

Development of Selective Growth Media for Denitrifying Bacteria using an Evolutionary Algorithm: a Strategy Outline

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Abstract

Denitrification is a facultative trait that is distributed over a broad range of phylogenetic groups. Diversity studies of denitrifying bacteria are focused on metabolic gene sequence analysis. The observed functional diversity is often interpreted as phylogenetic diversity. There is no extensive study that supports this. Therefore, an extensive study of functional gene sequences of cultured and correctly identified strains needs to be performed. In this study, a strategy outline is presented of a new culturing approach to obtain a more diverse and larger reference set of denitrifying strains, including so far previously unculturable representatives. Specific growth media for denitrifiers are developed using an evolutionary algorithm. Eleven parameters, each with a different set of values, are defined that can vary in order to obtain different growth media. The settings for the evolutionary algorithm and the definition of the fitness are described and substantiated.

Keywords

Denitrification, growth medium optimisation, evolutionary algorithm

INTRODUCTION

According to Zumft (1997), “the phenomenological definition of denitrification is the dissimilatory transformation of nitrate or nitrite to a gas species concomitant with energy conservation”. This process is the combination of nitrate and nitrite respiration, with subsequent NO reduction and N₂O respiration and is best described as a modular organisation. Seen as isolated processes, neither nitrate nor N₂O respiration are considered as denitrification *sensu stricto*.

The ability to denitrify is a facultative trait spread among a wide variety of physiological and taxonomic groups of nearly 130 bacterial species within more than 50 genera (Zumft, 1992). Denitrifiers are slightly more present in the alpha and beta classes of the Proteobacteria, but so far absent from the enterobacteria (which respire nitrate to nitrite and direct the further reduction of nitrite to ammonia), obligate anaerobes, and most Gram-positive bacteria (Tiedje, 1988). Denitrification also extends beyond the bacteria to the Archaea, where it is found among the halophilic and hyperthermophilic taxa. Most denitrifiers are aerobic heterotrophic organisms that transfer redox equivalents from the oxidation of a carbon source to an N oxide under anaerobic conditions.

Commonly, microbial communities are investigated through a 16S rDNA approach to avoid limitations in culturability. This approach is however unsuitable for community analysis of denitrifiers, since denitrification is widespread among phylogenetically unrelated groups. Therefore, the research concerning denitrifying bacteria has been focusing on the common metabolic functions such as nitrite and nitrous oxide reduction. These key steps of the denitrification process are catalysed by nitrite and nitrous oxide reductases, respectively

coded for by *nirS* or *nirK* genes and *nor* genes. Through the development of primers for *nir* and *nos* genes and the increase in speed of the sequencing analysis a lot of information is gathered (from the environment and pure cultures) on the presence and the sequence diversity of these functional genes (Braker *et al.*, 1998; Hallin & Lindgren, 1999; Braker *et al.*, 2000; Casciotti & Ward, 2001; Philippot L, 2002; Priemé *et al.*, 2002; Rösch *et al.*, 2002; Yan *et al.*, 2003; Throbäck *et al.*, 2004). At this moment, functional diversity is often interpreted as phylogenetic diversity, although no study has confirmed this approach. Indeed sequences of only a limited number of denitrifying strains spread over a few taxa are readily available in the public databases. To proof whether or not functional diversity based on comparative analysis of denitrification genes can be interpreted as a phylogenetic diversity and for a wide variety or only for a few taxa, further study of functional gene sequences of cultured and identified strains is needed. Further characterisation of cultured and so-called unculturable microorganisms harbouring denitrification genes can provide insights in the level of phylogenetic information comprised in molecular data of this part of the genome. This demands however the return to the classical microbiological approach of isolation, cultivation and study of denitrifying strains.

It is generally accepted that conventional cultivation-based techniques are insufficient for studying the diversity of naturally occurring prokaryotic communities since the majority of prokaryotes is believed to be unculturable by traditional techniques (Amann *et al.*, 1995). Cultivation of denitrifiers is generally carried out using complex media, due to the simplicity and acceptability for growth of many strains (Tiedje, 1988). Although defined media are known to be more satisfactory for the growth of many denitrifiers, tryptic soy agar (and to a lesser degree nutrient agar) supplemented with nitrate is routinely used for culturing campaigns (Braker *et al.*, 2000; Yoshie *et al.*, 2001). It may be fair to state that unculturable denitrifiers detected in culture-independent molecular diversity studies are in fact not-yet cultivated denitrifiers because a better-suited growth medium has not yet been developed or used.

Thus, one of the main drawbacks for isolation of denitrifiers is the lack of selective growth media. Selective growth media are composed of components that interact to enhance the chance to isolate bacteria with specified characteristics. The number of possible combinations of the media components is so extensive that optimisation is mostly 'educated guesswork', in which researchers try different amounts of various ingredients based on experience and knowledge. Because of the large number of parameters that may interact to determine the success of the growth medium, this approach is rarely rational. However, in order to optimise selective and defined microbial growth media, with various components, each with their own optimal concentration, only a fraction of the possible combinations can be tested in practice.

Genetic or evolutionary algorithms (EAs) (Holland, 1992) are adaptive computer programs based on the selection principles of evolution as described by Darwin. In nature, most organisms evolve by means of 2 primary processes: sexual reproduction and natural selection. The introduction of novel variations at genetic loci by mutation and the subsequent novel gene combinations introduced by recombination results in a fitness that is then subjected to the process of natural selection. Populations of living organisms are thus able to optimise fitness by exploring multiple possibilities for solutions. EAs can, based on the same two processes, provide solutions, for highly complex optimisation problems in various domains: medium optimisation (Weuster-Botz & Wandry, 1995), improvement of silage additives (Davies *et al.*, 2002), forest structure (Venema *et al.*, 2005), electricity estimations (Ozturk *et al.*, 2004), etc. The use of EAs in microbiology is until now rather limited. Recently, EAs

were used to find the optimal conditions for plasmid transfer (Boon *et al.*, 2005). Some main differences between EAs and conventional optimisation procedures are: (1) EAs use more than one starting point, (2) they use only pay-off information, and (3) they use probabilistic instead of deterministic rules. In the (micro)biological context, a key advantage of EAs over other methods of (medium) optimisation is that no model is assumed.

This paper outlines the strategy of the development of a growth medium for denitrifiers using an EA. The aim of the research is to obtain a selective medium with a high percentage and a high diversity of denitrifiers.

METHODS

Growth Medium

The mineral medium based on Stanier *et al.* (1966) was used. The media were buffered to different pHs. 10µM bromothymolblue (pK_i=7.0) was added to growth media with a pH of 6.5; 10µM phenol red was added to growth media with a pH of 7 or higher. Sodium acetate trihydrate, glycerol, sodium pyruvate, methanol, ethanol, glucose or sodium succinate were used as carbon source, while potassium nitrate or potassium nitrite was used as nitrogen source. The following solutions were used as supplements: the vitamin solution containing 4 mg 4-aminobenzoic acid, 2mg D-(+)-biotin, 10mg nicotinic acid, 5mg calcium D-(+)-panthothenate, 15mg pyridoxin hydrochloride, 4mg folic acid, 1mg lipoic acid in 100ml 10mM NAH₂PO₄, at pH 7.1 (Widdel & Bak, 1992; Kniemeyer *et al.*, 1999); a riboflavin solution containing 2.5 mg riboflavin in 100ml 25mM NAH₂PO₄, at pH 3.2 (Kniemeyer *et al.*, 1999); a thiamin solution containing 10 mg thiamin hydrochlorid in 100ml 25mM NAH₂PO₄, at pH 3.4 (Widdel & Bak, 1992; Kniemeyer *et al.*, 1999); and a cobalamin solution containing 50mg cyanocobalamin per liter distilled water (Widdel & Bak, 1992; Kniemeyer *et al.*, 1999). Trypticase soy agar was supplemented with 10mM KNO₃ and 10µM phenol red.

Isolation

A dilution series (10⁰ to 10⁻⁸) of a sample (e.g. activated sludge) was plated out on 15 different media per generation, as determined by the EA. The samples were incubated for two weeks under anaerobic conditions (less than 1.0mg/l O₂, composition gas mixture 8%CO₂/8%H₂/84%N₂) in an anaerobic chamber. For each growth medium, 20 isolates were picked up from the plate with the highest dilution showing growth and further purified and subcultured on the same medium. All isolates were incubated in liquid isolation medium for one week at isolation conditions before performing the reduction tests.

Reduction tests

Reduction tests of isolates were carried out using the Griese reagents described by Smibert and Krieg (1994).

FAME analysis

Cellular FAMES were prepared and extracted according to the standardized protocol of the Microbial Identification System (MIS; Microbial ID Inc.). The extracts were analysed on an Agilent 6890 gas-liquid chromatograph in combination with a flame ionisation detector, an automatic sampler, an integrator and a computer. For the pattern recognition and preliminary identification, the commercially available TSBA50 database was used.

Simple Evolutionary Algorithms for Optimisation

The Simple Evolutionary Algorithm for Optimisation (seao) software (Sys *et al.*, 2004) used here, is a library of functions for R (<http://www.r-project.org>) and can be downloaded, together with seao.gui (seao graphical user interface), from the website of the R archive network (<http://www.cran.r-project.org>). If one uses the graphical user interface, there is no need for knowledge of R. To use the full flexibility and possibilities of the software, one can use the command-line interface, but then a very basic knowledge of R (Ihaka & Gentleman, 1996) is necessary. R is GPL licensed (<http://www.gnu.org/licenses/gpl/html>, GNU General Public License 2.0, 1991, Free Software Foundation). The software requires R (with the standard R-libraries) and, if graphical user interface is used, TclTk as well as the tcltk R-library. R, seao, the tcltk R-library and TclTk are freely available for Unix, Linux as well as for Windows.

RESULTS AND DISCUSSION

Definition of EA Parameters

pH. Denitrification can occur over a wide range of pH values. It is relatively insensitive to acidity, it may be slowed down at a pH lower than 6. The optimal pH range for denitrification is accepted to be between 6.5 and 8.5 (Gerald, 2002). PH values were set at 6.5 to 8.

Temperature. Almost all denitrifiers are mesophilic (Zumft, 1992). Temperature values were set at 20°C and 37°C.

Carbon source. It is known that the choice of carbon source may lead to specific enrichment of nitrite-accumulating bacteria. Indeed the presence of fermentable substrates enhances growth of nitrate-respiring fermentative bacteria causing nitrite accumulation (Błaszczuk, 1993; Van Rijn *et al.*, 1996). Because of the toxicity for many bacteria for higher levels of nitrite, a correct choice of carbon source is very important to avoid this unwanted metabolic pattern.

Acetate donates electrons closer to nitrate reductase, in the upstream region of the respiratory chain to either ubiquinone or cytochrome b (Van Rijn *et al.*, 1996), but acetate as carbon source stimulates denitrification in activated sludge samples (Eilersen *et al.*, 1995). Methanol donates electrons closer to nitrite reductase, in a more downstream region, at the site of cytochrome c (Van Rijn *et al.*, 1996). Błaszczuk (1993) detected that, for *Paracoccus denitrificans*, the accumulation of nitrite decreased - the activity of nitrite reductases increased - with ethanol as a carbon source compared to methanol or sodium acetate. This was confirmed by Christensson *et al.* (1994). Succinate (Song & Ward, 2003), glycerol (Shoun *et al.*, 1998) and pyruvate are also successfully used as a carbon source for growing denitrifiers. Some denitrifying species of *Bacillus* and *Chromobacterium* can denitrify and ferment at the same time (Gerald, 2002), so glucose was also selected as a carbon source.

Molar C/N. The ratio of available carbon to electron acceptor controls whether nitrate partitions to dissimilatory nitrate reduction to ammonium (DNRA) or to denitrification (Tiedje, 1988). When the ratio is low, the organisms that gain the most energy per nitrate, i.e. the denitrifiers, have an advantage. We used a molar C/N ratio because it was easier to visualize the ratio and the calculations from the C/N ratio to the concentrations of carbon and nitrogen source were straightforward. The range of C/N was set from 1 to 25.

Nitrogen source. Nitrate and nitrite were used as nitrogen source for denitrification.

Nitrogen. When consulting the literature, one finds that nitrate is added to growth medium in a range between 1.5mM and 24.5mM (Shoun *et al.*, 1998; Bae *et al.*, 2002; Blaszczyk, 1993; Eilersen *et al.*, 1995; Körner & Zumft, 1989; Greene *et al.*, 2003; Van Rijn *et al.*, 1996; Kim *et al.*, 2003). Shoun *et al.* (1998) used 1.5mM nitrite. A range was set from 3mM to 18mM.

Salt. Some halotolerant and halophilic strains are known to denitrify (Denariaz *et al.*, 1991; Kim *et al.*, 2003). Two parameter values, 0% and 2% NaCl, were chosen.

Supplements. Blaszczyk (2003) reported that addition of riboflavin, thiamin, cobalamin, panthothetic acid, pyridoxin and nicotinic acid causes a reduction of doubling time of *Paracoccus denitrificans* when growing under denitrifying conditions. Some other *Paracoccus* species require vitamin supplements (biotin, thiamin, cobalamin) for growth in minimal media. Knemeyer *et al.* (1999) added a vitamin mixture, and cobalamin, thiamin and riboflavin to medium for isolation of denitrifying bacteria. Four supplements – solutions of vitamins, thiamin, cobalamin and riboflavin - were set as parameters with values of 0ml, 1ml or 2ml.

Combinations of the above parameters and their different values (**Table 1**) would result in 1197504 different growth media.

Table 1. Parameters of growth medium

Parameter	Value													
pH	6.5			7.0			7.5			8.0				
Temperature	20°C ^E						37°C							
C source	Acetate		Glycerol		Pyruvate		Methanol		Ethanol		Glucose		Succinate	
Molar C/N	1	2.5	5	7.5	10	12.5	15	17.5	20	22.5	25			
N source	nitrate						nitrite							
N	3 mM		6 mM		9 mM		12 mM		15 mM		18 mM			
Salt	0%						2%							
Vitamin solution ^A	0 ml					1 ml					2 ml			
Riboflavin ^B	0 ml					1 ml					2 ml			
Thiamin ^C	0 ml					1 ml					2 ml			
Cobalamin ^D	0 ml					1 ml					2 ml			

^{A-D}: For composition, see Methods

^E: Room Temperature

Evaluation of Growth Media: Ratio Denitrifiers and Diversity

As is shown before, adding a pH indicator to a growth medium is advantageous to monitor strains as denitrifiers (Mazoch & Kucera, 2002). It is generally known that denitrification causes an increase in alkalinity, thus the pH indicator causes the isolation medium to change colour. When this change to alkalinity was observed and the nitrate- and nitrite reduction tests were positive, denitrification was assumed. Because of this pH indicator, strains showing DNRA could also be eliminated (DNRA goes together with fermentation, which causes the pH to drop and acidifies the medium).

A selective growth medium for denitrifiers must allow growth of a large number of denitrifiers as diverse as possible. Thus, the fitness had to contain a component that represented the number of denitrifiers: ratio DEN was the ratio of the number of denitrifiers and the total number of isolates. The composition of the growth media had to avoid the selection for a specific group of bacteria. That is why the presumptive denitrifying isolates were preliminary identified in a fast way with fatty acid ethyl ester (FAME) analysis. This

diversity was then translated to a diversity index, the Simpson's reciprocal diversity index $1/D$, for each growth medium:

$$1/D = N(N-1) / \sum n_i(n_i-1)$$

with N = number of isolates, n_i = number of isolates belonging to genus i . The fitness of a medium expressed the success of the medium, which was an evaluation of the number of denitrifiers isolated and their diversity:

$$\text{Fitness} = \text{Ratio DEN} \times 1/D$$

This procedure was also carried out for supplemented TSA. In this way, the success (i.e. fitness) of the developed growth media could be compared with the success of the conventional cultivation approach.

Design of EA

Once the generations and the structure of the parameter set were defined, the fitness of each growth medium was determined and the next generation was calculated. In order to increase the fitness in a next generation four steps were taken (Sys *et al.*, 2004): (1) the batches that make up the new parent population of batches were combined and the best parameter sets were chosen to make a new batch of possible parameter sets, (2) parameters sets were selected, so that the parameter sets with the highest fitness had the highest chance to be selected, (3) the selected parameter sets were combined two by two having recombination (crossover), and finally (4) some parameter values were allocated a random value (mutation). The selection parameter was fitness-based and resulted in a score: the higher the score, the higher the chance that the set-up would be chosen as parent batch for the next generation. For the configuration and parameterisation of the seao for the experimental optimisation of medium composition the recommendations of Weuster-Botz (2000) were taken into account. P_M was set at 90%; this is the probability that each point in the variable space can be reached by cross over starting from the start population. The recommended population size or number of individuals per generation M was then given by (Weuster-Botz, 2000): $M > 1 + \log(1 - p_M^{1/l})(\log 0.5)^{-1}$ and $l = k \cdot j$, with k = number of variables, and j = the number of values for these variables. With p_M set at 90%, M had to be larger than 9.77. M was set at 15. Subsequent mutation followed a uniform distribution, which gave all possible values the same chance of being chosen, with a spread of 1.0 and a rate of 15. A low mutation rate results in convergence, a high mutation rate results in divergence. For the initial bath (first generation), the initial parameter values were generated by defining a number of set-ups based on purely random allele values for each set-up.

CONCLUSIONS

The culture-independent studies of denitrifiers in the environment leave us today with a significant gap between the available taxonomic information of denitrifiers and the sequence information of the key denitrification genes. (Phylogenetic) conclusions are drawn based on 'functional' trees without any insight in the distribution patterns of these functional sequences among bacterial taxa.

The above-described strategy wants to develop one or more growth media suitable for large cultivation studies on denitrification and create the basis for a clearer view on the taxonomic position of denitrifiers and the functional sequences they contain. Only simple techniques and free software are used to make this strategy universally applicable.

A case study using activated sludge as inoculum is ongoing at our lab. Preliminary results show an increase in the total number of isolates, in the number of denitrifying isolates, and in the average and maximum fitness after just three generations.

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