APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1995, p. 702–707 Vol. 61, No. 2
0099-2240/95/$04.00+0

The Abundance of *Zoogloea ramigera* in Sewage Treatment Plants

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Received 17 October 1994/Accepted 28 November 1994

***Zoogloea ramigera* has long been considered the typical activated sludge bacterium responsible for the formation of activated sludge flocs. On the basis of the results of a comparative sequence analysis, we designed three oligonucleotide probes complementary to characteristic regions of the 16S rRNAs of *Z. ramigera* ATCC 19544^T (T = type strain) and two misclassified strains, *Z. ramigera* ATCC 25935 and ATCC 19623. Dissociation temperatures were determined, and probe specificities, as well as the potential of probes for whole-cell hybridization, were evaluated by using numerous reference organisms. Several activated sludge samples were examined with these probes by using both the in situ and dot blot hybridization methods. Only the type strain probe hybridized to cells that accumulated in the typical branched gelatinous matrices, the so-called *Zoogloea* fingers. This probe revealed cells in most of the activated sludge samples studied. We found that relatively high levels of *Z. ramigera* cells (up to approximately 10% of the total number of cells) and typical morphology tended to be linked to overloading of sewage plants. The probe directed to rejected type strain *Z. ramigera* ATCC 19623 bound to only a few cells. Cells that reacted with the probe complementary to *Z. ramigera* ATCC 25935, which was originally isolated from a trickling filter, were not observed in activated sludge.**

The high levels of sensitivity and specificity of rRNA-targeted nucleic acid probes make them excellent tools for determinative, phylogenetic, and environmental studies in microbiology (3). Hybridization with such probes is an increasingly accepted method for monitoring populations of microorganisms, especially microorganisms that are difficult to cultivate (for reviews see references 1 and 2). It is a special attribute of rRNA-targeted probes that fluorescently labeled versions of them can be readily used for whole-cell and in situ hybridization (3, 7). This use is facilitated by the natural amplification of rRNA molecules in bacterial cells.

Over the last 20 years many sequences of bacterial 16S and 23S rRNAs have been determined; this has been done mainly to reconstruct the phylogeny of microorganisms (19, 39) and has resulted in a clearer understanding of natural relationships, especially relationships between distantly related groups. Often, the results of comparative sequence analyses have also supported suggestions that distantly related organisms were placed together in one species. One such example is the confused taxonomic situation in the species *Zoogloea ramigera*, a species in which the three most representative strains of the species belong to different phylogenetic groups (27, 29). This species was originally described on the basis of its characteristic formation of branched gelatinous matrices and has long been considered the bacterium that is responsible for floc formation in activated sludge (8, 9, 25, 26). Because of the biotechnological importance of *Z. ramigera*, numerous studies have been performed with presumed isolates of this species (9, 11, 30).

Assignment of new isolates to the genus *Zoogloea* often has been based only on the observation that flocs were formed during growth (6, 8, 10, 22). However, today floc formation is recognized as an unreliable phenotypic criterion that can be observed with many microorganisms (25, 34). On the basis of this simple phenotypic criterion, three phylogenetically distantly related strains, ATCC 19544^T (formerly strain 106^T [32]) (T = type strain), ATCC 25935 (formerly strain 115 [10]), and ATCC 19623 (formerly strain I-16-M [6]), were included in the same species. Assignment of these strains to *Z. ramigera* was a direct consequence of insufficient descriptions and the loss or lack of a pure culture of the type strain. The heterogeneous composition of the species did not facilitate the definition of a clear phenotypic profile (33) and complicated the identification of *Zoogloea*-like isolates (4, 12, 31). Probably as a result of the confusing taxonomy and incorrect identification, many strains have been deposited in culture collections as *Zoogloea* sp. However, it has been suggested that only isolates that clearly resemble neotype strain ATCC 19544 phenotypically should be considered genuine members of *Z. ramigera* (38). This suggestion is supported by chemotaxonomic data (13, 15, 18), as well as the results of comparative analyses of 16S rRNA primary structures (27, 29). Neotype strain *Z. ramigera* ATCC 19544 is affiliated with the beta subclass of the *Proteobacteria*, as is strain ATCC 25935. However, a relatively low level of rRNA similarity between these two strains (88%) (27) and the chemotaxonomic characteristics of these organisms indicate that they do not belong to the same genus. The former type strain *Z. ramigera* ATCC 19623 is even less closely related and is affiliated with the "*Rhizobiaceae*" group of the alpha subclass of the *Proteobacteria*.

Previously, *Z. ramigera* was identified in situ microscopically on the basis of the presence of finger-like zoogloeal structures (6, 10, 34). This criterion proved to be insufficient and led to contradictory results; the presence of finger-like gelatinous matrices could not be correlated with the presence of floc-forming isolates. Workers have described isolation of floc formers when no finger-like zoogloeal structures were present (38) and the lack of successful isolation of floc-forming strains when zoogloeal structures were present (4). Most of the problems involving the identification of *Z. ramigera* not only were related to the unsatisfactory taxonomy of the species, but also were due to the cultivation techniques used (26). The biases that occur in cultivation-dependent analyses of the microbial

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TABLE 1. Oligonucleotide probes

Probe	Target strain	Sequence	Sequence positions[a]	Exptl T_d (°C)[b]	Calculated T_d (°C)[c]	Hybridization temp (°C)[d]
ZAL	ATCC 19623	5′-CTTCCATACTCTAGGTAC-3′	647–664	45.7	47.6	50
ZRA	ATCC 19544^T	5′-CTGCCGTACTCTAGTTAT-3′	647–664	49.7	47.6	54
ZBE	ATCC 25935	5′-TGCCAAACTCTAGCCTTG-3′	646–663	50.2	49.6	56

[a] E. coli 16S rRNA numbering (5).
[b] Values determined experimentally for an [Na^+] of approximately 0.4 M.
[c] Values calculated mathematically (17).
[d] Recommended hybridization temperature in dot blot hybridization experiments.

communities in activated sludge have been well documented (36). Even when the cultivation conditions are well established, aggregation of cells in the activated sludge flocs may contribute to false data concerning the number of active cells (8, 35, 37).

It should be feasible to obtain more reliable data by using the cultivation-independent rRNA approach (23). Distinct sequence differences in the 16S rRNA molecules of three *Z. ramigera* strains allowed us to design specific oligonucleotide probes. In this study we used these probes to identify and quantify *Z. ramigera* cells in sewage treatment plant samples.

MATERIALS AND METHODS

Organisms and culture conditions. The following organisms were investigated in this study: *Z. ramigera* ATCC 19544^T, ATCC 25935, and ATCC 19623 and *Zoogloea* sp. strains WS 1830, WS 1831, WS 1846, WS 1847, WS 1848, and WS 1849. For probe specificity tests we used organisms belonging to the following phylogenetic groups: the alpha subclass of the *Proteobacteria* (*Agrobacterium tumefaciens* ATCC 23380^T, *Azospirillum brasilense* DSM 1690^T, *Bradyrhizobium japonicum* DSM 30131^T, *Brevundimonas diminuta* DSM 1635, *Paracoccus denitrificans* DSM 65^T, *Rhizobium leguminosarum* bv. viceae WS 1370, *Rhizobium leguminosarum* bv. trifolii WS 1379, *Rhizobium meliloti* DSM 30135^T, *Rhodobacter capsulatus* DSM 1710^T, *Rhodospirillum rubrum* DSM 107), the beta subclass of the *Proteobacteria* (*Alcaligenes denitrificans* subsp. *xylooxidans* WS 2166^T, *Alcaligenes eutrophus* DSM 531^T, *Alcaligenes faecalis* ATCC 8750^T, *Alcaligenes xylooxidans* subsp. *denitrificans* WS 1268, *Aquaspirillum metamorphum* DSM 1837^T, *Burkholderia cepacia* DSM 50181, *Comamonas testosteroni* DSM 50244^T, *Hydrogenophaga palleronii* DSM 63^T, *Rhodocyclus purpureus* LMG 7759^T), the gamma subclass of the *Proteobacteria* (*Acinetobacter calcoaceticus* LMG 1046^T, *Aeromonas hydrophila* WS 1406, *Erwinia carotovora* WS 1394, *Escherichia coli* DSM 30083^T, *Klebsiella pneumoniae* DSM 30104^T, *Providencia alcalifaciens* DSM 30120, *Pseudomonas aeruginosa* DSM 50071^T, *Pseudomonas putida* DSM 291^T, *Pseudomonas stutzeri* CCUG 11256^T, *Shewanella putrefaciens* LMG 2268^T), and gram-positive bacteria with high or low DNA G+C content (*Bacillus subtilis* ATCC 6633, *Enterococcus faecium* DSM 20477^T, *Enterococcus faecalis* DSM 20478^T, *Lactobacillus amylovorus* DSM 20552, *Lactobacillus casei* LMG 9091, *Lactobacillus fermentum* DSM 20052^T, *Lactococcus lactis* subsp. *cremoris* DSM 20069^T, *Micrococcus agilis* CCM 1744, *Staphylococcus hominis* ATCC 27844^T, *Streptococcus salivarius* DSM 20560^T, *Streptococcus thermophilus* DSM 20479^T, *Staphylococcus xylosus* DSM 20266^T).

Cells were cultured as recommended by the culture collections from which the strains were obtained. These culture collections were the American Type Culture Collection (ATCC), Rockville, Md.; the Culture Collection of the University of Göteborg, (CCUG), Göteborg, Sweden; the Deutsche Sammlung von Mikroorganismen (DSM), Braunschweig, Germany; the Laboratory for Microbiology Culture Collection of the University of Ghent (LMG), Ghent, Belgium; and the Weihenstephan Culture Collection (WS), Süddeutsche Versuchs- und Forschungsanstalts für Milchwirtschaft, Freising-Weihenstephan, Germany.

Cell fixation. Pure cultures were fixed as previously described (3). Activated sludge was fixed at the time of sampling by adding 4% paraformaldehyde to a final concentration of 3% and was kept at 4°C overnight. The cells were then washed in phosphate-buffered saline (PBS) and finally resuspended in PBS–96% ethanol (1:1, vol/vol).

Oligonucleotides. The sequences of the probes which we designed are shown in Table 1. The oligonucleotides were synthesized with a C6-TFA aminolinker [6-(trifluoracetylamino)-hexyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)phosporamidite] (MWG Biotech, Ebersberg, Germany) at the 5′ end. Labeling with tetramethylrhodamine-5-isothiocyanate (TRITC) (Molecular Probes, Eugene, Oreg.) and 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS) (Boehringer Mannheim, Mannheim, Germany) and purification of the oligonucleotide-dye conjugates were performed as described by Amann et al. (3). For dot blot hybridization experiments the oligonucleotides were labeled at their 3′ ends by using terminal transferase (Boehringer Mannheim), and [alpha-^{32}P]dCTP (Amersham, Braunschweig, Germany). Bacterial probe EUB (3) was used as a positive control in the dot blot hybridization analysis.

Dot blot hybridization. Nucleic acid extraction and immobilization on nylon membranes were carried out as described previously (14). A 1-μg portion of each nucleic acid extract obtained from a pure culture was deposited on a dot, and 3 μg of bulk nucleic acid extract obtained from each activated sludge sample was deposited on a dot. The membranes were prehybridized in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0), 7% sodium dodecyl sulfate (SDS), 10% Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), and 20 mM phosphate buffer (pH 7.0) and incubated for 5 h at the hybridization temperature (Table 1). Hybridization was performed in prehybridization solution containing 5 pmol of the labeled probe for 4 to 16 h. The membranes were washed twice with 2× SSC–0.1% SDS at the hybridization temperature for 10 min. After autoradiography, the hybrids were denatured in 0.1× SSC–0.1% SDS at 80°C for 10 min, and the bound nucleic acids could be used for additional hybridization experiments.

Determination of T_d. Experiments to determine dissociation temperatures (T_d) were performed in duplicate and always included a negative control. In all cases 1 μg of nucleic acid extract was bound to the membrane and hybridized as described above. The hybridization temperature was 35°C. After 16 h of incubation the filters were washed twice at the hybridization temperature with 2× SSC–0.1% SDS. The filters were then cut apart, and each dot was washed for 10 min in 1 ml of the same buffer; the temperature of the buffer was increased from 40 to 70°C in 2°C increments. The amount of radioactivity present in each supernatant (equivalent to the amount of probe released from the filter) was determined by scintillation counting. From these data we determined the T_d (the temperature at which one-half of the hybridized probe remained on the filter under the conditions described above).

Whole-cell hybridization. Whole-cell hybridization experiments and visualization by both conventional epifluorescence microscopy and confocal laser microscopy were performed as previously described (35).

RESULTS

Design and optimization of oligonucleotide-specific probes. On the basis of the 16S rRNA sequences of three phylogenetically distinct strains of *Z. ramigera* (ATCC 19544^T, ATCC 19623, and ATCC 25935) (27), we designed specific oligonucleotide probes. Probe ZRA is complementary to part of helix 23a of the 16S rRNA of *Z. ramigera* ATCC 19544^T. Probes ZBE (*Z. ramigera* beta subclass proteobacterium probe) and ZAL (*Z. ramigera* alpha subclass proteobacterium probe) target homologous regions of the 16S rRNAs of strains ATCC 25935 and ATCC 19623, respectively. Table 1 shows the probe sequences together with the corresponding target positions (*E. coli* 16S rRNA numbering) (5). A database comparison with 2,000 complete or almost complete accessible 16S rRNA sequences (16, 21) did not reveal any identical complementary sequences other than the sequences of the target organisms. All other bacterial sequences had at least one mismatch with the probe sequences. The difference alignment in Table 2 shows the locations of the mismatches in the noncomplementary *Z. ramigera* strains and other phylogenetically closely related target organisms.

Figure 1 shows denaturation curves for the three probes (ZAL, ZRA, ZBE) obtained with their respective target 16S rRNA molecules. For each probe the total activity recovered in each negative control profile was less than 5% of the total activity recovered when the homologous sequence was used.

TABLE 2. Difference alignment of 16S rRNA target regions

Organism	Target
Z. ramigera ATCC 19544^T	5′-GCAUAACUAGAGUACGGCAGA-3′
Z. ramigera ATCC 25935	5′-···AGG·····UU·····-3′
Z. ramigera ATCC 19623	5′-··GG··C·····U··A···-3′
Rhodocyclus purpureus	5′-···C·G·····-3′
Neisseria gonorrhoeae	5′-··GG·G···C···GU·U····-3′
Alcaligenes eutrophus	5′-···AGG·····U·U····-3′
Burkholderia cepacia	5′-·GCAGG·····U·····-3′
Comamonas testosteroni	5′-···AGG·····G·····-3′
Agrobacterium tumefaciens	5′-··GG··U··U··C··U··A···-3′
Rhizobium meliloti	5′-··GCA·U·····C·A·A···-3′
Rochalimaea quintana	5′-··G··GU··C···GU··A···-3′
Escherichia coli	5′-··GCA·G··U···CUC·U···-3′

For each probe we determined the T_d, which was defined as the temperature at which one-half of the hybridized probe remained bound to the target 16S rRNA. The experimentally determined T_d values were similar to estimated values calculated with the formula of Lathe (17) (Table 1). Dot blot hybridization experiments were performed at temperatures slightly above the T_d values. Using these conditions, we evaluated the specificities of the probes by performing dot blot hybridization experiments with nucleic acids isolated from 50 reference strains (see Materials and Methods). We observed practically no nonspecific hybridization of probes ZRA, ZAL, and ZBE even with closely related nontarget sequences.

The specificities and sensitivities of the fluorescent derivatives of the three probes were evaluated by gradually increasing the formamide concentration at a fixed hybridization temperature (46°C) and a fixed ionic strength (0.9 M NaCl), as previously described (20). For all three probes a formamide concentration of 35% yielded a good compromise between sensitivity and specificity. In mixtures containing fixed reference cells only the respective *Z. ramigera* strains were stained.

Rapid screening for "*Zoogloea*" strains. We used probes ZAL, ZRA, and ZBE to study the affiliation of six strains identified as *Zoogloea* sp. that were deposited in the Weihenstephan Culture Collection. The activated sludge isolates (28) were investigated by performing whole-cell and dot blot hybridization experiments. None of the three specific probes exhibited homology with any of the isolates. Hybridization with the probes specific for the alpha, beta, and gamma subclasses of the *Proteobacteria* (20) revealed that all six strains are members of the alpha subclass. Clearly, none of the six strains is affiliated with *Z. ramigera* sensu stricto or with misclassified strains *Z. ramigera* ATCC 19623 and ATCC 25935.

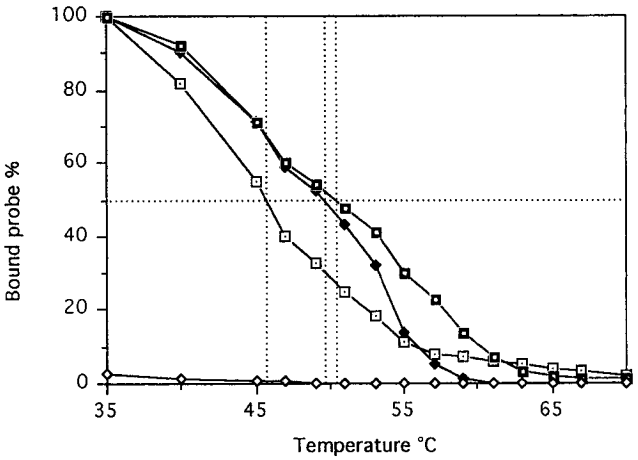

FIG. 1. Thermal denaturation profiles obtained with hybridized oligonucleotide probes and isolated rRNAs. Symbols: ⊡, probe ZAL and ATCC 19623; ◆, probe ZRA and ATCC 19544^T; □, probe ZBE and ATCC 25935; ◇, probe ZRA and ATCC 25935. The dotted lines indicate the T_d values of the three probes.

Monitoring of *Z. ramigera* in sewage treatment plants. We investigated samples obtained from 11 different sewage treatment systems in Germany and Spain. These systems differ with respect to size and treatment process. The samples were obtained from three high-capacity activated sludge plants, including the Grosslappen plant (Munich, Germany), the Dietersheim plant (Munich, Germany), and the El Pinedo plant (Valencia, Spain); three medium-capacity activated sludge plants, including the Palma 2 plant (Mallorca, Spain), the Freising plant (Freising, Germany), and the Grüneck plant (Grüneck, Germany); and four low-capacity activated sludge plants, including the Moosburg plant (Moosburg, Germany), the Zolling plant (Zolling, Germany), the Haag plant (Haag, Germany), and the Esporles plant (Mallorca, Spain). In addition, we obtained samples from the three first ponds at the Alaró lagooning sewage treatment plant (Mallorca, Spain). Representative results of the whole-cell hybridization and dot blot hybridization experiments are shown in Table 3 and Fig. 2, respectively.

Most of the samples obtained from the high- and medium-capacity sewage plants hybridized with probe ZRA (the *Z. ramigera* sensu stricto [*Z. ramigera* ATCC 19544^T] probe). In the sample obtained from aeration basin 1 of the Grosslappen plant, the number of cells that hybridized with probe ZRA was estimated to be approximately 10% of the total number of cells (Table 3). The dot blot hybridization results were in good agreement with the in situ observations (Fig. 2). In most cases the intensities of the dot blot signals agreed well with the level of cells determined by in situ hybridization. The exceptions were the samples obtained from the Freising plant and Palma 2 (Fig. 2, dots E1 and E2, respectively); these samples produced similar dot blot intensities, yet the frequencies of probe-positive cells were significantly different (Table 3). This again showed that dot blot values cannot be directly extrapolated to relative cell numbers (36). The in situ hybridization-positive cells in the Freising plant sample were brighter than the cells in the Palma 2 sample, indicating that the cellular rRNA content was higher and therefore that the metabolic activity was higher (7).

The secondary aeration basins at the Grosslappen and Dietersheim plants were characterized by lower frequencies and lower probe-conferred levels of fluorescence of ZRA-positive cells than the primary basins (Table 3). These results are consistent with the decreases in the amounts of available organic material in the basins. In the dot blot assay the level of hybridization of probe ZRA was below the threshold of detection (Fig. 2). The typical finger-like zoogloeal structures were observed only in the samples obtained from the Dietersheim and El Pinedo plants (Fig. 3). In all other samples the cells which we detected formed less characteristic aggregates. We did not detect *Z. ramigera* sensu stricto in the three low-capacity activated sludge systems tested (the Zolling, Haag, and Esporles plants) or in the three lagoons of the stabilization pond sewage system at the Alaró plant (Table 3 and Fig. 2).

With in situ probe ZAL we detected low numbers of cells in five activated sludge samples (samples obtained from both basins at the Grosslappen plant, both basins at the Dietersheim plant, and the Moosburg plant). These cells did not occur as aggregates in any of the samples. It has been reported previously that the target strain of this probe, *Z. ramigera* ATCC

TABLE 3. Results of hybridization experiments

Sample	Dot blot position[a]	Population equivalent (no. of people)	Results obtained with[b]:		
			Probe ZAL	Probe ZRA	Probe ZBE
Zolling	A1	5×10^3	−	−	−
Grosslappen basin 1	B1	2×10^6	+	+++	−
Moosburg	C1	3.4×10^4	+	+	−
Grüneck	D1	10^5	−	+	−
Freising	E1	1.1×10^5	−	+	−
Dietersheim basin 1	F1	10^6	+	++	−
Dietersheim basin 2	G1	10^6	+	+	−
Grosslappen basin 2	H1	2×10^6	+	+	−
Haag	A2	3×10^3	−	−	−
Alaró pond 1	B2	3×10^3	−	−	−
Alaró pond 2	C2	3×10^3	−	−	−
Alaró pond 3	D2	3×10^3	−	−	−
Palma 2	E2	3×10^5	−	++	−
Esporles	F2	3×10^3	−	−	−
El Pinedo	G2	7.3×10^5	−	++	−

[a] Position on the dot blot shown in Fig. 2.
[b] −, not detected; +, detected at low level (<1% of the estimated total number of cells); ++, between 1 and 10% of the estimated total number of cells; +++, >10% of the estimated total number of cells.

19623, does not exhibit flocculent growth (32). Dot blot hybridization of extracted nucleic acids with probe ZAL did not result in signals greater than the background level; this finding corroborated the low levels (probably less than 0.1%) of *Z. ramigera* ATCC 19623 in the sewage treatment systems examined.

The use of the specific probe ZBE, which was targeted to strain ATCC 25935, did not result in any positive signal in either whole-cell hybridization or dot blot hybridization experiments (Table 3 and Fig. 2).

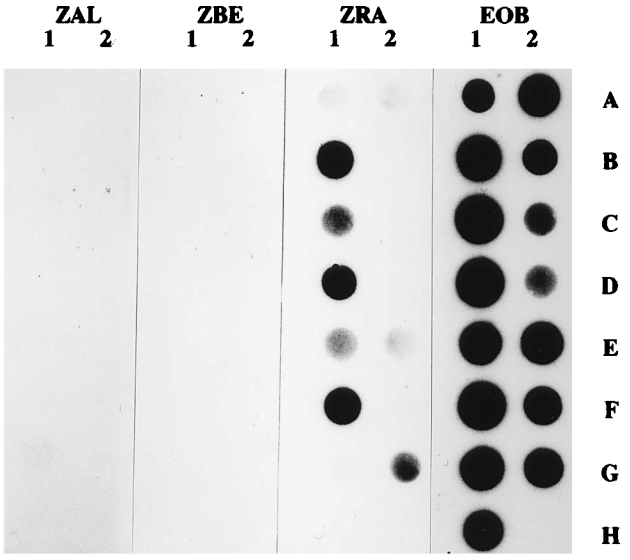

FIG. 2. Dot blot hybridization results obtained with the activated sludge samples. Dot A1, Zolling; dot B1, Grosslappen basin 1; dot C1, Moosburg; dot D1, Grüneck; dot E1, Freising; dot F1, Dietersheim basin 1; dot G1, Dietersheim basin 2; dot H1, Grosslappen basin 2; dot A2, Haag; dot B2, Alaró pond 1; dot C2, Alaró pond 2; dot D2, Alaró pond 3; dot E2, Palma 2; dot F2, Esporles; dot G2, El Pinedo; dot H2, negative control. The results were obtained by using the following specific oligonucleotide probes; ZAL, targeted to ATCC 19623; ZBE, targeted to ATCC 25935; ZRA, targeted to ATCC 19544^T; and eubacterial university probe (EOB).

DISCUSSION

The optimization of hybridization parameters is very important for the specificity and sensitivity of nucleic acid probes. Under carefully controlled conditions highly specific signals can be obtained. This is extremely important when researchers are working with complex environments, like activated sludge, where closely related nontarget species could be present. Furthermore, carefully choosing the right hybridization method and considering method-dependent consequences are crucial. Of all of the available methods, in situ hybridization has the potential to yield the most detailed results, including specific cell counts, spatial distribution of target cells, and information on the metabolic status of individual cells (for a review see reference 2). However, this technique is also prone to specific problems related to low abundance or accessibility of the intracellular rRNA molecules. In the case of *Z. ramigera*, the gelatinous matrices might form boundaries that hinder the penetration of probe molecules to the target cells and thereby result in false-negative results. Gelatinous matrices should, however, not influence the results of dot blot hybridization experiments, in which bulk nucleic acids are isolated prior to hybridization by rigid chemical and physical treatments. Therefore, in this study all samples were analyzed by dot blot and in situ hybridization methods.

The results obtained when we used probe ZRA with samples obtained from 11 sewage treatment systems indicated that cells that are closely related to the type strain, *Z. ramigera* ATCC 19544^T, are present in activated sludge in significant numbers. This strain was isolated initially by microdissecting zoogloeal fingers because of its relatively slow growth. Previous attempts in which standard cultivation techniques were used had failed. In situ, probe ZRA sometimes hybridized to the typical finger-like zoogloeae. This is important independent proof that the probe is specific. Using confocal laser scanning microscopy, we observed that the cells in the deeper layers of the matrix were also detected by the probe (data not shown). The observed hybridization of probe ZRA to smaller cell clusters should not be taken as an indication that nonspecific hybridization occurred, since Williams and Unz (38) found previously that the absence of finger-like zoogloeae does not mean that *Z. ramigera* is not present.

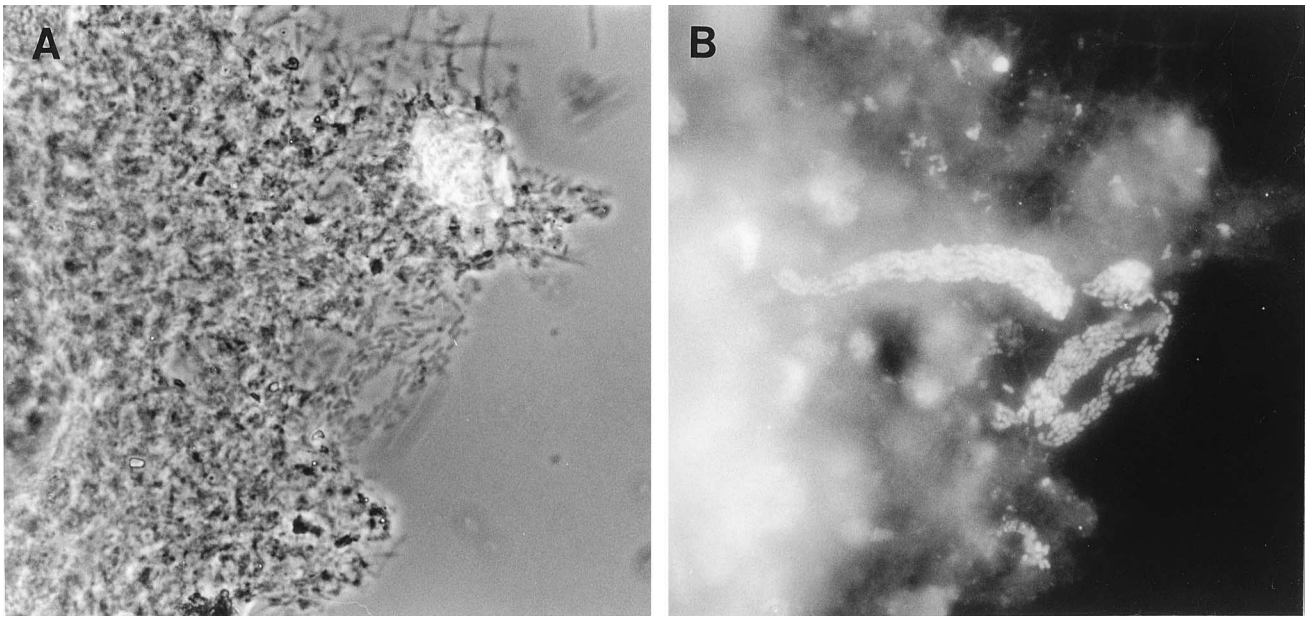

FIG. 3. In situ hybridization of an activated sludge sample obtained from the El Pinedo plant with tetramethylrhodamine-labeled probe ZRA. A phase-contrast micrograph (A) and an epifluorescence micrograph (B) of the same microscopic field are shown.

It has been suggested that *Z. ramigera* is a possible indicator organism for organic polluted environments and wastewater (38). In this study we could not detect *Z. ramigera* sensu stricto in three small activated sludge plants and in a lagooning system. Therefore, this organism seems not to be universally present in polluted waters. However, our results do not rule out the possibility that other members of the genus *Zoogloea* were present. The absence of *Z. ramigera* in the lagooning sewage treatment system at the Alaró plant is consistent with previous results obtained with cultivation techniques (24).

The low levels or lack of cells that hybridized with probes ZAL (*Z. ramigera* ATCC 19623) and ZBE (*Z. ramigera* ATCC 25935) in both the in situ and dot blot assays showed that these bacteria are not abundant in the sewage treatment plants which we examined. It remains to be seen if these target organisms occur at higher frequencies in other activated sludge systems. It could be that isolation of these organisms resulted from positive selection during enrichment and cultivation and not from high natural levels. However, in the last few years the microbial communities in activated sludge could have changed significantly because of changes in the composition and loading of sewage. Right now, it appears that *Z. ramigera* sensu stricto strain ATCC 19544^T is the only strain of the three *Z. ramigera* strains which we investigated that is abundant in modern activated sludge treatment systems.

Probe ZRA will be a useful tool for identification and in situ monitoring of genuine zoogloeae. This probe is a new addition to the set of probes that has been developed for wastewater bacteria (35, 37). To understand the real significance of *Z. ramigera* sensu stricto, more studies of activated sludge under different plant operating conditions will be necessary. The use of the specific oligonucleotides should help clarify the population dynamics in sewage treatment systems and should help identify genuine members of *Z. ramigera* and thus avoid further misunderstandings.

ACKNOWLEDGMENTS

We thank Hilde Lemmer and Rosa Aznar for providing activated sludge samples as well as useful discussions and Wolfgang Ludwig and Oliver Strunk for access to the TUM rRNA database, the arb editor and its probe functions.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (grant Am 73/2-2) and the Freistaat Bayern (FORBIO-SICH) to R.A. The postdoctoral fellowship granted to R.R.-M. by the Ministerio de Educación y Ciencia (Spain) is gratefully acknowledged.

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