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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. 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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† Supplemental material for this article may be found at http://aem.asm.org/.

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:

$$1/D = N \times (N - 1)/\Sigma [n_i \times (n_i - 1)] \quad (1)$$

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:

$$\text{Fitness} = \text{Ratio}_{den} \times 1/D \quad (2)$$

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 NaH_2PO_4 at pH 7.1; 0-, 1-, or 2-ml addition of riboflavin solution (17) containing 2.5 mg riboflavin in 100 ml 25 mM NaH_2PO_4 at pH 3.2; 0-, 1-, or 2-ml addition of thiamine solution (17) containing 10 mg thiamine hydrochloride in 100 ml 25 mM NaH_2PO_4 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 KNO_3 and 10 μM phenol red.

Isolation. A dilution series (10^0 to 10^{-8}) of activated sludge was spread plated (100 μl) 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% CO_2, 8% H_2, 84% N_2). 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 N_2O 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 N_2O to N_2. After a 1-week incubation, a gas sample (1 ml) was taken with a gas-tight syringe, and N_2O 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 μl 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 μl of BigDye buffer (5×), 1.5 μl sterile MilliQ water, and 3 μl (20 ng/μl) 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,

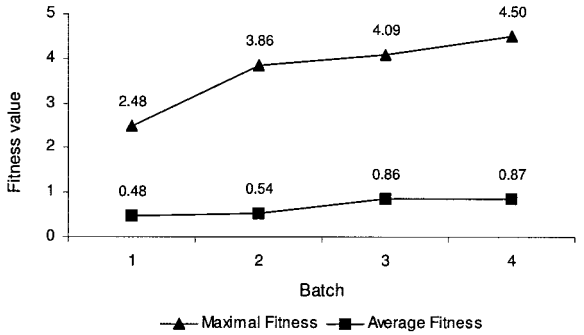


FIG. 1. Average and maximal fitness values for each batch of growth media. The fitness value of a growth medium represents the success of a combination of medium parameters in rendering a large (relative) number of denitrifying isolates that are highly diverse in genus assignments.

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. Thirty-one 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

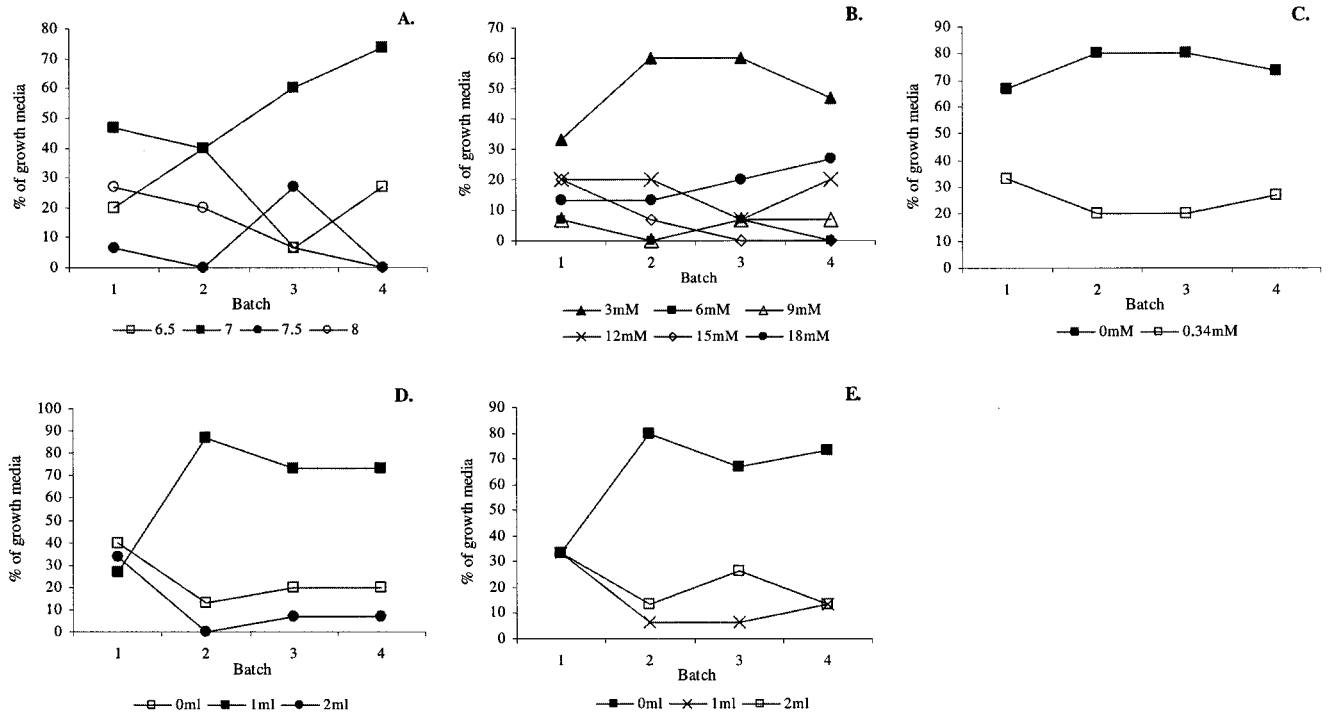


FIG. 2. Percentages of growth media with certain values for medium parameters for each batch. The experimental course of the following five medium parameters converged to one value: pH (A), nitrogen concentration (B), sodium chloride concentration (C), vitamin solution (D), and riboflavin solution (E). 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-

TABLE 1. Denitrifying organisms determined in this study

Taxonomic position (class, family, or genus)	Type strain with highest 16S rRNA gene sequence similarity to query sequence				Growth medium (no. of isolates)
	Species name	Strain number	% Sequence similarity	Accession number	
Alphaproteobacteria					
Brucellaceae					
Brucella	*Brucella ovis*	ATCC 25840T	99.5	L26168	G3M5 (1)
Ochrobactrum	*Ochrobactrum anthropi*	DSM 6882T	97.8–100	D12794	G1M3 (1), G1M14 (3), G2M6 (1), G2M7 (1), G3M4 (5), G3M5 (3), G3M7 (2), G4M3 (1)
	Ochrobactrum intermedium	LMG 3301T	99.2	U70978	G2M11 (1), G2M12 (1)
	Ochrobactrum tritici	DSM 13340T	100	AJ242584	G3M5 (2)
Methylobacteraceae					
Methylobacterium	*Methylobacterium suomiense*	DSM 14458T	95.1	AY009404	G2M4 (1)
Rhizobiaceae					
Rhizobium	*Rhizobium giardinii*	CIP 105503T	97.2	U86344	G3M12 (1)
	Rhizobium gallicum	MSDJ1109T	97.6	U86343	G1M15 (1)
	Rhizobium radiobacter	ATCC 19358T	97.2–100	AJ389904	G3M2 (1), G1M15 (1)
	Rhizobium sullae	DSM 14623T	97.6	Y10170	G1M15 (1)
Sinorhizobium	*Sinorhizobium morelense*	LC04T	97.0–97.2	AY024335	G1M1 (1), G2M11 (1), G2M12 (1)
Rhodobacteraceae					
Paracoccus	*Paracoccus aminophilus*	ATCC 49673T	97.6	AY014176	G1M3 (2)
	Paracoccus alcaliphilus	ATCC 51199T	97.6–97.8	AY014177	G1M3 (1), G2M7 (1), G3M4 (1), G3M7 (1), G3M12 (1), G4M15 (2)
	Paracoccus aminovorans	ATCC 49632T	97.4–99.7	D32240	G1M5 (2), G1M14 (3), G2M3 (3), G3M4 (1), G3M5 (1), G4M3 (1), G4M12 (5), G4M15 (3)
	Paracoccus carotinifaciens	E-396T	98.4–98.7	AB006899	G3M4 (1), G3M7 (2)
	Paracoccus pantotrophus	ATCC 35512T	100	Y16933	G3M5 (1), G3M14 (2)
	Paracoccus yeei	CCUG 46822T	97.8	AY014173	G3M4 (1), G3M12 (1), G3M13 (1)
	Paracoccus versutus	ATCC 25364T	99.9–100	AY014174	G1M7 (1), G2M4 (1), G2M7 (1), G3M5 (1)
Pannonibacter	*Pannonibacter phragmitetus*	DSM 14782T	100	AJ400704	G3M2 (1)
Betaproteobacteria					
Comamonadaceae					
Acidovorax	*Acidovorax avenae* subsp. *citrulli*	ATCC 29625T	98.1–98.3	AF078761	G2M6 (1), G2M7 (1)
	Acidovorax defluvii	DSM 12644T	99.4–100	Y18616	G2M15 (1), G3M2 (1), G3M10 (2), G3M11 (1), G3M12 (1), G3M13 (2), G4M13 (1), G4M14 (1)
	Acidovorax temperans	ATCC 49665T	97.8–99.5	AF078766	G1M1 (4), G1M8 (1), G2M15 (2)
Alicycliphilus	*Alicycliphilus denitrificans*	DSM 14773T	97.7–99.8	AJ418042	G1M1 (3), G3M1 (1)
Comamonas	*Comamonas aquatica*	ATCC 11330T	99.0–99.8	AJ430344	G3M1 (2)
	Comamonas denitrificans	ATCC 700936T	98.3–99.9	AF233877	G1M15 (1), G2M4 (1), G2M7 (2), G2M9 (1), G2M11 (1), G3M1 (2), G3M3 (2) G3M9 (1), G3M15 (1), G4M4 (1), G4M5 (3), G4M8 (3), G4M10 (17)
Diaphorobacter	*Diaphorobacter nitroreducens*	DSM 15985T	99.2–99.8	AB064317	G1M1 (3), G2M11 (2), G3M1 (1), G3M12 (1), G4M14 (1)
Neisseraceae					
Aquaspirillum	*Aquaspirillum metamorphum*	DSM 1837T	98.9	Y18618	G4M3 (1)
Rhodocyclaceae					
Azospira	*Azospira oryzae*	LMG 9096T	99.9–100	AF011347	G2M11 (1), G4M3 (1)
Azovibrio	*Azovibrio restrictus*	LMG 9099T	100	AF011346	G2M9 (1)
Dechloromonas	*Dechloromonas agitata*	ATCC 700666T	100	AF047462	G4M3 (4), G4M7 (2)
	Dechloromonas denitrificans	DSM 15892T	97.2–99.8	AJ318917	G4M3 (1), G4M6 (1)
Thauera	*Thauera aromatica*	DSM 6984T	99.2–99.3	X77118	TSA (2)
	Thauera aminoaromatica	DSM 14742T	99.5–100	AJ315677	G4M3 (5), G4M7 (2), G4M8 (2)
	Thauera chlorobenzoica	ATCC 700723T	99.4	AF123264	TSA (1)
	Thauera mechernichensis	DSM 12266T	98.0	Y17590	G2M13 (1)
	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)

Continued on following page

TABLE 1—*Continued*

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

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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