Molecular characterization of nocardioform actinomycetes in activated sludge by 16S rRNA analysis

Markus Schuppler,¹ Frank Mertens,² Georg Schön² and Ulf B. Göbel^{1†}

Author for correspondence: Ulf B. Göbel. Tel: +49 30 609 3247. Fax: +49 30 229 2741.

¹ Institut für Medizinische Mikrobiologie und Hygiene, Klinikum der Albert-Ludwigs-Universität Freiburg, Hermann-Herder-Straße 11, D-79104 Freiburg, Germany

² Institut für Biologie II/Mikrobiologie, Albert-Ludwigs-Universität Freiburg, Schänzlestraße 1, D-79104 Freiburg, Germany

The analysis of complex microbiota present in activated sludge is important for the understanding and possible control of severe separation problems in sewage treatment such as sludge bulking or sludge foaming. Previous studies have shown that nocardioform actinomycetes are responsible for these conditions, which not only affect the efficiency of sewage treatment but also represent a threat to public health due to spread of pathogens. However, isolation and identification of these filamentous, nocardioform actinomycetes is hampered by their fastidious nature. Most species are still uncultivable and their taxonomy is unresolved. To study the ecology of these micro-organisms at the molecular level, we have established a clone library of 16S rRNA gene fragments amplified from bulk sludge DNA. A rough indication of the predominant flora in the sludge was given by sequencing randomly chosen clones, which revealed a great diversity of bacteria from different taxa. Colony hybridization with oligonucleotide probe MNP1 detected 27 clones with 16S rDNA inserts from nocardioform actinomycetes and mycobacteria. The sequence data from these clones together with those from randomly chosen clones were used for comparative 16S rRNA analysis and construction of dendrograms. All sequences differed from those of previously sequenced species in the databases. Phenotypic characterization of isolates of nocardioform actinomycetes and mycobacteria cultivated in parallel from the same activated-sludge sample revealed a large discrepancy between the two approaches. Only one 16S rDNA sequence of a cultured isolate was represented in the clone library, indicating that culture conditions could select species which represent only a small fraction of the organisms in the activated sludge.

Keywords: 16S rRNA, activated sludge, nocardioform actinomycetes, uncultured bacteria

INTRODUCTION

The performance of a sewage treatment plant depends upon the microbial community of the activated sludge. Domestic sewage contains a large number of micro-organisms that play a major role in the biodegradation of

organic matter, the removal of inorganic compounds, the inactivation of human or animal pathogens, and the detoxification of pollutants. The composition of activated sludge flocs, consisting of clusters of filamentous and unicellular bacteria, determines the biodegradation efficiency and proper settling in the secondary clarifier. Growth imbalance of filamentous vs floc-forming bacteria leads to problems known as sludge bulking and sludge foaming. The latter condition not only reduces the efficiency of sewage treatment, but also represents a public health problem, due to the spread of pathogens in wind-blown scum (Blackall *et al.*, 1988), and the contamination of the receiving water with opportunistic pathogens, including mycobacteria or nocardioform actinomycetes such as *Rhodococcus equi*, *Nocardia asteroides* or *Gordona*

† Present address: Medizinische Fakultät (Charité), Humboldt-Universität Berlin, Institut für Mikrobiologie und Hygiene, Clara Zetkin Str. 96, D-10117 Berlin.

Abbreviation: EBPR, enhanced biological phosphorus removal.

The EMBL, GenBank and DDBJ accession numbers for the 37 nucleotide sequences from the 16S rDNA clone library reported in this paper are X78629 to X78665.

Table 1. Reference strains and results of identification by gas chromatography

Reference strains	Source*	GC-identification	Probability (%)
<i>Corynebacterium variabilis</i>	DSM 20536	No valid result	—
<i>Gordona aurantiaca</i> †	DSM 43246	No valid result	—
<i>Gordona rubropertinctus</i>	DSM 43197	<i>Nocardia otitidiscaviarum</i> / <i>G. rubropertinctus</i>	62/41
<i>Gordona rubropertinctus</i>	DSM 43248	<i>Gordona rubropertinctus</i>	65
<i>Gordona terrae</i>	DSM 43249	<i>Gordona rubropertinctus</i> / <i>Gordona terrae</i>	66/55
<i>Nocardia amarae</i>	DSM 43392	<i>Nocardia amarae</i>	65
<i>Rhodococcus equi</i>	ITH	Not determined	—
<i>Rhodococcus erythropolis</i>	DSM 43066	<i>Rhodococcus erythropolis</i>	60
<i>Rhodococcus erythropolis</i>	DSM 43135	<i>Rhodococcus erythropolis</i>	41
<i>Rhodococcus fascians</i>	DSM 20669	<i>Rhodococcus fascians</i>	65
<i>Rhodococcus rhodochrous</i>	DSM 43241	<i>Rhodococcus rhodochrous</i>	62
<i>Rhodococcus ruber</i>	DSM 43338	<i>Rhodococcus rhodochrous</i>	75
<i>Rhodococcus</i> sp.	DSM 427	No valid result	—

* DSM, Deutsche Stammsammlung für Mikroorganismen, Braunschweig, Germany; ITH, Institut für Tierhygiene, Freiburg, Germany.

† Renamed *Tsukamurella paurometabola*.

bronchialis (Lasker *et al.*, 1992). Several studies indicated that sludge foaming is often caused by an increase in the amount of nocardioform actinomycetes (Lemmer & Kroppenstedt, 1984; Sezgin *et al.*, 1988; Wanner & Grau, 1989). Since nocardioform actinomycetes and other Gram-positive bacteria with high G+C content occur preferentially in sewage treatment plants with enhanced biological phosphorus removal (EBPR) (Wagner *et al.*, 1994), plants of this type are especially susceptible to developing scum. Increasing eutrophication of natural waters is resulting in tougher wastewater legislation and an increasing number of EBPR plants. Thus, scum in wastewater treatment plants is likely to remain a problem in future. A thorough microbial analysis of activated sludge is therefore a prerequisite for the understanding and possible control of the sewage process and the problems which can arise.

Until recently, the analysis of natural microbial communities such as those in activated sludge relied on the cultivation of the resident micro-organisms as a prerequisite for subsequent identification. This classical approach, however, is hampered by the lack of information on appropriate culture conditions for many of the micro-organisms. Earlier studies indicate that only about 10% of a population is cultivable (Jones, 1977; Hoppe, 1978; Ferguson *et al.*, 1984; Atlas, 1983; Pickup, 1991). Recently, comparative sequence analysis of ribosomal RNA has been widely accepted as a tool for the identification and phylogenetic classification of bacteria (Woese *et al.*, 1985; Olsen *et al.*, 1986; Woese, 1987; Winker & Woese, 1991; Fox *et al.*, 1992). The great advantage of rRNA analysis and rRNA-based hybridization probes in microbial ecology is that the micro-organisms present in a natural community can be detected and characterized without prior cultivation (Göbel *et al.*,

1987; Giovannoni *et al.*, 1988; DeLong *et al.*, 1989; Amann *et al.*, 1990; Hahn *et al.*, 1992; Spring *et al.*, 1992).

Comparative 16S rRNA sequence analysis has been applied to studies of natural microbial communities and has resulted in the discovery of unexpectedly high levels of biodiversity (Pace *et al.*, 1986; Weller & Ward, 1989; Giovannoni *et al.*, 1990; Amann *et al.*, 1991; Schmidt *et al.*, 1991; Fuhrman *et al.*, 1992).

In this study we investigated the microbial diversity of an activated-sludge sample from a sewage treatment plant, with particular regard to nocardioform actinomycetes and mycobacteria, by comparative 16S rRNA sequence analysis. A library of *in vitro*-amplified 16S rRNA genes of activated-sludge bacteria was screened with an oligonucleotide probe in order to detect clones with 16S rDNA signature sequences of nocardioform actinomycetes and mycobacteria, and positive clones were sequenced and analysed. In parallel we isolated actinomycetes from the same activated-sludge sample and compared the partial 16S rRNA sequences of these cultured isolates with those obtained from the clone library.

METHODS

Micro-organisms. An activated-sludge sample was taken from a normally functioning municipal wastewater treatment plant with EBPR at Berlin-Marienfelde, Germany, in October 1992. Isolation of nocardioform actinomycetes was achieved by directly streaking the undiluted sludge onto agar plates with modified Czapek Dox medium (Difco) which is selective for actinomycetes (Lechevalier, 1989). After incubation at 20 °C for one week, colonies were picked at random, transferred to TSBA (trypticase soy broth) agar plates (BBL) and incubated for 2 d at 30 °C. Isolates were identified by gas chromatography (Miller, 1982) on a Hewlett-Packard HP5890 series II using the MIS

Table 2. Comparison of identification of culture isolates by gas chromatography and 16S rRNA sequence analysis

Isolate	GC-identification	Probability (%)	16S rRNA sequence analysis*	Similarity (%)
M2	<i>Nocardia amarae</i>	34	<i>Mycobacterium fallax</i>	88.4
M3	<i>Nocardia amarae</i>	41	<i>Mycobacterium fallax</i>	90.7
M7	<i>Rhodococcus chubuensis</i>	50	<i>Mycobacterium fallax</i>	93.9
M8	<i>Rhodococcus chubuensis</i>	61	<i>Mycobacterium fallax</i>	93.9
M12	<i>Rhodococcus globerulus</i>	76	<i>Mycobacterium chlorophenolicum</i>	91.6
M24	<i>Gordona rubropertinctus</i>	59	<i>Mycobacterium fallax</i>	93.9
M26	No valid result	—	<i>Mycobacterium farcinogenes</i>	85.3
M28	<i>Rhodococcus rhodochrous</i>	54	<i>Mycobacterium fallax</i>	88.6
M34	<i>Gordona rubropertinctus</i>	69	<i>Mycobacterium fallax</i>	93.9
M49	<i>Rhodococcus globerulus</i>	63	<i>Mycobacterium chlorophenolicum</i>	93.1

* Closest relationship to 16S rRNA sequences from EMBL or GenBank databases.

software. Reference strains included in this study are listed in Table 1.

Nucleic acid preparation. Part of the same activated-sludge sample which was used for isolation of the nocardioform actinomycetes was processed for nucleic acid preparation. The sample was centrifuged, washed twice in an excess of PBS (pH 7.0), and finally resuspended in PBS. DNA was recovered by adding 0.5 ml of the sample to approximately 0.5 ml zirconium beads (0.1 mm diameter) and 0.5 ml phenol. Bacteria were then mechanically disrupted by shaking the tube for 5 min in a Mini-Bead Beater (Biospec Products, Bartlesville, USA) at room temperature. After phase separation (10 min at 15000 g), the aqueous phase was extracted twice by adding 1 vol. phenol/chloroform (1:1, v/v) and 1 vol. chloroform/isoamyl alcohol (24:1, v/v). DNA was precipitated by adding 2 vols ice-cold ethanol (96%). The pellet was dried under vacuum for 10 min, dissolved in 50 µl TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) and purified twice on StrataClean resin (Stratagene).

PCR amplification. PCR was used to amplify 16S rRNA gene fragments from purified genomic DNA of the culture isolates as well as from the purified genomic DNA of the activated-sludge sample. The PCR mixture contained 1 × PCR buffer [50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris/HCl (pH 9.0), 10 µg gelatin ml⁻¹], 200 µM dNTPs, 0.3 µM each of primers RE-TPU1 [5'-CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA G-3'; corresponding to positions 8–27 in *E. coli* 16S rRNA (Brosius *et al.*, 1978); the underlined sequence represents restriction sites for *Eco*RI and *Sal*I] and RE-RTU3 [5'-CCC GGG ATC CAA GCT TG(T/A) ATT ACC GCG GC(G/T) GCT G-3'; corresponding to complementary positions 519–536 in *E. coli* 16S rRNA (Brosius *et al.*, 1978); the underlined sequence at the 5'-end represents restriction sites for *Bam*HI, *Hind*III and *Sma*I], 1.5 U *Taq* polymerase (Gibco BRL) and approximately 10–100 ng DNA in a final reaction volume of 100 µl. Amplification was performed in an automated thermal cycler (model 480, Perkin-Elmer-Cetus) by denaturing the samples at 95 °C for 2 min and subsequent amplification by 30 cycles of denaturing (92 °C for 1 min), annealing (50 °C for 1 min) and elongation (72 °C for 1.5 min). Amplification products were purified by agarose gel electrophoresis, eluted by centrifugation using small Mobicol columns (Mobitech), then precipitated with ethanol and resuspended in TE buffer. 16S rRNA gene fragments of culture isolates and type strains were amplified as described above, with the exception of using

primers TPU1 and RTU3 without polylinker tails. Direct solid-phase sequencing PCR was performed using biotinylated primers TPU1 or RTU3.

Cloning. Purified 16S rDNA PCR products and plasmid vector pUC19 (Pharmacia) were cleaved by a double digest with restriction endonucleases *Sal*I and *Bam*HI (Pharmacia) following the manufacturer's specifications. Cleaved products were purified twice on a StrataClean resin. Three ligation reactions using 50, 100 or 200 ng of vector pUC19 were performed. Each reaction contained 6 µl restriction-enzyme-digested PCR product, 1 U T4 DNA ligase (BRL) and 25 mM Tris/HCl buffer (pH 7.8) containing 10 mM MgCl₂, 1 mM DTT and 1 mM ATP, in a final volume of 10 µl. The ligation reactions were incubated at 16 °C overnight. Transformation was done by adding 5 µl ligation mix to 100 µl competent *E. coli* SURE cells (Stratagene) according to the manufacturer's recommendation. Aliquots (100 µl) of the cell suspension were plated onto LB agar medium (Gibco) containing 100 µg ampicillin ml⁻¹, 0.004% (w/v) of X-Gal (Boehringer Mannheim) and 0.5 mM of IPTG (BRL) and incubated overnight at 37 °C.

Colony hybridization. Recombinant clones were plated onto LB agar supplemented with 0.1 mg ampicillin ml⁻¹. The resulting colonies were transferred to positively charged Bio-dyne B nylon membranes (Pall). The bacterial colonies were lysed by standard methods (Sambrook *et al.*, 1989). The oligonucleotide probe MNP1 recognizing the target sequence 5'-AGC CTG GGA AAC TGG GTC TAA-3' [corresponding to positions 152–172 in *E. coli* 16S rRNA (Brosius *et al.*, 1978)] was labelled at the 5'-end with [γ -³²P]ATP (5 kCi mmol⁻¹, 185 TBq mmol⁻¹; Amersham) using T4 polynucleotide kinase (BRL). Hybridizations were carried out in glass tubes. Filters were prehybridized at 52 °C for 1 h using 20 ml hybridization buffer [5 × SSC, 5 × Denhardt's reagent (50 × Denhardt's reagent contains 5 g Ficoll, 5 g polyvinylpyrrolidone, 5 g BSA and H₂O to 500 ml), 0.5% (w/v) SDS] and 2 ml sheared herring sperm DNA (10 mg ml⁻¹). For the subsequent hybridization (1 h at 52 °C) 20 ml hybridization buffer, 200 µl tRNA (25 mg ml⁻¹) and labelled probe MNP1 (about 2.5 × 10⁶ c.p.m.) were added. After hybridization the membranes were washed at low stringency (54 °C for 3 min) and then at a slightly higher stringency (56 °C for 5 min) using washing buffer (5 × SSC, 0.2% SDS).

16S rDNA sequencing. Plasmid DNA was purified using the QIAprep-spin Plasmid Kit (QIAGEN) as indicated by the

Fig. 1. For legend see facing page.

manufacturer. Both insert strands were sequenced by a modified Sanger dideoxy chain-termination protocol using Sequenase (USB) according to the manufacturer's instructions. Biotinylated PCR products were directly sequenced by a solid-phase single-strand sequencing method. Briefly, biotinylated PCR products were incubated with streptavidin-coated paramagnetic beads (Dynabeads, Dynal) and strands were separated by addition of 8 µl 0.5 M NaOH. The tube was placed in a magnetic rack and the supernatant, containing the unbiotinylated strand, was removed. Purified, biotinylated single strands were sequenced using Sequenase, following the manufacturer's recommendations. Primers TPU1 and RTU3 without polylinker tails were used as forward and reverse primers, respectively.

16S rRNA sequence analysis. The 16S rDNA sequences were compared with all currently accessible (June 1994) 16S rRNA sequences in the EMBL and GenBank databases using the program FASTA of the HUSAR 3.0 (Heidelberg Unix Sequence Analysis Resources) program package (DKFZ, Heidelberg, Germany). The clone sequences were aligned with a set of published sequences of several reference bacteria on the basis of conserved primary sequence. The TREECON software package for construction and drawing of evolutionary trees was used for sequence analysis and construction of the dendrograms (Van de Peer & De Wachter, 1993). A 'Single Distance Matrix' was calculated from the first 500 bases of the 16S rRNAs including hypervariable regions, and corrected for superimposed mutations by the method of Jukes & Cantor (1969). The neighbour-joining method of Saitou & Nei (1987) was used for phylogenetic tree construction. The reliability of tree nodes was analysed by bootstrapping. One hundred bootstrap trees were generated, and confidence levels were determined.

RESULTS

Characterization of culture isolates

About 50 culture isolates grown from the activated-sludge sample on Czapek Dox agar were identified by gas chromatography. Isolates showing identical fatty acid profiles were classified in the same group. The 16S rDNA of one representative strain from each of these groups was partially sequenced. GC-identification and 16S rRNA analysis resulted in a different classification of the isolates and showed a high degree of discrepancy between the two approaches (Table 2).

16S rDNA clone library of activated sludge bacteria

Bulk DNA extracted from the activated-sludge sample was used as a mixed template for PCR-amplification of the first 500 bases, representing approximately one-third of the whole 16S rRNA gene. Cloning of the PCR products resulted in a 16S rDNA clone library. The cloning efficiency was 2.14×10^5 c.f.u. per µg vector DNA for the ligation reaction with 100 ng pUC19 DNA. About 3000 recombinant clones were randomly selected and transferred onto LB plates for further investigations.

Colony hybridization with probe MNP1

Partial 16S rDNA sequences (each about 500 bp) obtained by direct sequencing of PCR products from 13 nocardioform strains (Table 1) and 10 culture isolates (Table 2) were aligned. A sequence motif common to all 16S rDNA sequences from these strains and isolates was selected for the construction of oligonucleotide probe MNP1. A search in the EMBL and GenBank databases confirmed that this sequence was found within 16S rRNA genes of most members of the genera *Corynebacterium*, *Gordonia*, *Mycobacterium*, *Nocardia* and *Rhodococcus* and also within a few members of other genera of the high-G + C group of Gram-positive bacteria. The 16S rRNA sequences of *M. celatum*, *M. leprae*, *M. komossense*, *M. shimoidi*, *R. fascians* and *Tsukamurella paurometabola* showed one mismatch with probe MNP1. To identify the clones containing 16S rDNA inserts from nocardioform actinomycetes and mycobacteria, all recombinants of the gene library were screened by colony hybridization with radiolabelled probe MNP1. By using high-stringency conditions 27 out of 3000 clones were identified, all of which contained the full target sequence as determined by sequencing of the plasmid inserts.

Sequence analysis of the clone library

The composition of the 16S rDNA clone library from activated sludge was estimated by sequencing 24 randomly selected recombinant clones. Comparative sequence analysis of the resulting 16S rDNA sequences revealed a range of identities from Gram-positive and Gram-negative bacteria. None of the clone sequences showed identity with any of the 16S rRNA sequences found within EMBL or GenBank databases. The phylogenetic relationship of the bacteria represented in the clone library is represented in Fig. 1, which shows that most of the sequences were related to 16S rRNA sequences from members of the class *Proteobacteria*.

Sequence analysis of the 27 MNP1-positive clones resulted in the identification of 13 different 16S rDNA sequences. Several clones contained identical sequences, whereas others showed slight differences. About 400 bp of both strands at the 5'-end of 16S rRNA genes were sequenced. Comparative sequence analysis with rRNA sequences from the EMBL and GenBank databases revealed that all the sequences could be related to the group of nocardioform actinomycetes and mycobacteria (Fig. 2), with the exception of one sequence (SMKN1), which represented a high-G + C Gram-positive bacterium not belonging to that group. None of the sequences showed 100% identity with any of the sequences retrieved from databases. However, one clone sequence (SMKN35) was identical to that of an isolate (M49) grown in pure culture from the activated-sludge sample. Sequence SMKN41

Fig. 1. Dendrogram indicating the phylogenetic relationships among selected bacteria representing different phylogenetic taxa and randomly chosen clones (SMK). The dendrogram is rooted by outgroup *Methanococcus voltae*. The scale bar represents 10% difference in nucleotide sequence determined by taking the sum of the length of the horizontal line segments connecting two organisms. Numbers above each node are confidence levels (%) generated from 100 bootstrap trees (Van de Peer & De Wachter, 1993).

Fig. 2. Dendrogram indicating the phylogenetic relationships among selected Gram-positive bacteria and clones (SMKN) identified by probe MNP1. One representative from each group of identical clone sequences is shown (numbers in brackets indicate the number of identical sequences). The dendrogram is rooted by outgroup *Bacillus anthracis*. The scale bar represents 10% difference in nucleotide sequence determined by taking the sum of the length of the horizontal line segments connecting two organisms. The numbers above each node are confidence levels (%) generated from 100 bootstrap trees (Van de Peer & De Wachter, 1993).

was chimeric, consisting of two different 16S rDNA fragments, and it was used for further analysis after deleting the part of the 3'-end which did not match in the alignment.

DISCUSSION

Cultivable bacteria represent only a small proportion of natural microbial communities. Therefore, we have chosen the comparative sequence analysis of rRNA to analyse cultivable and uncultivable bacteria from activated sludge. Although this strategy has been successfully applied to characterize other microbial habitats, it should be stressed that this molecular genetic approach is not completely unbiased. Several points have to be considered for correct interpretation of the data. Assuming that all the bacteria present in an activated-sludge sample were uniformly disrupted by mechanical treatment to release DNA, and assuming further that this purified DNA from the different bacteria was amplified with the same efficiency, the resulting 16S rDNA clone library should represent a cross-section of all of the bacteria present in the activated-sludge sample. By restricting the number of clones analysed to about 3000, only those bacteria that occur in high numbers in the activated sludge have a good probability of being represented. A further source of error is the possible loss of amplified 16S rDNA fragments of bacteria that possess 16S rRNA genes containing internal restriction sites which may be digested prior to the cloning reaction. We attempted to circumvent this problem by using restriction enzymes *Bam*HI and *Sal*I, cleavage sites for which are only rarely found within 16S rRNA genes (Weisburg *et al.*, 1991; Liesack & Stackebrandt, 1992). However, because most of the 16S rRNA gene sequences isolated from the activated sludge were novel, possible cleavage of other PCR products cannot be excluded. Furthermore, it has been shown that the use of different cloning protocols can affect the composition of the resulting clone library (E. Stackebrandt, personal communication), producing altered distributions in the library of 16S rDNA sequences derived from different community members.

PCR artifacts such as the formation of hybrid molecules (chimeric sequences) can occur when amplifying genomic DNA from mixed cultures. This is especially true when low- M_r genomic DNA is used for cloning. The use of a bead-beater in genomic DNA preparation increases the probability of chimeric gene artifacts simply because DNA fragments resulting from this type of extraction are very small. The resulting chimeras may lead to an erroneous interpretation by describing sequences of organisms that do not actually exist in the sample investigated (Liesack *et al.*, 1991). Therefore, a critical analysis of the sequences using e.g. the program CHECK-CHIMERA (Olsen *et al.*, 1992), is necessary.

The finding that none of the partial 16S rDNA sequences from the randomly chosen recombinants of the 16S rDNA clone library was identical to any from the EMBL or GenBank databases suggests that only a few of the bacterial species in the activated sludge complex have

been described by classical bacteriological methodologies (Wayne *et al.*, 1987; Olsen, 1990; Ward *et al.*, 1990).

It should be noted that our analysis of the activated-sludge community was not intended to be comprehensive. The comparative sequence analysis of randomly selected recombinants from our 16S rDNA clone library represented only a small sample of the diverse micro-organisms present in the activated-sludge sample, but agreed with reports from other groups using 16S rRNA analysis to investigate complex microbial communities (Ward *et al.*, 1990; Schmidt *et al.*, 1991; Liesack & Stackebrandt, 1992).

It might be argued that complete 16S rRNA sequences would be necessary for a thorough analysis of the phylogeny of the species from which the cloned 16S rRNA gene fragments originated. However, it has been found that comparison of partial sequences is sufficient for an approximate phylogenetic assignment (Lane *et al.*, 1985; Schmidt *et al.*, 1991; Ward *et al.*, 1990; Choi *et al.*, 1994; Hillis *et al.*, 1994).

An indication of the biases of different methods was shown when the results of culture and molecular genetic analysis were compared. With the exception of one sequence (SMKN1), probe MNP1 detected only sequences of nocardioform actinomycetes and mycobacteria in the clone library from the activated-sludge sample, confirming the results of the database search. The finding that only 27/3000 recombinant clones were detected by this probe indicated that these sequences were not representative of the predominant flora in the activated-sludge sample tested. The comparison of the clone sequences with those from the cultured isolates revealed only one identical sequence, namely SMKN35 (cf. isolate M49). This may lead to the assumption that the cultured isolates represented only a small fraction of the organisms present in the activated sludge, obviously selected by the culture conditions.

Inconsistencies in the identification of the cultured isolates by 16S rDNA sequence analysis and gas chromatography probably reflect the bias associated with these methods, which is mainly due to the limited size of the databases used for identification. Better results and greater agreement would be achieved by expanding the databases.

The results reported in this paper from comparative sequence analysis suggest that most of the 16S rDNA sequences derived from nocardioform actinomycetes and mycobacteria in the activated sludge represent unknown and as yet uncultivated organisms. The identification, phylogenetic classification and successful cultivation of these nocardioforms is a prerequisite for the elucidation of their physiological role in activated sludge and determination of their true taxonomic position.

Concluding remarks

The observation that wastewater treatment plants with EBPR are especially susceptible to the development of scum (Wanner & Grau, 1989) correlates with the increased number of nocardioform actinomycetes and other

high-G + C Gram-positive bacteria in sewage treatment plants of this type. This observation and the fact that these micro-organisms contain polyphosphate inclusions indicate that they may play a greater role in biological phosphate removal than anticipated (Wagner *et al.*, 1994).

Fluorescently labelled rRNA probes have been applied successfully for the monitoring of bacterial populations in activated sludge as demonstrated by Wagner *et al.* (1994). The use of a whole set of specific oligonucleotide probes derived from the 16S rRNA sequences reported here would be of great help in the identification and enumeration of the organisms directly in sludge samples. With better knowledge of the taxonomic position of relevant nocardioforms in activated sludge, it should be possible to draw conclusions about their growth and nutritional requirements (Wanner & Grau, 1989). Rapid identification procedures and detailed knowledge of nutrition requirements will be important in controlling growth of these micro-organisms and in preventing the danger of scum formation in activated-sludge plants.

ACKNOWLEDGEMENTS

We would like to thank A. Wittmer for characterizing culture isolates from activated sludge by gas chromatography with the MIS-system, and S. Rensing for support with the application of the TREECON software. The support of Dr Y. Van de Peer for providing the TREECON software package and an alignment of 16S rRNA sequences is gratefully acknowledged. This work was supported in part by a grant from the state of Baden-Württemberg (LGFG).

REFERENCES

- Amann, R., Krumholz, L. & Stahl, D. A. (1990). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic and environmental studies in microbiology. *J Bacteriol* **172**, 762–770.
- Amann, R., Springer, N., Ludwig, W. & Schleifer, K. H. (1991). Identification *in situ* and phylogeny of uncultured bacterial endosymbionts. *Nature* **351**, 161–164.
- Atlas, R. M. (1983). Use of microbial diversity measurements to assess environmental stress. In *Current Perspectives in Microbial Ecology*, pp. 540–545. Edited by M. J. Klug & C. A. Reddy. Washington, DC: American Society for Microbiology.
- Blackall, L. L., Harbers, A. E., Greenfield, P. F. & Hayward, A. C. (1988). Actinomycete scum problems in Australian activated sludge plants. *Water Sci Technol* **20**, 23–29.
- Brosius, J., Palmer, L., Kennedy, J. P. & Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci USA* **75**, 4801–4805.
- Choi, B. K., Paster, B. J., Dewhirst, F. E. & Göbel, U. B. (1994). Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis. *Infect Immun* **62**, 1889–1895.
- DeLong, E. F., Wickham, G. S. & Pace, N. R. (1989). Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. *Science* **243**, 1360–1363.
- Ferguson, R. L., Buckley, E. N. & Palumbo, A. V. (1984). Response of marine bacterioplankton to differential centrifugation and confinement. *Appl Environ Microbiol* **47**, 49–55.
- Fox, G. E., Wisotzkey, J. D. & Jurtshuk, P., Jr. (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* **42**, 166–170.
- Fuhrmann, J. A., McCallum, K. & Davis, A. A. (1992). Novel major archaeobacterial group from marine plankton. *Nature* **356**, 148–149.
- Giovannoni, S. J., DeLong, E. F., Olsen, G. J. & Pace, N. R. (1988). Phylogenetic group-specific oligonucleotide probes for the identification of single microbial cells. *J Bacteriol* **170**, 720–726.
- Giovannoni, S. J., Britschgi, T. B., Moyer, C. L. & Field, K. G. (1990). Genetic diversity in Sargasso Sea bacterioplankton. *Nature* **345**, 60–63.
- Göbel, U. B., Geiser, A. & Stanbridge, E. J. (1987). Oligonucleotide probes complementary to variable regions of ribosomal RNA discriminate between *Mycoplasma* species. *J Gen Microbiol* **133**, 1969–1974.
- Hahn, D., Amann, R. I., Ludwig, W., Akkermans, A. D. L. & Schleifer, K.-H. (1992). Detection of micro-organisms in soil after *in situ* hybridization with rRNA-targeted, fluorescently labelled oligonucleotides. *J Gen Microbiol* **138**, 879–887.
- Hillis, D. M., Huelsenbeck, J. P. & Swofford, D. L. (1994). Hobgoblin of phylogenetics? *Nature* **369**, 363–364.
- Hoppe, H.-G. (1978). Relationships between active bacteria and heterotrophic potential in the sea. *Neth J Sea Res* **12**, 78–98.
- Jones, J. G. (1977). The effect of environmental factors on estimated viable and total populations of planktonic bacteria in lakes and experimental enclosures. *Freshwater Biol* **7**, 61–97.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. & Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. *Proc Natl Acad Sci USA* **82**, 6955–6959.
- Lasker, B. A., Brown, J. M. & McNeil, M. M. (1992). Identification and epidemiological typing of clinical and environmental isolates of the genus *Rhodococcus* with use of a digoxigenin-labeled rDNA gene probe. *Clin Infect Dis* **15**, 223–233.
- Lechevalier, H. A. (1989). Nocardioform actinomycetes. In *Bergey's Manual of Systematic Bacteriology*, vol. 4, pp. 2348–2404. Edited by S. T. Williams, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Lemmer, H. & Kroppenstedt, R. M. (1984). Chemotaxonomy and physiology of some actinomycetes isolated from scumming activated sludge. *Syst Appl Microbiol* **5**, 124–135.
- Liesack, W., Weyland, H. & Stackebrandt, E. (1991). Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. *Microb Ecol* **21**, 191–198.
- Liesack, W. & Stackebrandt, E. (1992). Occurrence of novel groups of the domain *bacteria* as revealed by analysis of genetic material isolated from an Australian terrestrial environment. *J Bacteriol* **174**, 5072–5078.
- Miller, L. T. (1982). A single derivatization method for bacterial fatty acid methyl esters including hydroxy acids. *J Clin Microbiol* **16**, 337–347.
- Olsen, G. J. (1990). Variation among the masses. *Nature* **345**, 20.
- Olsen, G. J., Lane, D. J., Giovannoni, S. J., Pace, N. R. & Stahl, D. A. (1986). Microbial ecology and evolution: a ribosomal RNA approach. *Annu Rev Microbiol* **40**, 337–365.
- Olsen, G. J., Overbeek, R., Larsen, N., Marsh, T. L., McCaughey, M. J., Maciukenas, M. A., Kuan, W.-M., Make, T. J., Xing, Y. & Woese, C. R. (1992). The ribosomal database project. *Nucleic Acids Res* **20**, 2199–2200.

- Pace, N. R., Stahl, D. A., Lane, D. J. & Olsen, G. J. (1986). The analysis of natural microbial populations by ribosomal RNA sequences. *Adv Microb Ecol* **9**, 1–55.
- Pickup, R. W. (1991). Development of molecular methods for the detection of specific bacteria in the environment. *J Gen Microbiol* **137**, 1009–1019.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstruction phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schmidt, T. M., DeLong, E. F. & Pace, N. R. (1991). Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. *J Bacteriol* **173**, 4371–4378.
- Sezgin, M., Lechevalier, M. P. & Karr, P. R. (1988). Isolation and identification of actinomycetes present in activated sludge scum. *Water Sci Technol* **20**, 257–263.
- Spring, S., Amann, R., Ludwig, W., Schleifer, K. H. & Petersen, N. (1992). Phylogenetic diversity and identification of nonculturable magnetotactic bacteria. *Syst Appl Microbiol* **15**, 116–122.
- Van de Peer, Y. & De Wachter, R. (1993). TREECON: a software package for the construction and drawing of evolutionary trees. *Comput Appl Biosci* **9**, 177–182.
- Wagner, M., Erhart, R., Manz, W., Amann, R., Wedi, D. & Schleifer, K. H. (1994). In situ monitoring of the genus *Acinetobacter* in activated sludge. *Appl Environ Microbiol* **60**, 792–800.
- Wanner, J. & Grau, P. (1989). Identification of filamentous microorganisms from activated sludge: a compromise between wishes, needs and possibilities. *Water Res* **23**, 883–891.
- Ward, D. M., Weller, R. & Bateson, M. M. (1990). 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* **345**, 63–65.
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P. & Trüper, H. G. (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.
- Weller, R. & Ward, D. M. (1989). Selective recovery of 16S rRNA sequences from natural microbial communities in the form of cDNA. *Appl Environ Microbiol* **55**, 1818–1822.
- Winker, S. & Woese, C. R. (1991). A definition of the domains *Archaea*, *Bacteria* and *Eucarya* in terms of small subunit ribosomal RNA characteristics. *Syst Appl Microbiol* **14**, 305–310.
- Woese, C. R., Stackebrandt, E., Macke, T. J. & Fox, G. E. (1985). A phylogenetic definition of the major eubacterial taxa. *Syst Appl Microbiol* **6**, 143–151.
- Woese, C. R. (1987). Bacterial evolution. *Microbiol Rev* **51**, 221–271.

Received 14 April 1994; revised 29 August 1994; accepted 21 September 1994.