Cultivation of Denitrifying Bacteria: Optimization of Isolation Conditions and Diversity Study[†]

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An evolutionary algorithm was applied to study the complex interactions between medium parameters and their effects on the isolation of denitrifying bacteria, both in number and in diversity. Growth media with a pH of 7 and a nitrogen concentration of 3 mM, supplemented with 1 ml of vitamin solution but not with sodium chloride or riboflavin, were the most successful for the isolation of denitrifiers from activated sludge. The use of ethanol or succinate as a carbon source and a molar C/N ratio of 2.5, 20, or 25 were also favorable. After testing of 60 different medium parameter combinations and comparison with each other as well as with the standard medium Trypticase soy agar supplemented with nitrate, three growth media were highly suitable for the cultivation of denitrifying bacteria. All evaluated isolation conditions were used to study the cultivable denitrifier diversity of activated sludge from a municipal wastewater treatment plant. One hundred ninety-nine denitrifiers were isolated, the majority of which belonged to the *Betaproteobacteria* (50.4%) and the *Alphaproteobacteria* (36.8%). Representatives of *Gammaproteobacteria* (5.6%), *Epsilonproteobacteria* (2%), and *Firmicutes* (4%) and one isolate of the *Bacteroidetes* were also found. This study revealed a much more diverse denitrifying community than that previously described in cultivation-dependent research on activated sludge.

For nearly 2 decades, molecular biology has provided the tools to successfully overcome the "great plate count anomaly" and allow the study of uncultured microbial diversity (3). The growing awareness that molecular methods cannot or, in very few cases, can only indirectly investigate the function of specific microorganisms in the environment has raised interest in new cultivation efforts and approaches once again (14, 15, 34). Simple adjustments to the classical cultivation approach, such as prolonging the incubation time and avoiding complex or nutrient-rich growth media, have successfully resulted in cultivation of previously uncultured bacteria (12, 30).

A physiological trait such as denitrification, the respiratory reduction of nitrate and nitrite to N_2O and nitrogen gas, is not limited to specific microbial taxa and is therefore studied independent of culture through the relevant functional genes (6, 25, 32, 38). To date, however, it is not clear to what extent, if at all, these functional genes contain phylogenetic information. Phillipot (22) showed that the phylogeny of *nir* and *nor* genes, coding for the key enzymes nitrite reductase and NO reductase in the denitrification pathway, does not always agree with the phylogeny of the 16S rRNA gene. New isolation and cultivation approaches are therefore imperative to provide the basis for further research on phylogenetic and functional gene diversity.

The isolation of specific physiological groups of bacteria, such as denitrifiers, requires knowledge of the interactions of a large number of medium components and growth conditions.

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Genetic or evolutionary algorithms (EAs) are heuristic optimization programs based on the Darwinistic principles of evolution by natural selection (10). An EA can aid in rationally deciding which fraction of all possible combinations of medium parameters needs to be tested in practice, with the advantage that it does not assume a model (10). Highly complex optimization problems in various domains as diverse as improvement of silage additives (8) and electricity estimations (21) have been resolved with EAs. In microbiology, their use so far has been limited to optimization of fermentation medium (36, 37) and conditions for transconjugant formation (5).

This paper discusses the optimization of the isolation conditions for denitrifying bacteria. The interactions between different medium parameters were investigated with an evolutionary algorithm. Using a minimal mineral medium as a basis, different combinations of medium parameters were applied as isolation medium for denitrifiers, with activated sludge of a municipal wastewater treatment plant (WWTP) as the inoculum, and the diversity of cultured denitrifiers was assessed.

MATERIALS AND METHODS

Inoculum. Activated sludge samples were taken at a municipal wastewater treatment plant with subsequent anoxic and aerated tanks (Bourgoyen-Ossemeersen, Ghent, Belgium). Samples (20 ml) were collected from an anoxic tank at the start of each new batch of growth media and immediately processed. Homogenization of the flocs was performed using a needle (diameter, 0.8 mm) and a 50-ml syringe. After homogenization, dilution series of the samples (10^{0} to 10^{-8}) were made and spread plated on the growth media.

EA experimental design. Each medium parameter can have different values, which can be different levels in concentration or temperature but also different sources of carbon or nitrogen. The combination of these values determines the composition of a growth medium. (The use of the term "growth medium" in this report refers to the composition of the medium and the culture conditions.) Different growth media were grouped into batches. Based on the success or fitness of the growth media from previous batches, a new batch was calculated by the EA. Therefore, the values of the medium parameters of the best scoring

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growth media were recombined in a new batch of growth media. As a result, the average fitness of each new batch should increase.

Eleven medium parameters with different values were selected as variables for the EA. The number of possible combinations of all parameters with their different values was 1,197,504. Each growth medium made up of a combination of medium parameter values was tested for suitability for isolating denitrifiers and was assigned a fitness value. The fitness value contained the following selection parameters: (i) the number of denitrifying isolates and (ii) the diversity of the denitrifying isolates. The first selection parameter was represented by the ratio between the number of isolated denitrifiers and the total number of isolates (Ratio_{den}) per growth medium. The second selection parameter required knowledge of the identity of the isolated denitrifiers. For this purpose, fatty acid methyl ester (FAME) analysis was chosen as a fast identification method. The observed diversity at the genus level was represented for each growth medium by Simpson's reciprocal diversity index 1/D, calculated as follows:

$$1/D = N \times (N-1)/\Sigma \left[n_i \times (n_i - 1)\right] \tag{1}$$

with N representing the number of denitrifying isolates per medium and n_i representing the number of denitrifying isolates per medium belonging to genus *i*. When only one denitrifier was isolated, the diversity index was 0; when all denitrifiers were assigned to the same genus by FAME analysis, the numerator in equation 1 was set to 1. A fitness value was calculated for each medium based on the results of both selection parameters, with both equally weighted, as follows:

$$Fitness = Ratio_{den} \times 1/D$$
(2)

The fitness of a given growth medium would increase if both the number of denitrifying isolates grown on this medium and the diversity of these denitrifying isolates increased. The combination of medium parameters with the highest fitness will therefore be most suited for use as a growth medium for denitrifiers.

Evolutionary algorithm. The Simple Evolutionary Algorithm for Optimization (seao) software (31) is available in an easy-to-use graphical user interface and can be freely downloaded (http://www.cran.r-project.org). The configuration and parameterization of the seao software for experimental optimization of the medium composition used the following settings: number of medium parameters, 11; number of growth media, 15; all previous batches were used for calculation of the next batch of growth media; the selection type was fitness based (rescaling = 0); recombination rate, 90%; and mutation followed a uniform distribution (i.e., all possible values have the same chance of being chosen), with a spread of 1.0 and a rate of 15. For the initial batch of growth media, the EA randomly combined medium parameter values into 15 different growth media.

Growth media. All growth media were based on the mineral medium described by Stanier et al. (29). The following 11 medium parameters with different values were selected for optimization with the EA: pH at 6.5, 7, 7.5, or 8; temperature at 20°C or 37°C; sodium acetate-trihydrate, glycerol, sodium pyruvate, methanol, ethanol, glucose, or sodium succinate as the carbon source; molar C/N ratio of 1, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, or 25; potassium nitrate or potassium nitrite as the nitrogen source; nitrogen concentration of 3 mM, 6 mM, 9 mM, 12 mM, 15 mM, or 18 mM; no addition of sodium chloride or a sodium chloride concentration of 0.34 M; 0-, 1-, or 2-ml addition of vitamin solution (17) containing 4 mg 4-aminobenzoic acid, 2 mg D-(+)-biotin, 10 mg nicotinic acid, 5 mg calcium D-(+)-panthothenate, 15 mg pyridoxine hydrochloride, 4 mg folic acid, and 1 mg lipoic acid in 100 ml 10 mM NaH₂PO₄ at pH 7.1; 0-, 1-, or 2-ml addition of riboflavin solution (17) containing 2.5 mg riboflavin in 100 ml 25 mM NaH₂PO₄ at pH 3.2; 0-, 1-, or 2-ml addition of thiamine solution (17) containing 10 mg thiamine hydrochloride in 100 ml 25 mM NaH₂PO₄ at pH 3.4; and cobalamin solution (17) containing 50 mg cyanocobalamin per liter distilled water. The following pH indicator was added (10 µM): bromothymol blue for growth media with a pH of 6.5 or phenol red for growth media with a pH of 7 or higher. Trypticase soy agar (TSA; Oxoid) was supplemented with 10 mM KNO3 and 10 µM phenol red.

Isolation. A dilution series (10^0 to 10^{-8}) of activated sludge was spread plated ($100 \ \mu$ l) on 15 different growth media per batch, as determined by the EA. The inoculated growth media were incubated for 2 weeks in an anaerobic chamber (gas composition, 8% CO₂, 8% H₂, 84% N₂). From each growth medium and supplemented TSA, 20 isolates were picked from the highest dilution still showing growth, further purified, and subcultured on the same medium (G4M3 was tested in triplicate).

Denitrification tests. All purified isolates were incubated in liquid isolation medium for 1 week under isolation conditions. Tests for nitrate and nitrite reduction were performed using Griess reagents (27). Selection for denitrifiers was based on the results of the reduction tests and the pH indicator (19). This

selection approach was validated by confirmation of the denitrifying activity of all isolates of the first batch with N₂O measurements. All isolates of the first batch presumed to denitrify were grown in 50-ml culture flasks with 10 ml liquid isolation medium. The headspace of the vials was replaced with filter-sterilized argon by evacuating five times and refilling. Acetylene (10%) was added to stop the reduction of N₂O to N₂. After a 1-week incubation, a gas sample (1 ml) was taken with a gas-tight syringe, and N₂O was measured with a gas chromatograph (Shimadzu GC-14B) equipped with an electron capture detector, a precolumn (1 m), and a Porapak column (2 m, 80- to 100-mesh).

FAME analysis. A qualitative and quantitative analysis of cellular fatty acid compositions was performed by the gas-liquid chromatographic procedure described by Sasser (26). The resulting profiles were identified with microbial identification software (MIDI) using the TSBA database, version 5.0 (MIDI, Newark, Del.). In batch 4, some denitrifiers could not be grown under the standard conditions (medium and incubation time) for FAME analysis. Genus identification was then obtained by 16S rRNA gene sequence analysis and used in the same way for the determination of diversity.

DNA extraction. DNA was extracted from each denitrifying isolate by the guanidium-thiocyanate-EDTA-sarkosyl method described by Pitcher et al. (23) for fast-growing strains and by alkaline lysis for slow-growing isolates. For alkaline lysis, one colony was suspended in an Eppendorf tube with 20 μ l of lysis buffer (2.5 ml 10% sodium dodecyl sulfate, 5 ml 1 M NaOH, 92.5 ml MilliQ water). After 15 min at 95°C, 180 ml MilliQ water was added, the tube was centrifuged for 5 min at 13,000 × g, and the supernatant was transferred to a new tube. DNA extracts were stored at -20° C until use.

16S rRNA gene sequence analysis. PCR amplification was performed as described by Heyrman and Swings (9). The PCR-amplified 16S rRNA gene products were purified using the Nucleofast 96 PCR system (Millipore). For each sequence reaction, a mixture was made using 3 µl purified and concentrated PCR product, 1 µl of BigDye Terminator RR mix, version 3.1 (Perkin-Elmer), 1.5 μ l of BigDye buffer (5×), 1.5 μ l sterile MilliQ water, and 3 μ l (20 ng/ μ l) of one of the six sequencing primers used. The primers for partial sequencing (reverse 358-339 and reverse 536-519) and the PCR program were previously described by Heyrman and Swings (9). The sequencing products were cleaned up as described by Naser et al. (20). Sequence analysis of the partial 16S rRNA gene (first 300 to 500 bp) was performed using an Applied Biosystems 3100 DNA sequencer according to protocols provided by the manufacturer. Sequences were assembled using BioNumerics 4.0 software (Applied Maths). A reliable identification was obtained by the following two steps: (i) a BLAST search (2) with the 16S rRNA gene sequence of an isolate retrieved 50 sequences with the highest sequence similarities to the query sequence and (ii) all type strains of all species of all genera mentioned in the BLAST report were compared in an exhaustive pairwise manner with the query sequence of each strain in BioNumerics 4.0. The strains were assigned to a genus based on the obtained 16S rRNA gene sequence similarities

Nucleotide sequence accession numbers. The nucleotide sequence data generated in this study have been deposited in the GenBank/EMBL/DDBJ databases under accession numbers AM083989 to AM084186.

RESULTS

EA experiment. An evolutionary algorithm was used to optimize the isolation conditions for denitrifiers. The influence of 11 medium parameters with different values and their combinations on the number and diversity of isolated denitrifying bacteria was examined. Sixty different growth media, i.e., combinations of medium parameter values, were investigated in four subsequent batches, with 15 growth media per batch. Activated sludge from a municipal wastewater treatment plant was used as the inoculum. An overview of the composition and the fitness results of each growth medium per batch is given in Table S1 in the supplemental material.

The success of a growth medium was determined as a fitness value (Fig. 1). This fitness selected for (i) a large number of denitrifying bacteria and (ii) a high diversity of denitrifying bacteria (see Materials and Methods). For the first batch, the EA randomly combined medium parameter values into 15 growth media. Batch 1 gave an average fitness of 0.48. In total,



FIG. 1. Average and maximal fitness values for each batch of growth media. The fitness value of a growth medium represents the success of a combination of medium parameters in rendering a large (relative) number of denitrifying isolates that are highly diverse in genus assignments.

269 isolates were examined and 34 were detected as denitrifiers. The maximal fitness of batch 1 (i.e., 2.48) was assigned to growth medium G1M1, with a nitrite concentration of 3 mM, a molar C/N ratio of 20, succinate as the carbon source, no sodium chloride or riboflavin added, the addition of 1 ml vitamin solution, 2 ml thiamine solution, and 2 ml cobalamin solution, a pH of 6.5, and incubation at 37°C. The EA calculated a second batch, selecting for those medium parameter values that contributed to high fitness in the previous batch. With batch 2, 217 isolates were examined, 33 isolates were detected as denitrifiers, and an average fitness of 0.54 was measured. The results of batches 1 and 2 appeared very similar, except for the maximal fitness, which increased to 3.86 in batch 2 (Fig. 1). Growth medium G2M11, giving the maximal fitness, differed from the best scoring medium of batch 1 only in the pH, which was 7 instead of 6.5. Some growth media in batches 1 and 2 showed no growth, not even from the undiluted activated sludge sample, while others showed growth, but with <20 colonies. This greatly limited the total number of isolates and, subsequently, the number of denitrifiers in these batches. Batch 3 was calculated based on the fitness results for batches 1 and 2. For the third batch, the average fitness increased to 0.86 (Fig. 1), 315 isolates were examined, and 56 denitrifiers were detected, which were clear increases for all three features compared to batches 1 and 2. The maximal fitness (i.e., 4.09) was found for growth medium G3M12, differing from the two former best scoring media in the values of most medium parameters, as follows: a pH of 7.5, ethanol as the carbon source, a low molar C/N ratio of 2.5, a nitrate concentration of 18 mM, 1 ml of thiamine solution, no cobalamin solution added, and an incubation temperature of 20°C. The EA calculated batch 4 based on the three preceding batches. Again, an increased number of denitrifying bacteria was isolated, with 69 denitrifiers from a total of 300 examined isolates. The maximal fitness of 4.50 was assigned to medium G4M3, which differed from G2M11 only in the use of nitrate instead of nitrite as a nitrogen source. This growth medium was arbitrarily chosen for testing in triplicate to investigate the reproducibility of the evolutionary algorithm. The fitness value differed between the three repeats due to a difference in diversity of the isolated denitrifiers (see Table S1 in the supplemental material). The average fitness value (i.e., 0.87) reached

a plateau in batch 4, which led to the decision to stop the EA. Supplemented TSA was tested in parallel with each batch. The average fitness value for supplemented TSA was 0.625.

Experimental course of medium parameters. A detailed look at the experimental course of each medium parameter defined by the EA revealed convergence to one optimal value for five medium parameters (Fig. 2). The percentage of growth media with the same medium parameter value is directly correlated with the parameter's contribution to high fitness in the preceding batches. Thus, a pH value of 7, a nitrogen concentration of 3 mM, the addition of 1 ml of vitamin solution, and the exclusion of sodium chloride and riboflavin solution contributed to the success of an elective growth medium for denitrifiers (Fig. 2). The other medium parameters diverged to different values. Both temperature values were equally selected over four batches, with an increasing preference for 20°C in batches 3 and 4. Cobalamin converged to either exclusion or the addition of 2 ml. For the nitrogen source, both nitrite and nitrate were equally selected, with an increasing preference for the latter in batch 4. For thiamine, all three possible values were equally selected. Although no optimal value could be determined, the carbon source and molar C/N ratio diverged to two (i.e., ethanol and succinate) and three (i.e., 2.5, 20, and 25) values, respectively, which were more favorable for isolation of denitrifiers than the other possible values. The best scoring growth medium in batches 1, 2, and 4 incorporated most or all of the optimal values determined for the medium parameters; only the composition of the best scoring medium in batch 3 deviated from these values.

Diversity of denitrifying populations in activated sludge. One hundred ninety-two denitrifying isolates were distinguished in a total of 1,101 isolates obtained on the 60 evaluated growth media, while 7 of 80 isolates obtained on supplemented TSA were able to denitrify. After FAME analysis, 198 denitrifying isolates were reliably identified to the genus level (Table 1) via partial 16S rRNA gene sequence analysis (no 16S rRNA gene amplicon could be obtained for one isolate). The majority of the denitrifiers belonged to the Betaproteobacteria (50.5%, or 100 isolates). Sixty-eight strains were assigned to the Acidovorax, Alicycliphilus, Comamonas, and Diaphorobacter genera of the Comamonadaceae and were isolated predominantly from growth media with ethanol or succinate as the carbon source, coupled with nitrate or nitrite as the nitrogen source, respectively. Thirtyone isolates were assigned to the Azospira, Azovibrio, Dechloromonas, Thauera, and Zoogloea genera of the Rhodocyclaceae, the majority of which were isolated on growth medium with succinate as the carbon source and a pH value of 7. One isolate belonged to the genus Aquaspirillum of the Neisseriaceae. The second biggest group of denitrifiers belonged to the Alphaproteobacteria (37.3%, or 74 isolates): 22 isolates belonged to the Brucella and Ochrobactrum genera of the Brucellaceae, 8 isolates belonged to the Rhizobium and Sinorhizobium genera of the Rhizobiaceae, 43 isolates belonged to the Paracoccus and Pannonibacter genera of the Rhodobacteraceae, and 1 isolate belonging to the genus Methylobacterium represented the Methylobacteraceae. The Gammaproteobacteria were represented by 11 isolates belonging to the genus *Pseudomonas* (5.6%). Four isolates (2%)belonging to Arcobacter represented the Epsilonproteobacteria. Eight isolates (4%) belonging to the Bacillus, Trichococcus, Enterococcus, Paenibacillus, and Staphylococcus genera represented



FIG. 2. Percentages of growth media with certain values for medium parameters for each batch. The experimental course of the following five medium parameters converged to one value: pH(A), nitrogen concentration (B), sodium chloride concentration (C), vitamin solution (D), and riboflavin solution (E). The percentage of growth media with the same value for a medium parameter is directly correlated with its contribution to high fitness in the preceding batches.

the *Firmicutes*. One isolate of the genus *Chryseobacterium* belonging to the *Flavobacteriaceae* represented the *Bacteroidetes*. No clear trends were observed in the compositions of the growth media used for isolation of members of the *Alpha-*, *Gamma-*, and *Epsilonproteobacteria* and *Firmicutes*.

DISCUSSION

Little is known about the denitrifying diversity present in activated sludge, as straightforward cultivation-independent approaches are not suitable and cultivation-dependent research is limited. Magnusson et al. (18) performed an isolation campaign on nutrient agar with activated sludge from five different municipal WWTPs and found only denitrifying proteobacteria belonging to the Rhodobacteraceae, Comamonadaceae, and Pseudomonadaceae. After applying 60 different defined isolation conditions, a much more important denitrifier diversity was found, although proteobacteria were still predominant. Denitrifying representatives of Alpha-, Beta-, Gamma-, and Epsilonproteobacteria, Firmicutes, and Bacteroidetes were found, and apart from genera classically known to harbor denitrifiers, such as Pseudomonas, Ochrobactrum, Comamonas, and Acidovorax, genera less frequently observed in cultivation studies of denitrifiers were also encountered. The Rhodocyclaceae were well represented, encompassing, besides the genus Thauera, the recently described genera Azospira and Azovibrio (24) and Dechloromonas (1, 11). Furthermore, possibly new species belonging to Thauera and Zoogloea were retrieved. Recent efforts to identify denitrifiers in activated sludge in a cultivation-independent manner by combining fluorescence in situ hybridization with microautoradiography (35) recognized the *Azoarcus-Thauera* group of the *Rhodocyclaceae* as probably the most abundant denitrifiers in industrial WWTPs. The genus *Arcobacter* was previously found in significant numbers in activated sludge (28), but its function was undetermined. In this study, four denitrifying *Arcobacter* strains were isolated, demonstrating that the genus can contribute to the denitrification process in activated sludge systems. The denitrifying potential of *Bacteroidetes* and *Firmicutes* strains, including *Bacillus, Paenibacillus, Staphylococcus, Trichococcus*, and enterococci, known from cultivation-independent studies to be numerically less important in WWTPs than the proteobacteria (13), was also established.

This study shows the applicability of an EA for the optimization of growth media. The progressive improvement of the average and maximal fitness values in each successive batch confirms the iterative nature of an EA. The maximal fitness value of each batch of newly designed media was significantly higher than the average fitness of supplemented TSA, which is still the standard growth medium for denitrifiers (33). Highly suitable elective growth media were developed, rendering between 40 and 80% denitrifiers. Comparable data are unavailable for cultivation-dependent studies on activated sludge; for soil, 10% of all isolates on supplemented nitrate broth were denitrifiers (7). After evaluations of 60 different combinations of medium parameters, the three best scoring growth media, G2M11, G3M12, and G4M3, can be recommended for the isolation of denitrifiers in the future.

The isolation conditions for denitrifiers were optimized heu-

Transmits position (class, funity, or genus) Type strain with highest 1/6 RNA gene sequence similarity of query sequence similarity Growth medium (no. of isolates) Constructions Species name Strain number ⁶ Sequence similarity Accession number Constructions Constructions Multiple Brucella Brucella oris ATCC 25840T 99.5 L26168 G3M5 (1) G3M3 (2), G3M3 (3), G3M7 (2), G3M4 (3), G3M4 (3), G3M7 (2), G3M4 (3), G3M4 (3), G3M7 (2), G3M4 (3), G3M4 (3), G3M7 (2), G3M4 (3), G3M7 (2), G3M4 (3), G3M7 (2), G3M4 (3), G3M7 (2), G3M4 (1), G3M7 (1), G3M4 (1), G3M4 (1						
(closs. funity, or genos) Species name Strain number ⁶ / ₁₅ Sequence annaber Accession annaber Colorent abcalant (pol 0: Solande) Alfpapproprinzations Bracella coris ATCC 2584IT 99.5 L20168 G3M5 (1) Ochrobactrum Ochrobactrum intermedium DSM 6321T 99.5 L20168 G3M5 (2) Ochrobactrum intermedium DSM 13340T 100 AI224254 G3M5 (2) Methylobacterine Methylobacterine intermedium DSM 13340T 100 AI224254 G3M5 (2) Methylobacterine intermedium DSM 1458T 95.1 AY00940 G2M4 (1) G3M7 (2), G3M3 (2) (1) Sinorhizobian Ritizobian molecular molecular MSO (1), G1M1 (2), G2M1 (2) (1) G3M3 (2) (1) G3M1 (2) (1) Sinorhizobian Sinorhizobian MSO (2), G1M1 (2), G2M1 (2) (2) G3M3 (2) (1) G3M3 (2) (1) G3M3 (2) (1) Sinorhizobian Sinorhizobian maldocar MSO (2), G3M3 (2) (2) G3M3 (2) (2) G3M3 (2) (2) Sinorhizobian Rolecone DSM (2), G3M1 (2), G3M1 (2) G3M3 (2) (G3M3 (2)	Taxonomic position	Type strain with highest 16S rf	Growth medium (no. of isolates)			
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Methylobacteraceae MethylobacteriumMethylobacteriumStatistica StatisticaStatistica Stat		Ochrobactrum tritici	DSM 13340T	100	AJ242584	G3M5 (2)
Methylobacterium Methylobacterium Methylobacterium Sinothicae CIP USSN [4485T 95.1 AY009404 GZM4 (1) Rhizobiane Rhizobian giardinii CIP USS03T 97.2 USS343 GIM15 (1) Rhizobian Rhizobian galleann MSD1109T 97.6 USS343 GIM1 (1) GIM15 (1) Sinorhizobian Rhizobian sulae DSM 1462ST 97.2-100 AJ38904 GIM1 (1) GIM1 (1) GZM4 (1) Sinorhizobian Sinorhizobian Sinorhizobian Sinorhizobian ATCC 49673T 97.6 YIU177 GIM3 (2) GIM1 (1) GZM4 (1) GIM3 (2) Paracoccus andiniphilus ATCC 49632T 97.4-99.7 D3240 GIM3 (2) GIM3 (2) GIM4 (2) GIM3 (2) GIM3 (2) GIM4 (1) GIM3 (2) GIM4 (1) GIM3 (2) GIM4 (1) GIM3 (2) GIM3 (1) GIM3 (2) GIM3 (2)<	Methylobacteraceae					
Nitobianeaee Brizobiam Rhitobiam giardini Ritobiam radioacter Ritobiam sullacen CIP 105503T MSD1109T 97.2 97.6 U86344 U86343 G3M12 (1) G3M2 (1), G3M12 (1) Sinorhitobiam GiM1 (1), G2M1 (1), G2M1 (1), G2M1 (1), G3M4 (1), G3M7 (1), G3M1 (Methylobacterium	Methylobacterium suomiense	DSM 14458T	95.1	AY009404	G2M4 (1)
Rhizobium Rhizobium galicum MSD1109T 97.2 U86344 GiM12 (1) Rhizobium radiobacter MSD1109T 97.6 U86344 GiM15 (1) Sinorhizobium Rhizobium radiobacter ATCC 1938T 97.2-100 AJ389904 GiM15 (1) Sinorhizobium Sinorhizobium morelense LC04T 97.0-97.2 AV024335 GiM1 (1), G2M1 (1), G2M12 (1) Biodobacteracea Paracoccus aclaiphilus ATCC 49673T 97.6-97.8 AV014176 GiM3 (2) GiM3 (2) Paracoccus aclaiphilus ATCC 49673T 97.4-99.7 D32240 GiM3 (2), G3M1 (2), G3M1 (2) GiM4 (1), G3M1 (2), G3M1 (2) Paracoccus acanoinipáciens E-396T 98.4-98.7 AB006393 G3M4 (1), G3M1 (2) G3M4 (1), G3M1 (2) Paracoccus versuitas ATCC 25512T 100 Y16933 G3M4 (1), G3M1 (2) G3M4 (1), G3M1 (2) G3M4 (1), G3M1 (2) Paracoccus versuitas ATCC 2564T 99.9-100 AY01474 G3M4 (1), G3M1 (2) G3M4 (1), G3M1 (2) <td< td=""><td>Rhizobiaceae</td><td></td><td></td><td></td><td></td><td></td></td<>	Rhizobiaceae					
Bhizobium salicum Rhizobium salicum autobacter Rhizobium salicum autobacter Rhizobium salicum autobacter Rhizobium salicum salicum Sinorhizobium morelenseMSDI110797.6U86343 97.2-100GIMI 5 (1) GIMI 5 (1) 	Rhizobium	Rhizobium giardinii	CIP 105503T	97.2	U86344	G3M12 (1)
Britzobium sulla ATCC 19381 97.2 -100 AJ38994 GM2 (1), GJM15 (1) Sinorhizobium sulla DSM 1462T 97.6 Y10170 GIM1 (1), GZM11 (1), GZM12 (1) Rodobacterizee Paracoccus aninophilus ATCC 4963T 97.6 AY014175 GIM3 (2) Paracoccus aninophilus ATCC 4963T 97.6 AY014175 GIM3 (1), GZM7 (1), G3M4 (1), Paracoccus aninophilus ATCC 4963T 97.6-97.8 AY014175 GIM3 (2), GZM7 (1), G3M4 (1), Paracoccus aninophilus ATCC 4963T 97.6-97.8 AY014175 GIM3 (2), GAM1 (2), Paracoccus caroninficiens E-396T 98.4-98.7 AB006899 G3M4 (1), G3M1 (2), Paracoccus caroninficiens E-396T 98.4-98.7 AY014173 GJM1 (2), Paracoccus versitus ATCC 253512T 100 Y16933 GJM1 (1), GZM1 (1), GZM1 (1), Paracoccus versitus ATCC 29625T 97.8 AY014173 GJM2 (1), GJM1 (2), Paracoccus versitus DSM 12644T 99.4-100 Y18616 G2M15 (1), G3M12 (1), GJM1 (2), GJM1 (1), GJM1 (2), <td></td> <td>Rhizobium gallicum</td> <td>MSDJ1109T</td> <td>97.6</td> <td>U86343</td> <td>G1M15 (1)</td>		Rhizobium gallicum	MSDJ1109T	97.6	U86343	G1M15 (1)
Britobium Rhobobacterizede Paracoccus aminophilus DSM 14621 ATCC 99673T 97.6 97.0-97.2 YU1/10 AV024335 GIMI (1), GZM1 (1), GZM1 (1), G3M7 (1), G3M1 (2), G3M1 (2), G3M1 (1), G3M1 (2), G3M1 (2), G3M1 (2), G3M1 (2), G3M1 (2), G3M1 (2), G3M1 (1), G3M1 (1), G3M1 (2), G3M1 (2), G3M1 (1), G3M1 (1), G3M1 (2), G3M1 (2), G3M1 (1), G3M1 (1), G3M1 (2), G3M1 (2), G3M1 (1), G3M1 (1), G3M1 (2), G3M1 (2), G3		Rhizobium radiobacter	ATCC 19358T	97.2–100	AJ389904	G3M2 (1), G1M15 (1)
Smorhizobium Rhadobacteraceae Paracoecus Smorhizobium Paracoecus aclaiphilus ATCC 49673T ATCC 51197 97.6 97.6 97.6 AY01417 AY014177 GIM1 (1), G2MI1 (1), G2MI1 (1), G2MI1 (1), G3M7 (1), G3M1 (1), G3M1 (1), G3M7 (1), G3M1 (1), G3M1 (1), G3M7 (1), G3M1 (1), G3M1 (2), G3M4 (1), G3M7 (1), G3M1 (2), G3M4 (1), G3M1 (2), Paracoecus partinophus E-396T 98.4-98.7 AB006893 (1), G3M1 (1), G2M1 (2), G3M4 (1), G3M1 (2), G3M3 (1), G3M1 (1), G2M1 (1), G2M1 (1), G3M2 (1), G3M1 (2), G3M4 (1), G3M1 (2), G3M3 (1), G3M3 (1), G3M1 (1), G2M1 (1), G3M2 (1), G3M1 (2), G3M3 (1), G3M1 (1), G3M1 (2), G3M1 (1), G3M1 (1), G3M1 (2), G3M1 (1), G3M1 (1), G3M1 (2), G3M1 (1), G3M1 (1), G3M1 (1), G3M1 (2), G3M1 (1), G3M1 (1), G3M1 (1), G3M1 (1), G3M1 (1), G3M1 (1), G3M1 (2), G3M1 (1), G3M1 (1), G3M1 (2), G3M1 (1), G3M1 (1), G3M1 (1), G3M1 (1), G3M1 (1), G3M1 (1), G3M1 (1), G3M1 (2), G3M1 (1), G3M1 (1)	a . 1 . 1 .	Rhizobium sullae	DSM 14623T	97.6	Y10170	G1M15 (1)
Rindabactericade ParacoccusParacoccus aninophilus Paracoccus alcaliphilusATCC 49673T ATCC 51199T97.6 97.6-97.8AY014176GIM3 (2) GIM3 (1), G2M7 (1), G3M4 (1), GIM3 (1), G3M12 (1), G3M1 (1), GIM3 (1), G3M3 (1), GIM3 (1), G3M1 (1), G3M3 (1), GIM1 (1), G3M1 (1), G3M1 (1), GIM1 (1), GIM1 (1), G3M1 (1), GIM1 (1), G3M1 (1), GIM1 (2), GIM1 (1), GSM1 (1), GIM1 (2), GIM1 (1), GSM1 (1), GIM1 (2), GIM1 (1), GSM1 (1), GIM1 (2), GIM1 (2), GIM1 (1), GIM1 (2), <b< td=""><td>Sinorhizobium</td><td>Sinorhizobium morelense</td><td>LC04T</td><td>97.0–97.2</td><td>AY024335</td><td>G1M1 (1), $G2M11$ (1), $G2M12$ (1)</td></b<>	Sinorhizobium	Sinorhizobium morelense	LC04T	97.0–97.2	AY024335	G1M1 (1), $G2M11$ (1), $G2M12$ (1)
Paracoccus Paracoccus andiophulus ATCC 49631 97.6 AY014177 GIM3 (2) Paracoccus andiophulus ATCC 49632T 97.6–97.8 AY014177 GIM3 (1), G3M7 (1), G3M4 (1), G3M7 (1), G3M1 (2), G3M4 (1), G3M4 (1), G3M5 (2), GIM1 (2), G3M4 (1), G3M4 (1), G3M5 (2), GIM1 (2), G3M4 (1), G3M5 (1), G3M1 (2) Paracoccus anotinifaciens E-396T 98.4–98.7 AB006899 G3M4 (1), G3M7 (2) Paracoccus pantorophus ATCC 25512T 100 Y16933 G3M5 (1), G3M14 (2) Paracoccus versulus ATCC 25364T 99.9–100 AY014174 G3M4 (1), G3M12 (2), G3M13 (1) Paranonibacter Pannonibacter phragmitetus DSM 14782T 100 AJ400704 G3M2 (1) Betaproteobacteria Commonadaccea Acidovorax avenae subsp. ATCC 29625T 98.1–98.3 AF078761 G2M6 (1), G2M7 (1), G3M11 (2), G3M13 (2), G3M11 (2), G3M11 (2), G3M11 (2), G3M13 (2), G3M11 (2), G3M13 (2), G3M3 (2) Alicycliphilus Acidovorax temperans ATCC 49665T 97.8–99.5 AF078766 GIM1 (3),	Rhodobacteraceae	D ' 1'1	ATTCC 40(72T	07.6	A \$201 417C	
$ \begin{array}{c} Paracoccus accipinitis & ATCC 511911 & 97.8-97.8 & AY0141/7 & GIM3 (1), GAM7 ($	Paracoccus	Paracoccus aminophilus	ATCC 496/31	97.6	AY014176	GIM3(2)
Paracoccus aminovorans ATCC 49632T 97.4-99.7 D32240 GMM (1), GMM (2), GMM (3), GMM (1), GMM (3), CMM (3), GMM (1), GMM (1), GMM (1), GMM (1), GMM (1), GMM (1), GMM		Paracoccus alcaupnilus	ATCC 511991	97.6-97.8	AY0141//	GIM3(1), G2M7(1), G3M4(1),
Partnervectus antiniportantsATCC 49052197.8–95.7D52240GIMS (1), GIMIS (1), GZMIS (3), GAMI2 (1), GZMIS (3), GAMI2 (3), GAMIS (1), GAMI2 (3), GAMIS (1), GAMI2 (3), GAMIS (3), GAMI2 (3), GAMIS (3), GAMI2 (3), GAMIS (3), GAMI2 (3), GAMIS (3), GAMIS (1), GAMIS (3), Paracoccus yees Paracoccus yees Paracoccus versutusE-396T98.4–98.7AB006899GIMS (1), GAMIS (3), GAMI2 (1), GAMIS (1), GAMI2 (1), GAMIS (1), GAMI2 (1), GAMI3 (1), Paracoccus versutusParancoccus versutusATCC 35512T100Y16933GAMS (1), GAMIS (1), GAMIS (1), GAMI3 (1), GAMIS (1), GAMI3 (1), GAMIS (1), GAMI3 (1), GAMIS (1), <td></td> <td>Danagoogua aminouonana</td> <td>ATCC 40622T</td> <td>074 007</td> <td>D22240</td> <td>$G_{1M5}(2)$ $G_{1M14}(2)$ $G_{2M2}(2)$</td>		Danagoogua aminouonana	ATCC 40622T	074 007	D22240	$G_{1M5}(2)$ $G_{1M14}(2)$ $G_{2M2}(2)$
$ \begin{array}{c} \mbox{Constraint} (1), \mbox{Constraint} (2), Constrai$		Furucoccus uminovorans	ATCC 490521	97.4-99.7	D32240	$G_{1MI3}(2), G_{1M14}(5), G_{2M15}(5), G_{2M14}(1), G_{2M15}(1), G_{2M15}(1)$
$ \begin{array}{c} Paracoccus carotinifaciens \\ Paracoccus pantotrophus \\ Paracoccus versuitus \\ Paracocus ver$						$G_{4}M_{12}(5)$ $G_{4}M_{15}(2)$
Paracoccus partococcus partorophusDistrictDistrictDistrictDistrictParacoccus yeeiCCUG 46822T97.8AY01473G3M4 (1), G3M14 (2),Paracoccus yeeiCCUG 46822T97.8AY01473G3M4 (1), G2M4 (1),ParnonibacterPannonibacter phragmitetusDSM 14782T100AY0473G3M5 (1)PannonibacterPannonibacter phragmitetusDSM 14782T100AY0476G3M2 (1)BetaproteobacteriaComamonadaccaeComamonadaccaeG2M15 (1), G3M2 (1), G3M10 (2),G3M11 (1),AcidovoraxAcidovorax avenae subsp. citrulliATCC 29625T98.1-98.3AF078761G2M6 (1), G2M7 (1)AcidovoraxAcidovorax defluviiDSM 12644T99.4-100Y18616G2M15 (1), G3M2 (1), G3M13 (2), G3M11 (1), G3M13 (2), G3M11 (1), G3M13 (1),AlicycliphilusAlicycliphilus denitrificansDSM 14773T97.8-99.5AF078766G1M1 (4), G1M8 (1), G2M7 (2), G3M1 (1),Comamonasaquatica Comamonas aquatica Comamonas aquatica Comamonas aquaticaATCC 11330T99.0-99.8AJ480344G3M1 (2)DiaphorobacterDiaphorobacter nitroreducensDSM 15985T99.2-99.8AB064317G1M1 (4), G4M3 (1), G3M12 (1), G3M11 (2), G3M3 (2)Neisseraceae Acovitrio AzovitrioAzovitrio AzovitrioAzovitrio AzovitrioAzovitrio AzovitrioAzovitrio AzovitrioAzovitrio AzovitrioG4M3 (1), G4M3 (1)Neisseraceae Acovitrio Azovitrio AzovitrioAzovitrio AzovitrioAzovitrio AzovitrioAzovitrio		Paracoccus carotinifacians	E 306T	08/087	A B006800	$G_{3M4}(1)$ $G_{3M7}(2)$
Paracoccus period Paracoccus versutusCCUG 468221 CCUG 468221100 99.9-100AY014173 GM4173GM41 (1), GM112 (1), GM113 (1), GM12 (1), GM112 (1), GM113 (1), GM135 (1)PannonibacterPannonibacter phragmitetusDSM 14782T100AJ400704GM2 (1)Betaproteobacteria Comamonadaccae AcidovoraxArtCC 29625T98.1-98.3AF078761G2M6 (1), G2M7 (1)Betaproteobacteria Comamonadaccae Acidovorax defluviiDSM 12644T99.4-100Y18616G2M15 (1), G3M2 (1), G3M13 (2), G3M11 (1), G3M12 (1), G3M13 (2), G4M13 (1), G4M14 (1)Alicycliphilus Comamonas Comamonas denitrificans Alicycliphilus Comamonas denitrificansATCC 49665T97.8-99.5AF078766G1M1 (4), G1M8 (1), G2M15 (2) G1M1 (4), G1M8 (1), G2M15 (2) G1M1 (3), G3M1 (1)DiaphorobacterDiaphorobacter nitroreducens AccoptraDSM 15985T99.2-99.8AB064317G1M1 (2), G3M1 (2), G3M3 (2) G3M9 (1), G3M12 (1), G3M1 (2), G3M3 (2) G3M9 (1), G3M12 (1), G3M1 (2), G3M3 (2) G3M9 (1), G3M12 (1), G4M4 (1), G4M4 (1), <b< td=""><td></td><td>Paracoccus pantotrophus</td><td>ATCC 35512T</td><td>100</td><td>X16033</td><td>$G_{3M5}(1), G_{3M14}(2)$</td></b<>		Paracoccus pantotrophus	ATCC 35512T	100	X16033	$G_{3M5}(1), G_{3M14}(2)$
Paracoccus versatusATCC 25364T99.9-100AY014174GIMT (1), G2M4 (1), G2M7 (1), G3M5 (1)PannonibacterPannonibacter phragniietusDSM 14782T100AJ400704G3M2 (1)Betaproteobacteria Comamonadaccae AcidovoraxAcidovorax avenae subsp. citrulliATCC 29625T98.1-98.3AF078761G2M6 (1), G2M7 (1)AcidovoraxAcidovorax defltwiiDSM 12644T99.4-100Y18616G2M15 (1), G3M1 (2), G3M10 (2), G3M11 (1), G3M12 (1), G3M11 (2), G3M13 (2), G4M13 (1), G4M14 (1)AlicycliphilusAcidovorax temperans Alicycliphilus denitrificansATCC 49665T97.8-99.5AF078766G1M1 (4), G1M8 (1), G2M17 (2), G3M1 (2), G3M1 (2), G3M1 (2), G3M1 (2), G3M1 (2), G3M1 (2), G3M1 (2), G3M1 (2), G3M3 (2)DiaphorobacterDiaphorobacter nitroreducensDSM 15985T99.2-99.8AB064317G1M1 (3), G2M11 (1), G3M1 (2), G3M1 (2), G3M1 (2), G3M3 (2)Neisseraceae Acouibrio AzovibrioAcuaspirillum metamorphum DSM 1589TDSM 1585T99.2-99.8AB064317G1M3 (1) G3M1 (1), G3M1 (1), 		Paracoccus veei	CCUG 46822T	97.8	AY014173	$G_{3M4}(1), G_{3M12}(1), G_{3M13}(1)$
PannonibacterPannonibacter phragmitetusDSM 14782T100AJ400704G3M5 (1)Betaproteobacteria Comamonadaceae AcidovoraxAcidovorax avenae subsp. citrulli Acidovorax defluviiATCC 29625T98.1–98.3AF078761G2M6 (1), G2M7 (1)Betaproteobacteria Comamonadaceae AcidovoraxAcidovorax avenae subsp. citrulli Acidovorax temperansATCC 29625T98.1–98.3AF078761G2M6 (1), G3M12 (1), G3M10 (2), G3M11 (1), G3M13 (2), G4M13 (1), G4M13 (1), G4M13 (1), G3M13 (2), G4M13 (1), G4M14 (1)Alicycliphilus Comamonas Comamonas Comamonas aquatica Comamonas aquatica Comamonas denitrificans Comamonas denitrificansATCC 49665T97.8–99.5AF078766G1M1 (4), G1M8 (1), G2M15 (2) G1M1 (3), G3M1 (1)Diaphorobacter Aquespirillum Rhodocyclaceae AcospiraDiaphorobacter nitroreducens AcospiraDSM 15785T99.2–99.8AB064317G1M1 (3), G3M1 (1), G3M12 (1), G3M1 (2), G3M3 (2) G3M3 (2)Neisseraceae Acospira Acospira Dechloromonas deinitificansDSM 1837T98.9Y18618G4M3 (1)Neisseraceae Acospira Acospira Dechloromonas deinitificansDSM 1837T98.9Y18618G4M3 (1)Neisseraceae Acospira Acospira Dechloromonas agiata Thauera Thauera aminacoromatica Thauera acomatica Thauera mecherichensis DSM 1882T99.9–100AF011347G2M11 (1), G4M3 (1) G2M11 (1), G4M6 (1)Dechloromonas Thauera mecherichensis Thauera mecherichensis Thauera mecherichensis DSM 14743T95.9–90.3X77118TSA (2) TSA (1)Thauera Thauera mecherichensis<		Paracoccus versutus	ATCC 25364T	99 9-100	AY014174	$G_{1M7}(1), G_{2M4}(1), G_{2M7}(1)$
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Incontrol	Azovibrio	Azovibrio restrictus	LMG 9099T	100	AF011346	$G_{2M9}(1)$
Dechloromonas denitrificans Dechloromonas denitrificansDSM 15892T DSM 15892T97.2–99.8 99.2–99.3AJ318917 AJ315677G4M3 (1), G4M6 (1)ThaueraThauera aromatica Thauera aninoaromaticaDSM 16892T DSM 14742T99.2–99.3 99.5–100X77118 AJ315677TSA (2)Thauera chlorobenzoica Thauera mechernichensis Thauera phenylaceticaDSM 12266T DSM 14743T99.4AF123264 95.0–99.5TSA (1)Thauera selenatis ZoogloeaATCC 55363T Zoogloea ramigera99.1 ATCC 19544TY17591 96.4TSA (1)	Dechloromonas	Dechloromonas agitata	ATCC 700666T	100	AF047462	$G_{4M3}(4) G_{4M7}(2)$
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Thauera phenylacetica DSM 14743T 95.0–99.5 AJ315678 G2M13 (1), G3M15 (1), G4M6 (2), TSA (1) Thauera selenatis ATCC 55363T 99.1 Y17591 TSA (1) Zoogloea Zoogloea ramigera ATCC 19544T 96.4 X74913 G4M6 (1)		Thauera mechernichensis	DSM 12266T	98.0	Y17590	G2M13 (1)
Thauera selenatisATCC 55363T99.1Y17591TSA (1)ZoogloeaZoogloea ramigeraATCC 19544T96.4X74913G4M6 (1)		Thauera phenylacetica	DSM 14743T	95.0-99.5	AJ315678	G2M13 (1), G3M15 (1), G4M6 (2),
ZoogloeaThauera selenatisATCC 55363T99.1Y17591TSA (1)Zoogloea ramigeraATCC 19544T96.4X74913G4M6 (1)						TSA (1)
Zoogloea Zoogloea ramigera ATCC 19544T 96.4 X74913 G4M6 (1)		Thauera selenatis	ATCC 55363T	99.1	Y17591	TSA (1)
	Zoogloea	Zoogloea ramigera	ATCC 19544T	96.4	X74913	G4M6 (1)

TABLE 1.	Denitrifying	organisms	determined in	n this study
TIDEE I.	Demanying	organionio	acterimited i	i this study

Continued on following page

Taxonomic position (class, family, or genus)	Type strain with highest 16S rR				
	Species name	Strain number	% Sequence similarity	Accession number	Growth medium (no. of isolates)
Gammaproteobacteria Pseudomonadaceae					
Pseudomonas	Pseudomonas aeruginosa Pseudomonas alcaligenes Pseudomonas mendocina Pseudomonas nitroreducens Pseudomonas putida Pseudomonas stutzeri	ATCC 10145T ATCC 14909T ATCC 25411T ATCC 33634T ATCC 12633T ATCC 17588T	99.9–100 100 95.3–95.6 99.1–99.3 98.9 100	AF094713 Z76653 AJ308310 D84021 AJ308313 AF094748	G1M1 (1), G2M11 (1) G2M6 (2), G2M7 (1) G3M9 (1), TSA (1) G1M10 (1), G3M9 (1) G4M9 (1) G2M11 (1)
Epsilonproteobacteria Campylobacteraceae Arcobacter	Arcobacter cryaerophilus Arcobacter skirrowii Arcobacter nitrofigilis	CCUG 17801T ATCC 51132T ATCC 33309T	99.3–99.8 94.6 95.4	L14624 L14625 L14627	G4M6 (2) G4M6 (1) G4M6 (1)
Firmicutes Bacillaceae					
Bacillus	Bacillus clausii Bacillus mojavensis	ATCC 700160T ATCC 51516T	99.7 98.6–98.7	X76440 X68416	G4M3 (1) G1M4 (1), G1M8 (1)
Carnobacteraceae Trichococcus	Trichococcus flocculiformis	DSM 2094T	100	AJ306611	G4M6 (1)
Enterococcus	Enterococcus casseliflavus	ATCC 25788T	99.2–100	AF039903	G1M5 (1) G2M11 (1)
Paenibacillaceae Paenibacillus Stanhulococcacae	Paenibacillus agaridevorans	DSM 1355T	98.6	AJ345023	G3M12 (1)
Staphylococcuceue Staphylococcus	Staphylococcus hominis subsp. hominis	ATCC 27844T	99.9	L37601	TSA (1)
Bacteroidetes Flavobacteriaceae					
Chryseobacterium	Chryseobacterium gleum	ATCC 35910T	94.8	AY468449	G2M6 (1)

TABLE 1—Continued

ristically. Convergence of a medium parameter to one value indicates no interaction with other medium parameters. The EA determined that five medium parameters converged to one optimal value. Because of their independence of the overall medium composition, these parameters can be fixed at these values in further optimization studies while other medium parameters are varied. Although halotolerant and halophilic denitrifiers are known (16), the exclusion of sodium chloride appeared to increase the isolation of denitrifiers. This observation may be correlated with the use of activated sludge as the inoculum. Riboflavin did not result in an enhanced retrieval of denitrifiers, which contradicts an earlier report on the reduction of the doubling time for Paracoccus denitrificans when riboflavin was added under denitrifying conditions (4). The same study showed an increase in the nitrite reductase activity, thus decreasing the accumulation of nitrite, with ethanol as the carbon source. The suitability of ethanol as a carbon source for denitrifiers was also confirmed here. In contrast to previous optimization studies in microbiology with EAs (5, 8, 36), the reproducibility of fitness was assessed. The observed nonreproducibility of the genus diversity determination was probably attributed to (i) the limited number of investigated strains per growth medium due to logistics and time, (ii) the use of FAME analysis for genus identification, and/or (iii) other possible parameters not included in the EA.

Weuster-Botz (37) stated that "a combination of highly directed random searches to explore the *n*-dimensional variable space with a genetic algorithm, and subsequent application of classical statistical experimental design is recommended for media development." The work reported here can be seen as the initial step for elective medium design and development for denitrifying bacteria and provides the basis for further cultivation-dependent research on denitrifiers. Furthermore, through this study, new growth media are available that favor the growth of denitrifiers exhibiting high natural diversity. Also, a large set of denitrifying isolates has been obtained that can be further subjected to research concerning denitrification, e.g., functional gene sequence analysis. Similar large-scale cultivation studies could have future value for physiologically interesting bacterial groups that are difficult to study, e.g., filamentous or nitrifying bacteria.

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