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# The presence of embedded bacterial pure cultures in agar plates stimulate the culturability of soil bacteria

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ABSTRACT

Traditional methods for bacterial cultivation recover only a small fraction of bacteria from all sorts of natural environments, and attempts have been made to improve the bacterial culturability. Here we describe the development of a cultivation method, based on the embedment of pure bacterial cultures in between two layers of agar. Plates containing either embedded *Pseudomonas putida* or *Arthrobacter globiformis* resulted in higher numbers of CFUs of soil bacteria (21% and 38%, respectively) after 833 h of incubation, compared to plates with no embedded strain. This indicates a stimulatory effect of the bacterial pure cultures on the cultivation of soil bacteria. Analysis of partial 16S rRNA gene sequences revealed a phylogenetical distribution of the soil isolates into 7 classes in 4 phyla. No difference was observed at the phylum or class level when comparing isolates grouped according to embedded strain. The number of isolates belonging to the same class as the embedded strain was reduced in comparison to that of plates with no embedded strain, indicating that intercellular signalling was unlikely to cause the observed stimulatory effect. Significantly higher fractions of isolates with less than 97% sequence homology to known sequenced isolates in GenBank were recovered from plates with embedded strains than from those without, which indicate a higher number of potential novel soil isolates. This approach for cultivation is therefore a feasible alternative or supplement to traditional cultivation on agar plates in order to enhance bacterial culturability.

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## 1. Introduction

For several decades, it has been clear that cultured bacteria represent only a small fraction of the bacterial population present in nature (Jannasch and Jones, 1959; Torsvik et al., 1990). Despite of this and the appearance of a large array of molecular tools, the culture-dependent methods have remained fundamental tools in microbial ecology. This is because these methods are still the best suited for the study, not only of bacterial identity, but also of their function. Therefore, several studies have focused on the development of modified media from the notion that our inability to culture bacteria is caused by our inability to mimic the conditions under which bacteria live in nature. Not surprisingly, only few bacteria are adapted to grow and divide on high-nutrient agar plates at environmentally high temperatures at a pace that produces a visible colony after a few days.

Recently, several studies have retrieved higher numbers and/or an altered diversity of bacterial colonies by changed media compositions or incubation conditions, such as low nutrient concentrations (Aagot et al., 2001; Davis et al., 2005; Hobel et al., 2004), long incubation time (Davis et al., 2005), addition of signal compounds (Bruns et al., 2002), incubations on soil suspensions (Ferrari et al., 2005) and altered medium pH (Johnson, 1995; Sait et al., 2006). In addition to these factors, evidence has been reported where the growth of organisms was stimulated by the concomitant growth of other organisms (Johnson, 1995; Kaeberlein et al., 2002). By incorporating acidophilic heterotrophic bacteria in agar plates, the plate efficiencies and growth rates of iron-oxidizing acidophilic bacteria were improved (Johnson and McGinness, 1991) and isolates of the genera *Lewinella* (MSC1) and *Arcobacter* sp. (MSC2) were readily cultivated in growth chambers, but reported only to grow on Petri dishes in co-culture (Kaeberlein et al., 2002). Likewise, a Japanese research consortium has described several new anaerobic syntrophic taxa. As an example, the growth of a thermophilic, strictly anaerobic filamentous bacterium was significantly stimulated when co-cultured with a hydrogen-utilizing methanogen (Sekiguchi et al., 2001). In studies of biofilm consortia, growth stimulation of one bacterial species by the presence of another has been reported several times (Burmølle et al., 2006, 2007; Filoche et al., 2004; Stewart et al., 1997). Recently, it was even demonstrated how a bacterial strain evolved by simple mutations, as a consequence of the association with another bacterial species in a biofilm, in order to adapt to the conditions of the coexistence (Hansen et al., 2007). On the other hand, the growth of organisms can be inhibited by the presence of other organisms. Several results have shown that the effect of colony density on the colony number per gram sample decrease gradually with increasing inoculum density,

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probably as a result of mutual competition (Jensen, 1962; Olsen and Bakken, 1987), and some bacteria produce compounds that inhibit others (Rao et al., 2005).

To further study the extent of these phenomena, we decided to use two bacterial cultures as stimulating and/or selective agents for the isolation of soil bacteria. Our aims were 1) to construct an agar plate system to stimulate and/or inhibit the culturability of bacteria extracted from soil and 2) to investigate to what extent such factors played a role when using well-known bacteria and standard agar media regarding the CFUs and the diversity of the bacterial isolates.

## 2. Materials and methods

### 2.1. Bacterial strains and cultivation conditions

Bacterial strains *Pseudomonas putida* SB5 (*P. putida*, Burmølle et al., 2007) and *Arthrobacter globiformis* DSM20124 (*A. globiformis*) were transferred from glycerol at −80 °C to R2A plates (Burmølle et al., 2007). The strains were incubated at 20 °C for 5 days. A loop full of culture was transferred to tubes with 5 ml 1/10 tryptic soy broth (TSB, Merck, 3 g/l,) and incubated overnight at 20 °C, 150 rpm. After this, cells were centrifuged at 5000 ×*g* for 10 min and washed twice in 5 ml Winogradsky's salt solution (WSS, 250 mg $K_2HPO_4$, 125 mg $MgSO_4 \cdot 7H_2O$, 125 mg NaCl, 2.5 mg $FeSO_4 \cdot 7H_2O$, 2.5 mg $MnSO_4 \cdot 7H_2O$ pr l, pH = 7.2). Finally, the pellet was re-suspended in WSS and the concentration of CFUs was adjusted to approx. $10^3$ CFU/ml by measuring $OD_{600}$ and using a previously established conversion factor. Aliquots of 100 µl of the bacterial suspensions were spread onto 1/100 TSA, which consists of 0.3 g/l TSB, 18 g Agar–Agar (Merck), $dH_2O$ ad 1000 ml and 12.5 ml of a 4 mg/ml Nystatin (Sigma) solution added after autoclaving. Tubes with 15 ml 1/100 TSA each were autoclaved and cooled to 50 °C and poured onto plate-spread plates leaving a uniform solid top agar layer. From general estimations of the weight and nutrient content of bacterial cells, it was calculated that the contribution of organic matter in the form of the embedded bacterial cells was negligible. After incubation at 20 °C for 7 days, the surface of the top agar was carefully checked for visible colonies, in order to ensure that colonies were only growing in between the two layers of agar. The number of embedded CFUs in each plate after incubation was 348 ± 32 and 117 ± 6 for *P. putida* and *A. globiformis*, respectively.

### 2.2. Soil sampling and cultivation of soil bacteria

Soil samples were taken from the brink of a small stream (55:36:16 N, 11:35:00 E) covered mainly by *Aegopodium podagraria*. Soil was sieved (2 mm mesh size) and stored at 15 °C for 6 days. Soil characteristics was analysed at the University of Århus, Foulum, Denmark and were: coarse sand (500–2000 µm) 7.3; coarse sand (200–500 µm) 18.9; fine sand (125–200 µm) 15.7; fine sand (63–125 µm) 9.1; fine sand (50–63 µm); coarse silt (20–50 µm); silt (2–20 µm); clay (<2 µm); humus 12.0; total N 0.51; total C 7.04. A subsample of 10.0 g soil was transferred to a Blue Cap bottle with 90.0 ml WSS. The bottle was placed horizontally on an orbital shaker (350 rpm, 5 min), raised and left for settling for 5 min. Ten millilitres of the supernatant was transferred to a tube, and tenfold dilution series in WSS were made. From dilutions $10^{-4}$, $10^{-5}$ and $10^{-6}$, 100 µl aliquots of the soil suspension were spread onto the top agar and incubated at 20 °C.

Three series of plates were included, one with no embedded bacteria (NE), one with *A. globiformis* embedded (AgE), and equivalently one with *P. putida* (PpE). Initially, each series contained five replicates, but this was reduced to four for the AgE- and PpE-series, due to contamination of the surface of the plates prior to inoculation of the soil suspension. During incubation, colonies larger than 0.3 mm on the surface of the top agar were enumerated under a stereo microscope after 77, 126, 144, 211, 332, 481, 668 and 833 h. For all three series, the numbers of CFUs were determined on the basis of counts from plates with $10^5$ diluted samples.

After 44 days, 50 of the previously counted colonies from each replicate of each of the three series were sub-cultured, resulting in 250 isolates from plates without embedded bacteria, and 200 from plates with AgE or PpE. The colonies were selected randomly by placing a grid under the plate and picking the colony closest to the intersection of the lines in order to avoid selection for the large or coloured colonies. To assure purity, colonies were transferred to agar plates using a loop. Colonies from all the series were streaked onto 1/100 TSA plates. In addition, colonies from plates with AgE and PpE were streaked onto 1/100 TSA plates with the corresponding bacterial strain embedded.

### 2.3. Sequencing and phylogeny analysis

Pure cultures were grown in 1/100 TSB with gentle shaking (75 rpm) until cells were visible. DNA was purified from these cell suspensions by mild sonication for 5 min in a Branson ultrasonic water bath, by boiling for 5 min, transfer to ice for 2 min, mild sonication for 5 min and centrifugation at 5900 ×*g*). 100 µl of the supernatant was stored for use as template DNA for the PCR amplification of part of the 16S rRNA gene. PCR mix (50 µl) contained 1× Phusion HF buffer (Finnzymes Oy, Espoo, Finland), 0.2 mM dNTP (Roche, Basel, Switzerland), 0.4 µM of the primers 27F and 1492R (Lane, 1991), 1 µl DNA, 1 IU Phusion polymerase, and 0.01 mg/ml BSA (New England Biolabs, Ipswich, MA). PCR conditions were as follows: 98°C for 2 min; 35 cycles of 98°C for 10 s, 60°C for 20 s, 72°C for 90 s; 72°C for 6 min. Amplified DNA was verified on agarose gels and purified using PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequencing mix consisted of 1× DYEnamic $^{tm}$ET terminator sequencing premix, 0.2 µM primer — 518R (5′-ATT ACC GCG GCT GCT CC-3′, Muyzer et al., 1993) and 200 ng DNA. Sequencing reactions were performed as follows: 94 °C for 3 min; 30 cycles of 94 °C for 20 s, 49 °C for 15 s, 60 °C for 1 min; 30 cycles of 92 °C for 20 s, 49 °C for 15 s, 60 °C for 2 min; 60 °C for 5 min. Following a Sephadex™ G-50 purification (GE healthcare), products were sequenced by using a Megabase 1000 sequencer. Some of the PCR products were sequenced by Macrogen Inc. (Seoul, South Korea).

Obtained sequence chromatograms were checked by use of the FinchTV program (version 1.4.0). Obtained high-quality sequences from the different treatments, AgE, PpE and NE, were blasted against sequences available in the GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify closely related sequences. A 97% similarity threshold was set to assign if the sequences were related to unculturable bacteria. Then, the sequences were uploaded in The Ribosomal Database Project 10 (RDP, http://rdp.cme.msu.edu/index.jsp; Cole et al., 2009) to identify number of clusters at 97% similarity and to classify them into different bacterial groups. The closely related sequences in the GenBank together with sequences obtained from the different culture conditions were aligned using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) and phylogenetic trees were constructed using the *neighbor-joining method* available in the same software.

### 2.4. Statistical analysis

The chi square test was performed for comparison of the diversity between the isolates from the different plates. Normality test failed for Two-way ANOVA, so cell counts were analysed by One-way ANOVA (or ANOVA on ranks when normality test failed) to compare between agar treatments for each time of isolation. We used $P < 0.05$ as the level of significance.

## 3. Results and discussion

### 3.1. Stimulation of culturability of soil bacteria

The accumulated numbers of colonies appearing on the agar plates with and without embedded strains are presented in Fig. 1. From the plate counts performed after 481 h of growth and onwards, we observed a significantly higher number of CFUs on PpE-plates when compared to that of NE-plates ($P<0.05$). In addition, there were significantly more CFUs on AgE- than on NE-plates after 668 h and onwards ($P<0.05$). At the point of termination of plate observation (after 833 h of incubation), the number of CFUs was enhanced by 21% on AgE-plates and by 38% on PpE-plates compared to the CFUs on NE-plates. Thus, the presence of embedded *A. globiformis* and *P. putida* in agar plates has a stimulating effect on the culturability of soil bacteria.

In the present experimental design, pure bacterial cultures were embedded in the agar plates, allowing all excreted, diffusible products to reach the soil bacteria on the top agar. Several studies have previously suggested a dependency of some bacterial strains on such diffusible compounds or the presence of other bacteria for growth and cultivation (Bruns et al., 2002; Ferrari et al., 2005; Filoche et al., 2004; Kaeberlein et al., 2002). *A. globiformis* and *P. putida* were selected to be embedded in the plates, as they are originally soil isolates, and since *P. putida* has previously been observed to cause synergy regarding biofilm formation and planktonic growth (Burmølle et al., 2007, and unpublished data). A relatively dilute agar was chosen so colony formation would progress at a pace allowing a long incubation and thereby enhancing the possibility that the embedded bacteria affected the growth of the plate-spread bacteria.

### 3.2. Diversity of isolated bacteria

In order to investigate if and how the presence of embedded strains influenced the diversity of the isolated soil bacteria, the 16S rRNA gene of a total of 262 isolates (78, 87 and 97 from AgE-, PpE- and NE-plates, respectively) was partially sequenced and analysed. The obtained sequences were checked and only sections of high quality were used for further analysis, resulting in final sequences ranging from 202 to 501 bp. We are aware that complete 16S rRNA sequences would probably result in more deep and accurate isolate identification and classification, but as a tool for accessing and comparing the overall diversity of the three groups of isolates, we believe that the partial sequences are sufficient.

By use of a cut-off value of a maximum of 97% sequence homology, the number of clusters obtained from AgE- and PpE-plates was (44/78, 56%) and (51/87, 59%), respectively, which was more that than obtained from NE-plates (46/97, 47%). However, this difference was statistically not significant ($P>0.05$).

RDP classifier allowed classification of the isolates into four phyla and seven phylogenetic classes. The majority of bacteria growing on all types of plates belonged to the class Flavobacteria in the Bacteroidetes phylum (Fig. 2). Fig. 3A, B, and C show the relatedness of the sequences obtained from the different treatments to sequences of closely related species of the phyla Actinomycetes, Firmicutes, Proteobacteria and Bacteroidetes. Each of these phyla has distinct clusters except Firmicutes, which was clustered with sequences of Proteobacteria. When the 16S rRNA libraries obtained from different culture treatment were compared using the Library Compare at RDP 10, it was found that the number of sequences belonging to the order Actinomycetales were statistically higher in the library obtained from NE-plates compared with the library obtained from AgE-plates ($P<0.05$). Additionally, a higher number of sequences belonging to the order Caulobacterales were present in the library obtained from PpE-plates compared to the library obtained from NE-plates ($P<0.05$).

We observed no significant differences in the classification of the isolates from the three different isolation procedures (Fig. 2), indicating that the cultivation method did not select for specific phylogenetic types of bacteria. However, a reduction was observed in the number of isolates belonging to the same class as the embedded strain when compared to NE-plates; Fewer Actinobacteria were isolated on AgE-plates and likewise for Gammaproteobacteria on PpE-plates, only the first was statistically significant ($P<0.05$). These results indicate that the observed stimulation of the culturability of soil bacteria by the embedded strain do not rely on a production and detection of signalling compounds, as such signalling is known to primarily stimulate cells of similar or closely related species (Riedel et al., 2001). Some types of signal molecules (e.g. AI-2) are produced and detected by many different species, but these compounds always affect members of the

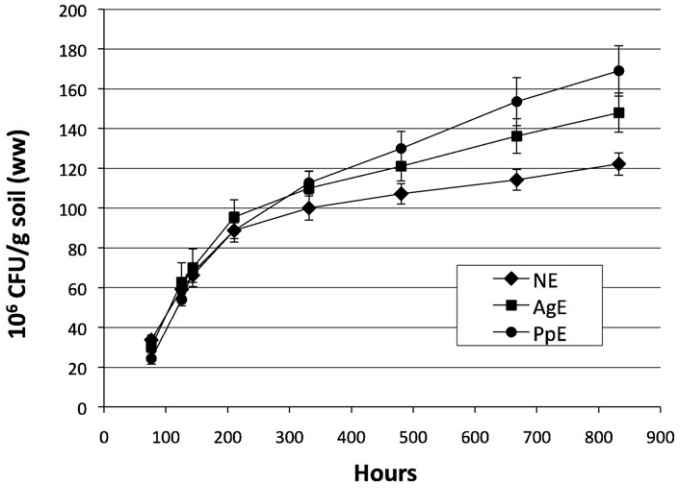


**Fig. 1.** The number of CFUs isolated from a soil suspension on agar plates with either *P. putida* SB5 (PpE, circles) or *A. globiformis* DSM20124 (AgE, squares) embedded, or with no embedded strain (NE, diamonds). The plates with the embedded strains were incubated at 20 °C for 7 days and the soil suspension was spread on the top agar. Plates were once again incubated at 20 °C, and CFUs were enumerated after 77, 126, 144, 211, 332, 481, 668 and 833 h of growth. Significantly higher numbers of CFUs were observed on *P. putida*-plates after 481, 668 and 833 h, and on *A. globiformis*-plates after 668 and 833 h of incubation. Error bars represent standard errors of the means of four (*A. globiformis*, *P. putida*) or five (no strain) replicates.

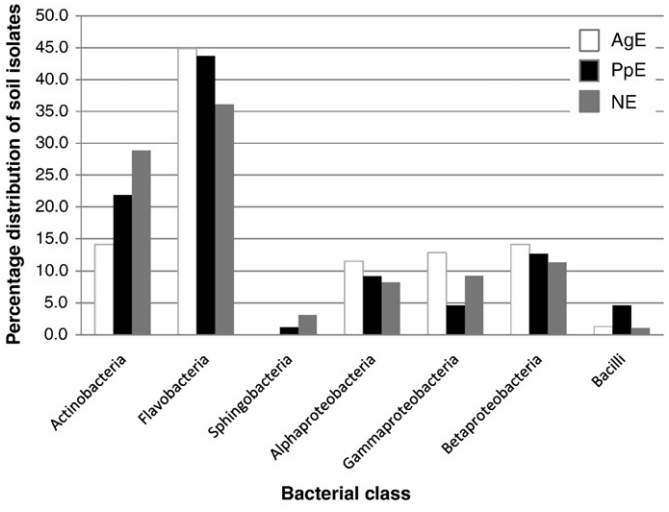


**Fig. 2.** The percentage distribution in phylogenitic classes of soil isolates from agar plates with *A. globiformis* DSM20124 (AgE, white), *P. putida* SB5 (PpE, black) or no embedded strain (NE, grey). Partial sequences of the 16S rRNA gene of 78, 87 and 97 isolates, respectively, were grouped according to embedded strain and classified by use of the RDP 10 Database, where the classification was generated. There was no significant difference in the distribution of isolates when comparing the three groups, but a reduction in the number of isolates belonging to the same class as the embedded strain (Actinobacteria: *A. globiformis*, Gammaproteobacteria: *P. putida*) was observed.

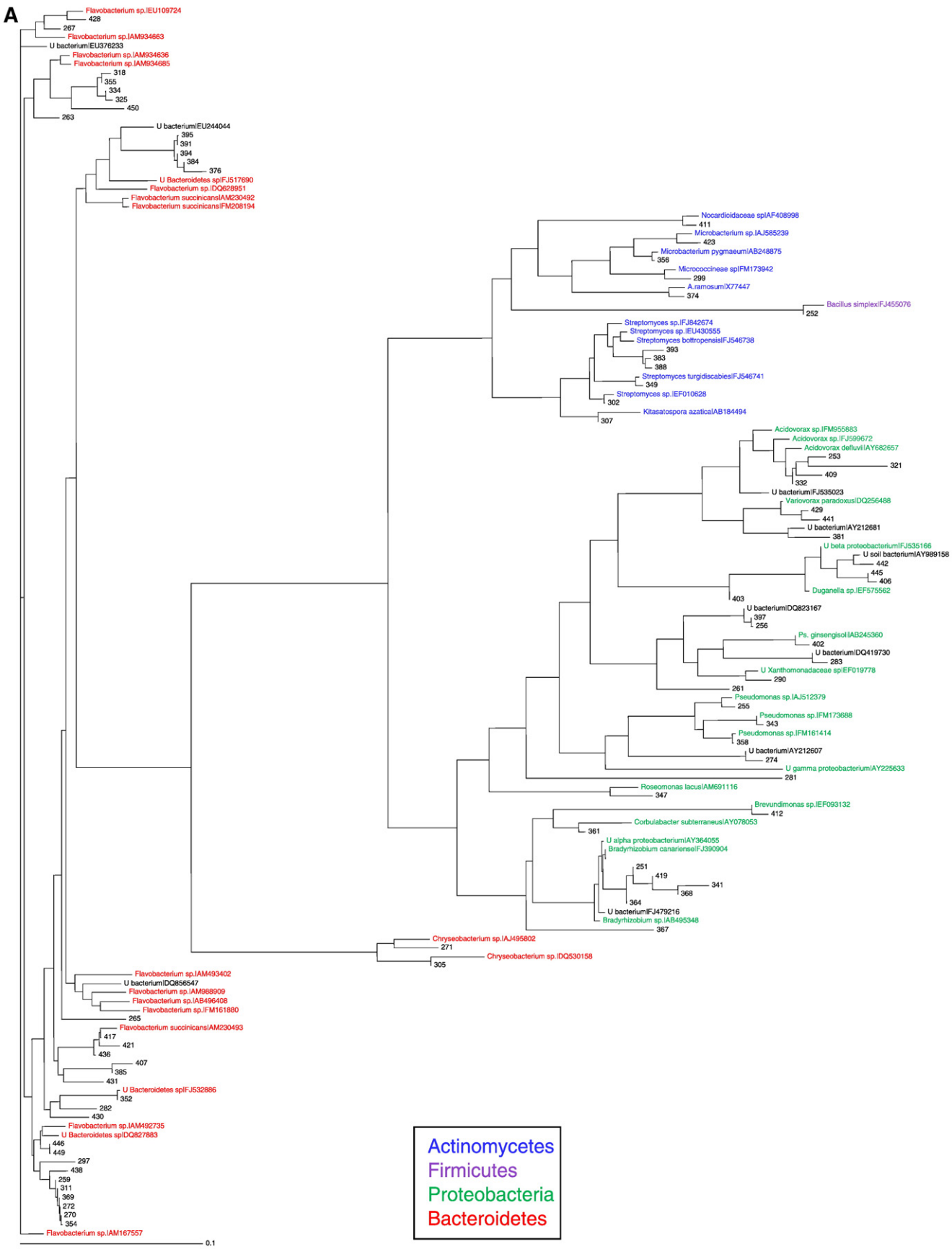


**Fig. 3.** The relatedness of sequences obtained from agar plates with *A. globiformis* DSM20124 (3A), *P. putida* SB5 (3B) or no embedded strain (3C) to sequences of closely related species of the phyla Actinomycetes (blue), Firmicutes (purple), Proteobacteria (green) and Bacteroidetes (red). The phylogenetic trees were constructed using the neighbor-joining method in the BioEdit software (http://www.mbio.ncsu. edu/BioEdit/BioEdit.html) on closely related sequences in the GenBank.

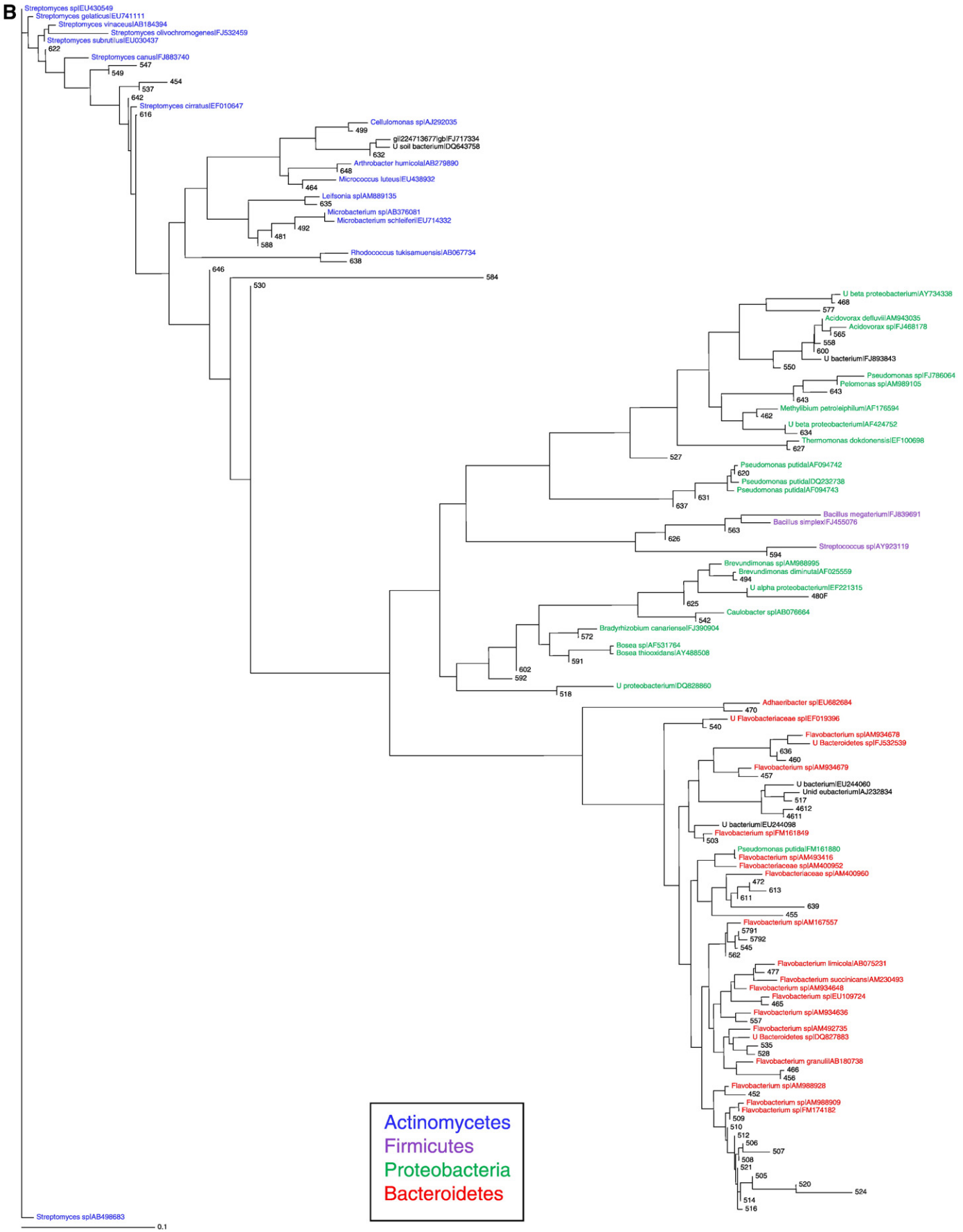


**Fig. 3** (*continued*).

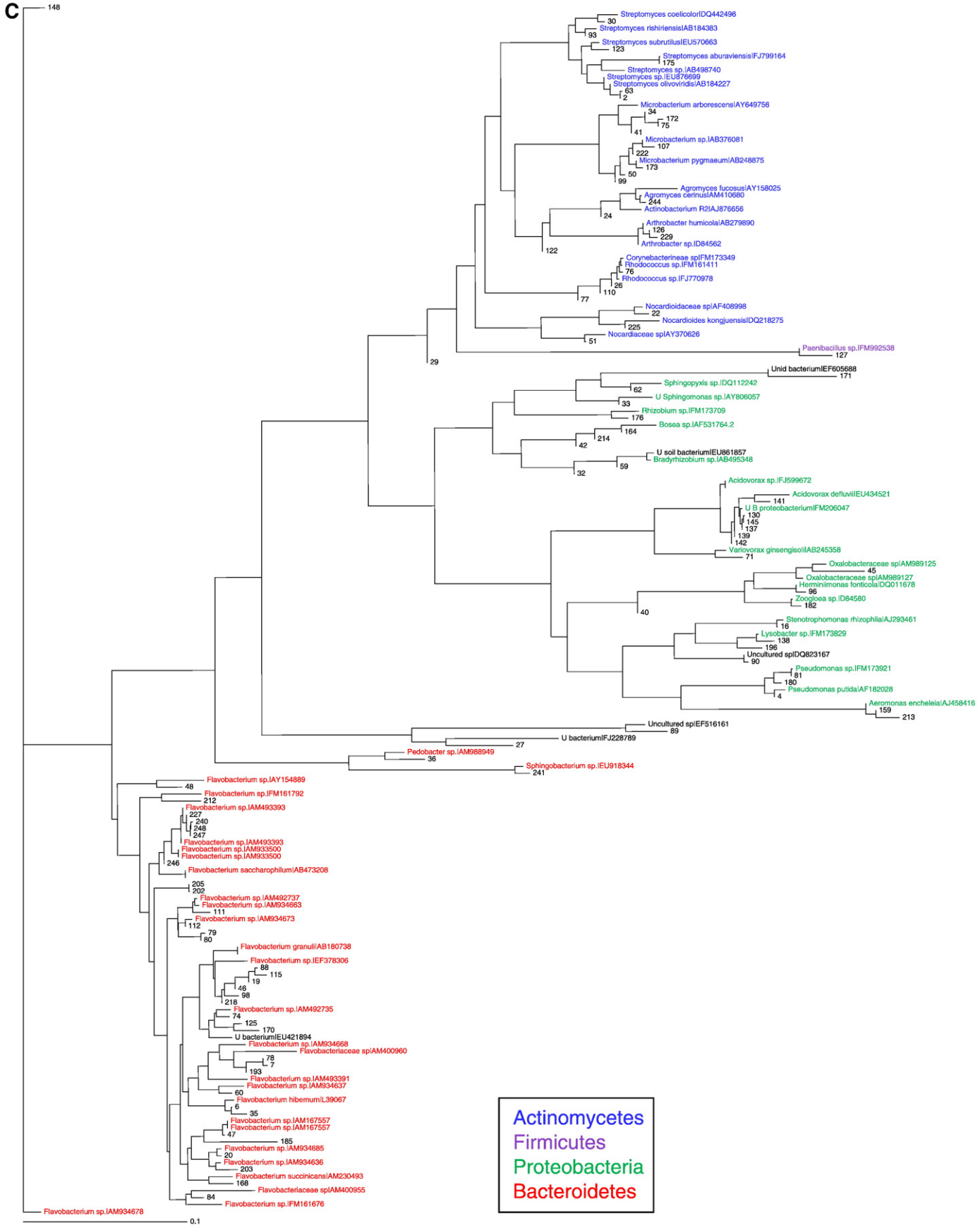


**Fig. 3** (*continued*).

producing species (Reading and Sperandio, 2006; Xavier and Bassler, 2003). Thus, the fact that we recover lower numbers of isolates closely related to the embedded strain indicates that bacterial signalling is not causing the observed stimulation of the culturability of the soil bacteria, which may rather be due to alteration of the medium pH, a shift towards even more oligotrophic conditions or removal of compounds present in the agar that inhibit the growth of some bacteria. By use of a similar approach, Johnson and McGinness (1991) improved the culturability of acidophilic iron-oxidizing bacteria by embedding acidophilic heterotrophs in the underlayer of double-layered agar plates. This was believed to lower the concentration of inhibitory organic compounds in the media, which then stimulated the culturability of the iron-oxidizers, and in fact a reduced concentration of monosaccharides in the medium was detected. In future experiments, we will examine whether further dilution of TSA medium and use of other types of gelling agents will result in effects similar to those observed in the present study. Alternatively, the enhanced culturability of soil bacteria in the presence of embedded strains is caused by one or several metabolic by-products that are produced by the embedded strain and able to diffuse in the agar. Thereby these products stimulate colony formation by the soil bacteria on the top agar. As previously mentioned, this type of growth stimulation has several times been reported for bacterial biofilm communities (Burmølle et al., 2006; Filoche et al., 2004; Hansen et al., 2007; Stewart et al., 1997). A very likely possibility is that not only one of the above-mentioned conditions causes the growth stimulation, but rather a combination of several. This then leads to an acclimatisation of the soil bacteria, improving their ability of colony formation on agar plates, also without the presence of an embedded strain. The fact that we did not observe any differences in the number of strains that could be sub-cultured on NE and AgE/PpE-plates (data not shown) supports this.

### 3.3. Novelty of isolated bacteria

As a measure of the novelty of the isolates, in relation to known, cultured isolates in the RDP, we compared the similarity scores provided by RDP from the three groups of isolates. We used a cut-off value of a maximum of 97% sequence homology, as this is commonly used to classify sequences from bacterial isolates as belonging to different species (Stackebrandt and Goebel, 1994). The numbers of novel isolates with similarity scores below 97% were 29/78 (37%), 30/87 (34%) and 20/97 (21%) from AgE-, PpE- and NE-plates, respectively. Thus, higher fractions of novel isolates, which has formerly not been cultivated, and in some cases not even detected in 16S rRNA gene clone libraries, were obtained from plates with embedded strains compared to those without and the difference was statistically significant ($P<0.05$). These novel isolates might prove interesting for future characterization of new bacterial taxa. The results indicate that not only did the method developed in this study result in an enhanced number of CFUs of soil bacteria, also more novel isolates were obtained. Because both embedded strains, used in this study, stimulated culturability, we expect this to be the case for several strains and will, in the near future, screen numerous pure cultures for similar or even more profound stimulatory effects on the culturability of bacteria from soil and other environments.

## 4. Concluding remarks

Several cultivation methods have been presented and suggested as alternatives to the plate cultivation method, including growth chambers and microcolony growth on filters (Ferrari et al., 2005; Kaeberlein et al., 2002). These methods have shown to enhance the culturability and diversity of bacteria, but in spite of this, the agar plate approach is most frequently used due to its simplicity, low cost and the independency of equipment, all allowing several dilutions of sample to be analysed in many replicates. In the cultivation approach presented in this study, the advantageous features of the agar plate approach are preserved, although with an extended plate preparation time, and the number of CFUs isolated from a soil suspension was enhanced, indicating a stimulatory effect of the embedded pure cultures on the culturability of the soil bacteria. The presence of an embedded strain did not result in a selective pressure favouring isolation of specific phylogenetical groups, but higher numbers of novel isolates were obtained. Therefore, this approach is a feasible and reasonable alternative — or supplement — to traditional agar plate cultivation. In addition, our plate design may be used to test the effect of specific gene products (e.g. antibiotics, signal molecules) on culturable bacterial communities.

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