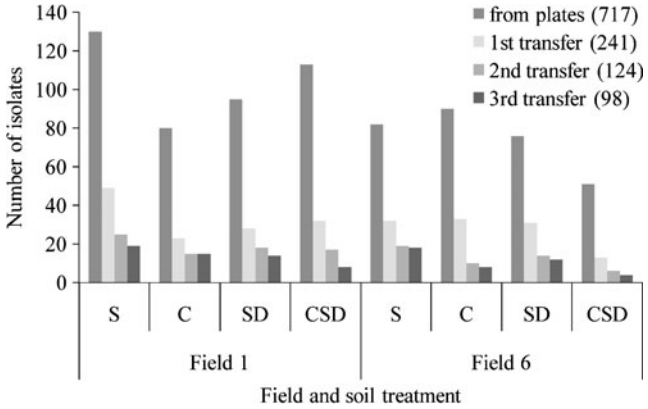

Figure 1 Distribution of bacterial isolates on low-C agar medium over the differently treated soils after subsequent transfer steps to fresh low- and high-C agar media. Only those isolates that exclusively grew on low-C agar medium were used in the succeeding transfer step. S, C, SD, and CSD stand for soils treated with, respectively, slurry, compost, slurry and dung, and compost, slurry plus dung

indicates that soil treatment has an effect on the abundance of the dominant groups of oligotrophs in the differently treated soils, but not on the oligotroph species diversity (only after the first transfer step). S-treated soils were the ones lowest in Ctot and DOC, whereas in CSD-treated soils both variables had higher values (Table 1), indicating that the amount of carbon present in soils affect the most dominant oligotrophic groups in the different soils.

Identification and Characterization of Selected True Oligotrophic Isolates

For better identification, larger stretches (approximately 1,400 bp in size) of the 16S rRNA genes of 17 selected isolates (at least one from each genus) obtained on low-C agar medium after the third transfer step were compared, and their growth rates were determined. One isolate had probably died in the freezer and could not be reactivated on

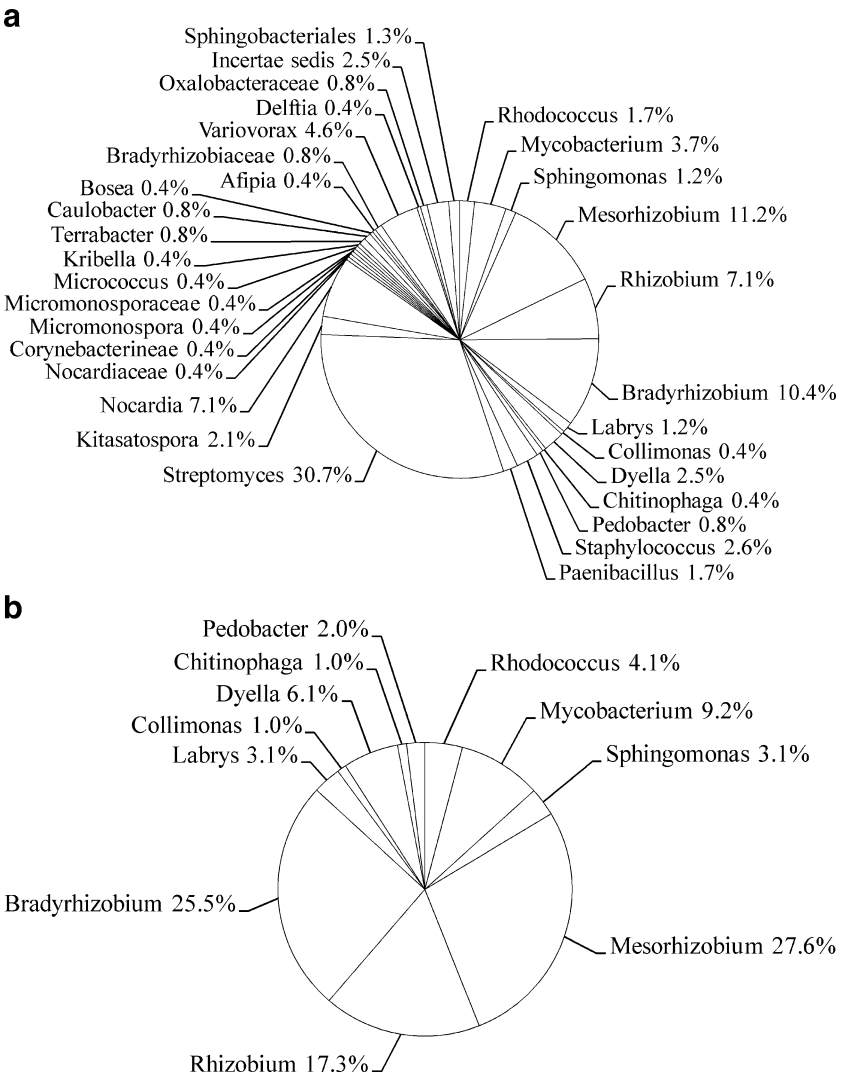

Figure 2 Taxonomic identity of isolates from all treated soils that exclusively grew on low-C agar medium after the first (**a**) and third (**b**) transfer step

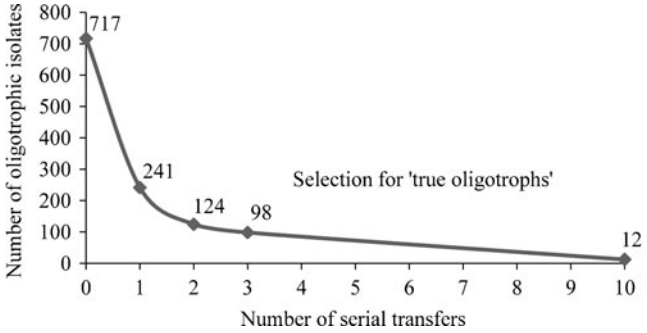

Figure 3 Number of oligotrophic bacterial isolates from soil selected for exclusive growth on low-C agar medium after repeated transfers to low- and high-C agar media. The number of true oligotrophs in the graph is an estimate derived from the number of true oligotrophs (2) obtained from the selected subset of 16 isolates after the third transfer step that were further tested for growth on low-C and high-C agar media up to the tenth transfer step

low-C medium. From the remaining 16 isolates, 15 showed nearest matches at similarity levels of 96% or higher with culturable type strains in the RDP database, whereas one (denoted as IS204) matched at a similarity level of 91.6% with *Pedobacter roseus*. Nine of the selected oligotrophs were identifiable within the class of *Alphaproteobacteria*, two within the *Betaproteobacteria*, one within the *Gammaproteobacteria,* three within the *Actinobacteria*, and one within the *Sphingobacteria*. One isolate showed a nearest match with an uncultured bacterium at a lower level (91.4%), indicating that this isolate may represent a hitherto uncultured bacterial group. Therefore, it can be concluded that the majority of low-C isolates closely resembled already described bacteria from soils.

Growth rates of these 16 isolates on low- and high-C agar media were measured. Because not all isolates were able to grow in a uniform suspension in liquid media, it was decided to estimate growth rates from colony diameters on agar surfaces. Estimated growth rates of the isolates ranged between 0.1 and 0.6 mm day^{-1} on low-C agar medium and between 0 and 1.0 mm day^{-1} on high-C agar medium, demonstrating that there were still broad-range bacteria among these isolates (Table 4). Growth rates on the high-C agar medium were mostly similar or higher than on the low-C agar medium. In three cases, the growth rates on the low-C agar medium was higher than on the high-C agar medium, while two of these isolates did not grow at all on the latter medium. These two isolates (IS183 and IS204) were only able to grow on low-C agar medium up to the tenth transfer step. Apparently, these two isolates, showing nearest matches with *Rhizobium alamii* and *P. roseus* type strains, can be regarded as "true" oligotrophs as they most likely do not possess the capacity to grow at higher nutrient levels in the agar medium. One of these two true oligotrophic isolates matched even more closely to a hitherto uncultured bacterium.

Discussion

Many broad-range and a few true oligotrophic bacteria were recovered from soils that were amended with different organic substrates. These soil treatments are realistic in organic farming where organic amendments like compost, manure, and slurry are commonly applied for crop growth and disease control [41]. Agronomic cultural practices were shown to affect the microbial community structure in soils most likely as a result of changes in chemical composition of the soils [41, 42]. Here, we demonstrated that the carbon status in soils treated with different organic amendments affected the numbers of broad-range and oligotrophic bacteria isolated from these soils.

Soils that have been managed organically for several years and received solid composted farm yard manure are expected to be relatively low in available nutrients [40]. Therefore, it was hypothesized that oligotrophs would be

Table 3 Distribution of bacteria isolated on low-C agar medium after the first transfer step over different taxonomic groups and Shannon diversity values for the differently treated soils from fields 1 and 6 at the Droevendaal Experimental Farm of Wageningen UR, the Netherlands

Number of oligotrophs and diversity	S[a]		C		SD		CSD	
	1	6	1	6	1	6	1	6
Total number of isolates	42	38	35	25	34	19	21	27
Alphaproteobacteria	15	16	12	8	16	6	6	2
Bradyrhizobium	8	3	3	2	5	2	1	1
Mesorhizobium	4	6	3	6	2	4	2	0
Rhizobium	3	5	3	0	5	0	1	0
Shannon diversity (H')[b]	0.724	0.788	0.786	0.578	0.836	0.767	0.655	0.558

[a] S, C, SD, and CSD denote soils treated with, respectively, slurry, compost, slurry, and dung, and compost, slurry plus dung in field 1 and field 6

[b] Calculated on the basis of all genera present among the low-C isolates from the different soils

Table 4 Taxonomic identity and colony growth of a selected subset of bacterial isolates that grew exclusively on low-C agar medium at the third transfer, but not necessarily after additional transfers on low- and high-C agar media

Isolate	Nearest match with nontype strains	Similarity (%)	Nearest match with type strains	Colony growth[a]		
				Similarity (%)	Growth rate (o/c), mm day^{-1}	App (days)
Alphaproteobacteria						
IS6	*Rhizobium sp. CCBAU 85046*	99.6	*Rhizobium huautlense*	97.2	0.1/0.3	1/3
IS63	*Rhizobium sp. CIAM1414*	99.8	*Mesorhizobium ciceri*	98.9	0.1/0.2	1/2
IS19	*Mesorhizobium loti LMG 6123*	98.5	*Mesorhizobium septentrionale*	98.3	0.3/0.3	1/1
IS119	*Mesorhizobium sp. USDA 4322*	99.9	*Mesorhizobium septentrionale*	98.9	0.2/0.2	1/1
IS183	*Rhizobium sullae*	97.8	*Rhizobium alamii*	97.4	0.1/0	1/–
IS252	*Mesorhizobium amorphae CCBAU 45139*	99.8	*Mesorhizobium amorphae*	98.9	0.1/0.3	1/1
IS354	*Bradyrhizobium japonicum*	99.6	*Bradyrhizobium japonicum*	99.0	0.6/1	1/1
IS152	*Labrys methylaminiphilus DSM 16812*	97.6	*Labrys monachus*	96.9	0.2/0.2	2/1
IS42	*Sphingomonas* sp. *kmd_118*	99.5	*Sphingomonas asaccharolytica*	97.8	0.2/0.7	1/1
Betaproteobacteria						
IS184	*Betaproteobacterium EC4*	98.4	*Duganella zoogloeoides*	97.6	0.1/0.3	1/1
IS343	*Collimonas* sp. *wged41*	99.4	*Collimonas fungivorans*	98.4	0.3/1	1/1
Gammaproteobacteria						
IS173	*Dyella marensis CS5-B2*	98.9	*Dyella koreensis*	98.0	0.5/0.5	2/1
Actinobacteria						
IS39	*Mycobacterium sp. IMER-B1-12*	98.5	*Mycobacterium septicum*	96.7	0.5/0.4	1/1
IS100	*Rhodococcus tukisamuensis*	99.4	*Rhodococcus tukisamuensis*	99.4	0.5/0.7	1/1
IS316	*Rhodococcus maanshanensis*	98.0	*Rhodococcus maanshanensis*	98.0	0.4/0.8	1/1
Sphingobacteria						
IS204	Uncultured *Sphingoterrabacterium* sp.	97.0	*Pedobacter roseus*	91.6	0.1/0	2/–

[a] Measured after three transfer steps: o/c indicates oligotrophic/copiotrophic agar medium

App first appearance of visible colonies

dominant in such soils. Indeed, the initial number of isolates grown on low-C agar medium was higher than that on high-C agar medium in our soils, and the number of low-C isolates was reduced at higher carbon concentrations in these soils, whereas the culturable copiotrophs were not. Based on these culturable cell counts, we could accept our hypothesis. Most likely, it is the reduction in the amount of available carbon that favors growth of broad-range and oligotrophic bacteria in these soils rather than the increased amount of total carbon that favors growth of hydrolytic copiotrophic bacteria and possibly suppression of oligotrophs. Our observations were in line with observations made by Hu and coworkers [17], who demonstrated that putative oligotrophic bacteria peaked in their growth after the copiotrophs. Copiotrophic bacteria were more numerous at high carbon availability levels, while populations of putative oligotrophic bacteria declined at high carbon levels [17]. Under these circumstances, this latter group of bacteria would likely be outcompeted by copiotrophs. However, as soon as carbon availability declines, the copiotrophs become arrested in growth and activity whereas oligotrophs remain active at lower carbon levels [34], will gain advantage over copiotrophs, and will proliferate.

Qualitative analysis of the isolates on low-C agar media characterized by 16S rRNA gene comparisons in the four differently treated soils revealed that there were no major differences in the predominant groups of isolates, i.e., *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium* spp., among these soils, i.e., about 70% of the isolates after the third transfer. These three genera belong to the *Alphaproteobacteria*, which were shown before to dominate the oligotrophic bacterial community in soils and seawater, especially those belonging to *Rhizobium* and *Bradyrhizobium* spp. [10, 22, 33]. Two *B. japonicum* strains could be reisolated from different field soils, 16 and 20 years after they were introduced [27], indicating that members of the *Bradyrhizobium* group of species are well adapted to circumstances prevailing in soil. Most of these genera contain nitrogen-fixing organisms. As our low-C agar medium was low in carbon as well as nitrogen, there could have been a selection for N_2-fixing bacteria on our plates, although we did not check the N_2-fixing activity of the

isolates. The ability of many oligotrophic bacteria to fix nitrogen was demonstrated by Japanese researchers a long time ago [30].

Two of our isolates closely matched members of the genus *Rhodococcus*. A representative of the same group was isolated from soil before and characterized as an "extreme oligotroph" [28]. One of our isolates showed a close match to *Collimonas* sp. (*Betaproteobacteria*). *Collimonas* species were also isolated from dune sand, which is an environment extremely low in available nutrients [14, 21]. Remarkably, the isolates obtained by Hoppener-Ogawa and coworkers [14] all were recovered on an agar medium relatively high in nutrients, whereas our *Collimonas* sp. was recovered on low-C agar medium and showed exclusive growth on this medium after three subsequent transfers. However, it did grow on our high-C agar medium after subsequent transfers, indicating that it was a broad-range organism. A high variation in physiology and taxonomy may be present among members of this genus.

Further testing for exclusive growth on low-C agar medium by repeated transfers to fresh media revealed an even stronger contribution of the *Alphaproteobacteria* to the total oligotrophic community in these soils [47]. It must therefore be concluded that the *Alphaproteobacteria* are the most important group among the oligotrophs selected within the constraints of the experimental set up, i.e., by selection for growth on agar media including low concentrations of glucose and casamino acids as sole nutrient sources.

For isolation and quantification of oligotrophic bacteria from any natural sources, there are currently no other methods besides cultivation on media with very low concentrations of readily utilized sources of carbon plus some growth factors, which were provided by enzymatic casein hydrolysate in our study. Any cultural method is selective, and in our case the main selective factor was the carbon concentration. However, it is clear that the common approach for isolation of oligotrophs is not selective enough for the vast majority of the strains obtained in our study. Semenov [34] proposed that the vast majority of microorganisms are "polyfunctional," i.e., they possess a "wide reaction range" for organic carbon concentrations in their surroundings, which is in line with the observations made in our study. Fewer microorganisms possess narrow reaction ranges, either towards low or high organic carbon concentrations [34]. The two isolates, denoted as "true" oligotrophs and identified as *R. alamii* and *P. roseus*, persistently showed exclusive growth at low nutrient availability levels, and these must belong to the group possessing a "narrow" reaction range.

The close match with an uncultured bacterium and a distant match with *P. roseus* type strain of one of the two true oligotrophs indicates that, among this group, new or hitherto uncultured bacteria can be found. Recently, isolates identified as *Verrucomicrobia* subdivision 1 and belonging to a hitherto uncultured group present in the potato rhizosphere were obtained on the same low-C agar medium but then amended with catalase or potato root exudates [26]. This medium offers great opportunities for the recovery of new species from soil environments, especially among the ones that exclusively grow thereon. The other true oligotrophic isolate resembled *Rhizobium sullae* and *R. alamii*, indicating that this isolate may be a plant symbiont. Representatives of *R. alamii* were isolated from the root environment of *Arabidopsis thaliana* [2], and those of *R. sullae* originated from *Hedysarum coronarium* L [36]. This isolate and also the ones belonging to the genera *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium* that lost their exclusive growth on low-C agar medium are important members of the isolates obtained in this study and may play an important role in plant symbiotic N-fixation mentioned above.

It can be concluded that the group of low-C bacteria recovered from the soils in this experiment, whether they could grow at a broad or narrow range of carbon concentrations, can play important roles in soil functioning. These bacteria may not only be important for nitrogen fixation but also for biological control of plant pathogens. For example, it was shown that some *Collimonas* species were mycophagous, i.e., they can live on fungi [21], and may be responsible for the control of soil-borne phytopathogenic fungi. It is clear that bacteria isolated on low-C agar media belong to a fascinating group of soil bacteria, possibly affecting plant growth. Thus far, these bacteria have hardly been exploited for improved plant growth, and this aspect will be further tested in later studies.

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