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

Mette Burmølle, Kaare Johnsen, Waleed Abu Al-Soud, Lars Hestbjerg Hansen, Søren Johannes Sørensen *

Section of Microbiology, Department of Biology, University of Copenhagen, Denmark

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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.

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,

* Corresponding author. Sølvgade 83H, 1307 Copenhagen K, Denmark. Tel.: +45 35 32 20 59; fax: +45 35 32 20 40.
E-mail address: sjs@bio.ku.dk (S.J. Sørensen).

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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.