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Whole-Cell Hybridization of *Methanosarcina* Cells with Two New Oligonucleotide Probes

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Two new oligonucleotide probes targeting the 16S rRNA of the methanogenic genus *Methanosarcina* were developed. The probes have the following sequences (*Escherichia coli* numbering): probe SARCI551, 5′-GAC CCAATAATCACGATCAC-3′, and probe SARCI645, 5′-TCCCGGTTCCAAGTCTGGC-3′. In situ hybridization with the fluorescently labelled probes required several modifications of standard procedures. Cells of *Methanosarcina mazeii* S-6 were found to lyse during the hybridization step if fixed in 3% formaldehyde and stored in 50% ethanol. Lysis was, however, not observed with cells fixed and stored in 1.6% formaldehyde–0.85% NaCl. Extensive autofluorescence of the cells was found upon hybridization in the presence of 5 mM EDTA, but successful hybridization could be obtained without addition of this compound. The mounting agent Citifluor AF1, often used in conjugation with the fluorochrome fluorescein, was found to wash the labelled probes out of the cells. Stable labelling could be obtained with rhodamine-labelled probes when the specimen was mounted in immersion oil, and high hybridization intensities of the *Methanosarcina* cells were found even in the presence of biomass from an anaerobic reactor. The inherent high autofluorescence of the biomass could be lowered by use of a highly specific narrow-band filter. The probes were found to be specific for *Methanosarcina* and useful for detection of this genus in samples from anaerobic reactors.

In 1988, Giovannoni et al. presented the use of oligonucleotide probes for characterization of microbial cells in environmental samples by in situ hybridization (14). This approach relies on the rapidly increasing records of biopolymer sequences of microorganisms that not only have provided new insight into the relatedness of all organisms (50) but also constitute a valuable tool for ecological studies on microbial environments (5, 8, 31, 32).

A substantial number of papers have presented different uses of in situ hybridization as well as improvements of the methods. An important milestone has been the introduction of fluorochrome-labelled oligonucleotide probes that allow for the simultaneous examination of cell morphology and phylogenetic affiliation (7, 8, 21, 46) as well as cell activity (12, 30).

Raskin and coworkers (33–36) introduced the oligonucleotide approach to the area of methanogens. These authors described the development of an array of probes encompassing almost all known taxa of methanogens as well as the feasibility of these probes for characterization of reactor samples. This work mostly employed hybridization to samples of purified RNA, slot blotted to membranes. The sum of the methanogen 16S rRNA quantified by probes specific for the known taxa of methanogens closely equalled the total amount of *Archaea* 16S rRNA in the samples (33). This showed that the known methanogenic taxa most likely comprise all methanogens present in the anaerobic reactors under study. No members of the domain *Archaea* other than methanogens are likely to exist in anaerobic digesters.

Methanogenesis from acetate accounts for approximately 70% of the methane produced from complex substrates in biogas reactors (11, 23). This reaction is thus a key process for the use of anaerobic digestion for removal of organic matter as well as energy recovery from waste. Cells belonging to the genus *Methanosarcina*, known to utilize acetate for methane production, have been shown to be common in anaerobic reactors (1, 2, 22, 40). Ribosomal probes for detection of this genus are, therefore, a very attractive tool for microbial characterization of methanogenic processes.

To extend the collection of oligonucleotide probes for in situ characterization of methanogens in anaerobic reactors, we designed and tested two new probes for the genus *Methanosarcina*. In situ hybridization of cells of this genus was, furthermore, found to succumb to a number of methodical problems. In this paper, we describe the problems and the solutions that were developed.

MATERIALS AND METHODS

Microbial strains. The microorganisms used in this study together with their culturing conditions are presented in Table 1. All strains were from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) unless otherwise stated. The cultures were harvested during the exponential growth phase to ensure maximum ribosome content (12, 43). *Methanosarcina thermophila* TM-1 and *Methanosarcina mazeii* S-6 were grown with high substrate concentrations together with elevated concentrations of calcium and magnesium (Table 1) to ensure that they grew as single cells rather than as packets (3, 51).

The biomass sample used was homogenized granules originating from a mesophilic lab-scale upflow anaerobic sludge blanket reactor fed with glucose.

Oligonucleotide probes. Two new probes complementary to the small subunit of the rRNA of the genus *Methanosarcina* were designed after visual inspection of a sequence alignment of 42 strains of the domains *Archaea* and *Bacteria*, including 28 strains of methanogens. The nucleotide sequences of the probes are (*Escherichia coli* numbering) for SARCI551, 5′-GACCCAATAATCACGATC AC-3′, and for SARCI645, 5′-TCCCGGTTCCAAGTCTGGC-3′. By use of the standardized oligonucleotide nomenclature defined by Alm et al. (4), the probe names are S-G-Sarci-0551-a-A-20 and S-G-Sarci-0645-a-A-19, respectively. For convenience, the short forms of the names are used in this paper. Probes for radioactive labelling were obtained from the Laboratory of Biotechnology, Uni-

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TABLE 1. Microbial strains used for this work

Strain	Strain used for:		Culturing conditions	DSM no.[a]
	Whole-cell hybridizations	Slot blot hybridizations		
Escherichia coli B/6	X	X	LB medium; 37°C	—[b]
Pseudomonas putida		X	LB medium; 37°C	291
Desulfobulbus propionicus Lindhorst	X		Modified UASB medium[c]; 37°C	2032
Desulfovibrio desulfuricans subsp. *desulfuricans* Essex 6		X	DSM medium 63[a]; 37°C	642
Archaeoglobus fulgidus 7324[d]		X	Marine medium with lactate[e]; 37°C	8774
"*Methanobacterium*" sp. strain Marburg[f]	X		BA medium[g]; H_2-CO_2; 60°C	2133
"*Methanobacterium*" sp. strain CB12[f]	X		BA medium; H_2-CO_2;60°C	3664
Methanococcus vannielii SB[h]	X	X	McC[i]; H_2-CO_2; 37°C	1224
Methanococcus voltaei PS[h]	X	X	McC; H_2-CO_2; 37°C	1537
Methanosarcina barkeri 227		X	Modified BA medium[j]; 37°C	1538
Methanosarcina mazeii S-6	X	X	Modified BA medium[k]; 37°C	2053
Methanosarcina thermophila TM-1	X	X	Modified BA medium[l]; 45°C	1825
Methanococcoides methylutens TMA-10[h]	X	X	MSH[m]; trimethylamine; 30°C	2657
Methanosaeta concilii GP6	X	X	DSM medium 334[a]; acetate; 37°C	3671
Methanosaeta concilii Opfikon		X	DSM medium 334[a]; acetate; 37°C	2139

[a] See DSM catalog of strains (13).
[b] —, mutant of strain B, DSM catalog no. 499. The mutant strain is no. 1.08.0002 in the Culture Collection of the Department of Microbiology, University of Bergen, Bergen, Norway.
[c] The UASB medium described in reference 42 with bicarbonate and carbon dioxide omitted.
[d] Strain kindly provided by Roald K. Nilsen, Department of Microbiology, University of Bergen.
[e] The marine medium described in reference 10.
[f] Exact taxonomic position of this strain remains to be assessed (44).
[g] BA medium, the BA medium described previously (9), as modified in reference 18, amended with 0.5 g cysteine-HCl per liter.
[h] Strain kindly provided by Bente-Lise P. Lillebø, Department of Microbiology, University of Bergen.
[i] McC, the McC medium described previously (49).
[j] This modified BA medium contained 0.5 g of cysteine-HCl per liter, 11 mM $MgCl_2$, 10.3 mM $CaCl_2$, 2 g of yeast extract per liter, 1 g of tryptone per liter, and 4 or 6 ml of methanol per liter. One culture used was grown on BA medium with 0.5 g of cysteine-HCl per liter, 50 mM trimethylamine, 1 g of yeast extract per liter, 5.5 mM $MgCl_2$, and 3.0 mM $CaCl_2$.
[k] This modified BA medium contained 0.5 g of cysteine-HCl per liter, 50 mM trimethylamine, 1 g of yeast extract per liter, 5.5 mM $MgCl_2$, and 3.0 mM $CaCl_2$.
[l] This modified BA medium contained 0.5 g of cysteine-HCl per liter, 100 mM trimethylamine, 1 g of yeast extract per liter, 150 mM $MgCl_2$, and 5 mM $CaCl_2$.
[m] MSH is MSHCO2 medium (28) with the following modifications. The bicarbonate buffer system was established by the addition of 0.67 g of NaOH per liter and 2 g of $NaHCO_3$ per liter and gassing with a H_2-CO_2 (8:2) mixture. The pH was adjusted to 7.2 to 7.3. The organic additions were 2 g of Casamino Acids per liter, 2 g of casein hydrolysate peptone per liter, 0.1 g of 2-mercaptoethanesulfonic acid per liter, 0.1 g of cysteine-HCl per liter, and 0.5 ml of the vitamin solution described previously (48) per liter. The trace elements were 1 ml of the trace element solution per liter and 0.5 ml of the iron stock solution described previously (48) per liter.

versity of Bergen, Bergen, Norway. Fluorochrome-labelled probes were purchased from Hobolth DNA Syntese (Hillerød, Denmark). These were labelled with lissamine rhodamine (Molecular Probes) via an amino linker and purified by high-performance liquid chromatography. In addition, the probe for the archaeal domain, S-D-Arch0344a-A-20 (34, 36), labelled with a fluorescein amidite (Hobolth DNA Syntese), and the probe for the bacterial domain, S-D-Bact0338a-A-18 (6, 36), labelled with rhodamine (kindly provided by R. I. Amann, Technische Universität München), were used as positive controls. The NON probe, complementary to the S-D-Bact0338a-A-18 probe (47) and labelled with lissamine rhodamine (Hobolth DNA syntese), was used as a negative control.

Whole-cell hybridizations. The cells harvested for whole-cell hybridization were fixed with 3% (wt/vol) paraformaldehyde and resuspended in Tris buffer and ethanol as described by Raskin et al. (35). The cell suspensions were stored at 4 or −20°C. *Methanosarcina mazeii* S-6 was fixed by washing with saline-formaldehyde (1.6% formaldehyde in 0.85% NaCl), suspended in the same solution, and stored at 4°C.

The hybridization was performed on six-well Teflon-coated slides purchased from Struers KEBO lab, Albertslund, Denmark (catalog no. 113.700-309). The hybridization followed the procedure described by Poulsen et al. (31) with the following modifications. The slides were treated with poly-L-lysine for adhesion of the specimen as described in the instructions of the manufacturer (Sigma Diagnostics catalog no. P8920). For cells of *Methanosarcina mazeii* S-6, which adhered strongly to glass slides, the lysine coating was omitted. The hybridization buffers contained different concentrations of formamide (see below) and were adjusted to pH 7.2. Approximately 20 ng of probe was applied to each well. The humid chambers were glass petri dishes sealed with Parafilm M. The first washing solution (31) had the same concentration of formamide as the hybridization mixture, and the first wash lasted for 20 min. Between the first and second washes, the slides were rinsed with distilled water.

After final air drying, the slides were mounted in immersion oil or, in the cases with the fluorescein-labelled probe (S-D-Arch0344a-A-20), with Citifluor AF1 (Citifluor Products, Chemical Laboratory, The University, Canterbury, Kent, United Kingdom).

The whole-cell hybridizations were recorded with a charged-coupled device (CCD) camera, the AT200 CCD camera system (Photometrics Ltd., Tucson, Ariz.), connected to a Zeiss Axioplan microscope equipped with an HBO 100W/2 burner for epifluorescence illumination and a Plan Apochromat 100×/1.4 oil objective. The exposure time was 1 s. The camera was controlled with the PMIS Image Processing Software version 3.0 (Photometrics), and the acquired images were processed with the Image-Pro Plus software version 1.3 (Media Cybernetics, Silver Spring, Md.). The microscope was focused on the autofluorescence of the cells illuminated with UV light (390 to 420 nm; Zeiss filter set 18) where cells showed some autofluorescence. Hybridizations with rhodamine-labelled probes were visualized with the Zeiss filter set 15 (green excitation light around 546 nm), while the Zeiss filter set 09 (blue excitation around 450 to 490 nm) was used to visualize fluorescein-labelled probes.

The whole-cell hybridizations with the NON probe and with biomass samples were evaluated with the epifluorescence microscope setup described by Poulsen et al. (31). For work on biomass samples, a narrow-band bypass filter of 590 ± 10 nm was used in concert with filter set 15 for reduction of the inherent autofluorescence (31). With the narrow-band filter, the exposure time was 5 s. The images were processed for printing by use of the Photoshop software version 3.0 (Adobe Systems Inc., Mountain View, Calif.).

The whole-cell hybridizations comparing cells of *Methanosarcina mazeii* S-6 fixed with 3% paraformaldehyde and formaline-saline were evaluated with a Leica DMIRBE epifluorescence microscope equipped with a Kappa ZODXC air CCD camera, a Leica 100-W mercury burner, filter package N2.1 513610, and a Leica PL FLOUTAR 100×/1.30 oil objective. The exposure time was 1 s. The acquired images were processed with Microvision software version 1.1:82b (Department of Chemistry, Danish Technological Institute, Tåstrup, Denmark).

The hybridization intensity was assessed for a number of cells in each image by measuring the pixel intensity in the point of highest intensity of each cell manually defined with the pointing device. When a high variance in the hybridization intensities of the individual cells was found, the measurements were done on the brightest cells only.

The best-suited concentration of formamide in the hybridization mixture was determined by measuring hybridization intensities obtained with the following formamide concentrations: 0, 5, 10, 15, 20, 30, 50, and 70%. For subsequent hybridizations, the formamide concentration giving half the maximum intensity was chosen; this corresponded to the use of dissociation temperatures (T_ds) of the probes in slot blot hybridizations (see below).

Hybridizations with the probes S-D-Arch0344a-A-20 and S-D-Bact0338a-A-18

were performed without formamide in the hybridization buffer, while hybridizations with the NON probe were done at the same formamide concentrations as those used for the *Methanosarcina* probes.

Purification of rRNA and slot blot hybridizations. RNA from a number of microbial strains (Table 1) was extracted by the hot phenol method described by Moran et al. (27) with the DNase step omitted. Cells of *Methanosarcina barkeri* 227 were mixed vigorously with glass beads to disintegrate the cell clumps and break the cell wall (the *Methanosarcina* cell wall is not sensitive to lysozyme treatment [20]). The RNA extracted was visualized with UV light after gel electrophoresis in a minigel apparatus (GNA-100; Pharmacia, Uppsala, Sweden) in 1% agarose as described by Moran et al. (27) and staining with ethidium bromide (1 μg/ml). All equipment and solutions for work with purified RNA were autoclaved for 1 h to inactivate RNases. Glassware was subsequently baked at 120°C overnight. Samples of RNA were blotted onto Hybond-N hybridization transfer membranes (Amersham) with the use of Minifold I Dot/Slot system SRC 96-D with slot well plate SRC 60/5-D (Schleicher & Schuell, Dassel, Germany). The procedure followed the instructions from the manufacturer (39). The samples were diluted in 10× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and denaturated at 65°C for 15 min. An amount of 500 ng of RNA was applied to each slot. The concentration of RNA was determined by the absorbance at 260 nm (A_{260}; an A_{260} of 1 is equivalent to 33 μg/ml). Subsequent to RNA application, the membranes were baked for 2 min in a microwave oven for cross-linking of the RNA.

Prior to labelling, the probes were precipitated with 0.3 M sodium acetate–ethanol, washed with 64% ethanol, and dried to remove ammonium from the preparation. Labelling of the probes with ^{32}P was done with T4 polynucleotide kinase in the bacteriophage T4 polynucleotide kinase buffer described by Sambrook et al. (38). To label 20 pmol of oligonucleotide, 0.8 U of the enzyme (Promega) was used in conjugation with 30 pmol of [γ-^{32}P]ATP (Amersham). After 1 h at 37°C, the reaction was terminated by the addition of 100 μl of 0.17× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) with 0.1% sodium dodecyl sulfate (SDS), and the probe was recovered by filtration through Sephadex G-50. To elute the probe, 0.17× SSPE with 0.1% SDS was used. The amounts of label in the oligonucleotide fractions were measured in a Tri-Carb liquid scintillation analyzer (Packard Instrument Company) with the use of Hydroluma scintillation fluid (Lumac/3M bv; Schaesberg). The labelling efficiencies were 26 and 40% of the oligonucleotides present for probes SARCI551 and SARCI645, respectively.

An amount of prehybridization as well as hybridization buffer corresponding to 0.25 ml per slot was used. The prehybridization buffer was 6× SSPE with 0.1% SDS, 10× Denhardt's reagent, and 100 μg of sonicated herring DNA (Sigma D6898) per ml or 50 μg of herring sperm DNA per ml in concert with 50 μg of baker's yeast tRNA (Sigma R-8759). The DNA was denaturated at 100°C for 5 min before it was added to the mixture. The membranes were prehybridized at 42°C for a minimum of 3 h with agitation. The hybridization buffer was 6× SSPE with 1% SDS. Probe was added to 1×10^6 to 5×10^6 cpm/ml. The hybridization was allowed to proceed for approximately 16 h, whereupon the filters were washed twice in 6× SSPE with 0.1% SDS at room temperature for 15 min, once at the hybridization temperature for 3 min, and finally for 15 min at room temperature without SDS.

The membranes were finally wrapped in plastic food wrap and incubated on a sheet of Kodak X-Omat AR film on top of an intensifying screen (Sigma I-5136) for 3 to 5 h.

For quantification of the hybridizations, the individual slots were cut from the membranes and counted in Hydroluma scintillation fluid as described above.

The T_ds of the probes were deduced from hybridizations of blotted RNA from *Methanosarcina barkeri* 227 performed at nine different temperatures between 39 and 65°C. The T_d is defined as the temperature at which the hybridization intensity is reduced to half the value at maximum intensity (43). These temperatures were used for all subsequent slot blot hybridizations with the two probes.

Comparison with reported 16S rRNA sequences. The specificities of the probe sequences were tested with the CHECK_PROBE service at the Ribosomal Database Project, University of Illinois, Urbana (25) (release 5.0, 17 May 1995). This database contained sequences from the small-subunit rRNA of 2,810 prokaryotic strains, including 73 methanogens.

RESULTS

In situ hybridization protocol. In a series of experiments, single cells of *Methanosarcina mazeii* hybridized with rhodamine-labelled SARCI551 or SARCI645 were mounted in Citifluor AF1. Citifluor AF1 is a glycerol-containing mounting agent, optimized for use with the fluorochrome fluorescein, leading to highly increased fluorescence intensity. Citifluor AF1 was, however, found to wash the probe from the cells, leading to a decreasing hybridization intensity and increasing background staining during inspection (data not shown).

Some of the cells of *Methanosarcina mazeii* S-6 changed shape during the hybridization and washing procedure and appeared as enlarged and poorly defined bodies when fixed as described by Raskin et al. (33). Especially in an experiment in which the hybridization and washing buffer contained 50 mM $NaPO_4$ (pH 7.2), 0.1% SDS, 5 mM EDTA, and 500 mM NaCl, all the cells exhibited this change of morphology. If, on the other hand, the cells were fixed only by washing with saline-formaldehyde, most cells appeared as whole, well-defined irregular cocci with a visible hybridization response (data not shown). The EDTA in the hybridization buffer was, however, found to enhance the autofluorescence of the *Methanosarcina* cells substantially (data not shown). All the whole-cell hybridizations of *Methanosarcina mazeii* S-6 reported in this work were, therefore, done on cells fixed with saline-formaldehyde and hybridized without EDTA in the hybridization buffer.

It should be noted that the hybridization intensities of individual *Methanosarcina* cells differed greatly within the same hybridization experiment. Moreover, cells were normally found in small clusters in which the individual cells were difficult to locate in the acquired images.

The combined hybridization of *Methanosarcina mazeii* S-6 cells with probe SARCI551 and SARCI645 was tested. Only an insignificant increase in the hybridization intensities was found: the value obtained from the dual hybridization was 1,324 (standard deviation [SD] = 220; $n = 20$), while the intensities obtained with the probes used individually were 1,142 (SD = 160; $n = 10$) and 873 (SD = 122; $n = 10$), respectively. The background fluorescence of unlabelled cells was 263 (SD = 11; $n = 10$).

The performance of the *Methanosarcina* probes was tested on cells of *Methanosarcina mazeii* S-6 in the presence of a biomass sample. This sample was granular sludge from an upflow anaerobic sludge blanket reactor, which did not contain *Methanosarcina* cells so that the hybridization found could be attributed to the added cells of *Methanosarcina mazeii*. The absence of *Methanosarcina* cells in the sample was verified by use of a specific antibody against *Methanosarcina mazeii* S-6. This antibody has been found to detect all strains of *Methanosarcina* tested to date (41). The results from an in situ hybridization of a biomass sample are presented in Fig. 1. Added cells of strain S-6 were readily seen upon hybridization with SARCI551 or SARCI645. As demonstrated in Fig. 1, the biomass sample showed extensive autofluorescence at the excitation wavelength for the rhodamine labelling, and many distinct cell morphotypes were visible. Applying a narrow-band emission filter increased the signal-to-noise ratio significantly. The absence of unspecific binding of probe to the biomass sample was confirmed in a hybridization experiment with the NON probe (data not shown).

Specificities of the probes. Figure 2 shows the whole-cell hybridization intensities obtained at different concentrations of formamide. Formamide concentrations of 20 and 15% were used in all subsequent experiments with SARCI551 and SARCI645, respectively.

Whole-cell hybridizations performed on 10 microbial strains (Table 2) demonstrated the specificities of the probes. The probes hybridized extensively only with the two *Methanosarcina* strains tested. All cells were found to hybridize with probe S-D-Arch0344a-A-20 or S-D-Bact0338a-A-18, showing that the rRNA was accessible to hybridization. *Methanococcoides methylutens*, *Methanococcus vannielii*, and *Methanococcus voltaei* hybridized weakly with both of the *Methanosarcina* probes (Table 2), but a control experiment with an equal amount of the NON probe in parallel to probe 645 showed uniform hybridization intensities with the NON probe and with SARCI645. Cells of *E. coli* B6, *Methanosaeta concilii* GP6, "*Methanobacterium*" sp. strain Marburg, and "*Methanobacte-*

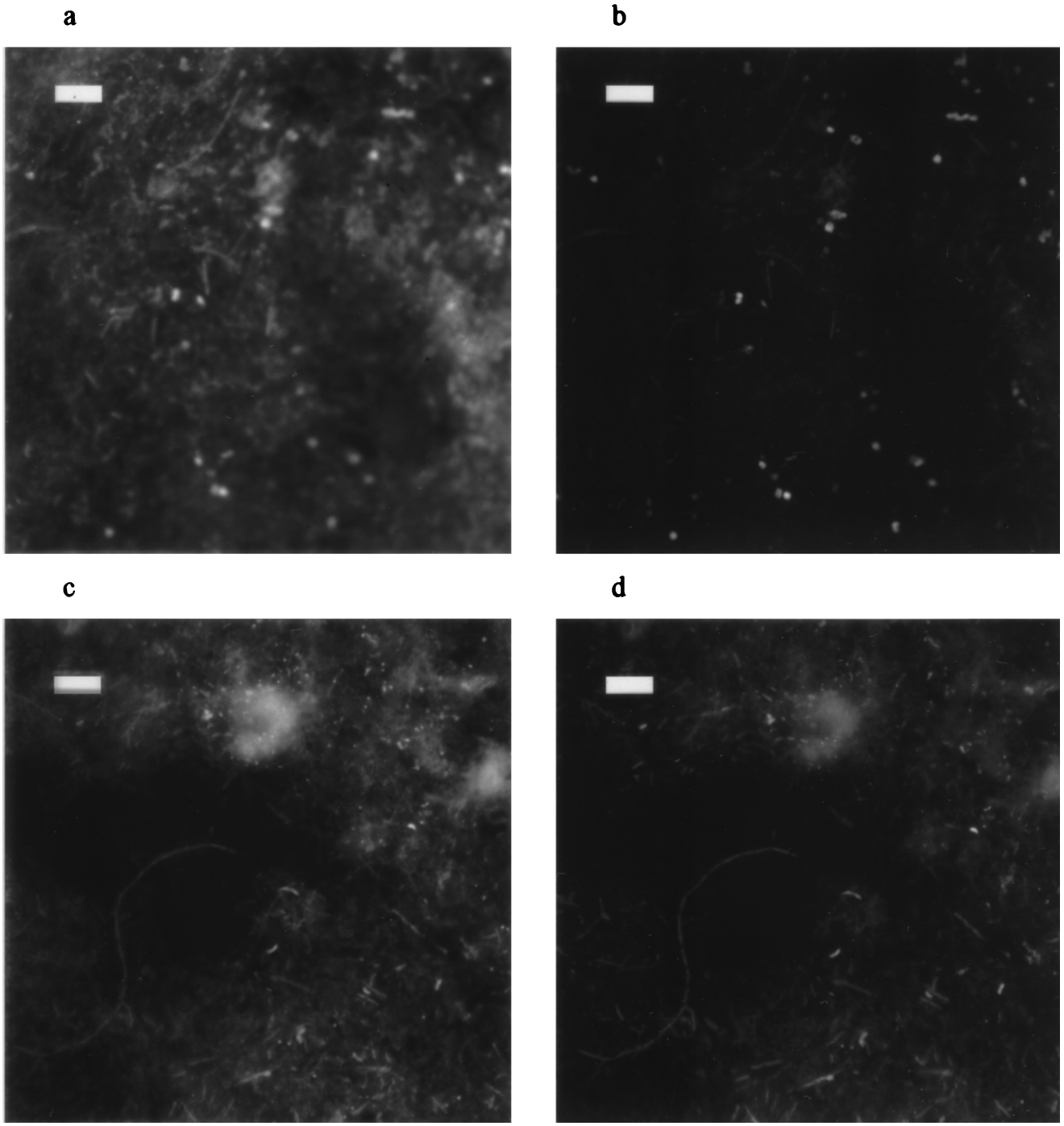

FIG. 1. Epifluorescence micrographs showing in situ hybridization of a biomass sample with single cells of *Methanosarcina mazeii* S-6 added. Bars, 10 μm. The images were exposed and processed to enhance the contrasts between labelled cells and the inherent fluorescent material. The light intensities in the different images are thus not comparable. (a and b) Hybridization with probe SARCI551. Micrographs of the same field were acquired without (a) and with (b) a narrow-band filter selective for wavelengths emitted by the fluorochrome label. (c and d) Negative control without probe. Micrographs of the same field were acquired without (c) and with (d) a narrow-band filter selective for wavelengths emitted by the fluorochrome label. The images were processed by use of Adobe Photoshop software version 3.0.

rium" sp. strain CB12 did not bind the NON probe nor did they give any response with the *Methanosarcina* probes.

It was found that *Methanosarcina thermophila* TM-1 hybridized more intensively with probe SARCI551 than with SARCI645, while the two probes gave comparable hybridization intensities with *Methanosarcina mazeii* S-6 (Table 2).

From the hybridizations on blotted RNA, T_ds of SARCI551 and SARCI645 of 50 and 53°C, respectively, were found. Figure 3 shows the specificity test performed with membrane-fixed rRNA from a selection of 12 prokaryotes. The hybridizations were performed at the respective T_ds of the probes. Probe SARCI551 hybridized strongly with rRNA from the three *Methanosarcina* species but not to RNA from any of the other strains tested. Probe SARCI645 hybridized strongly with the

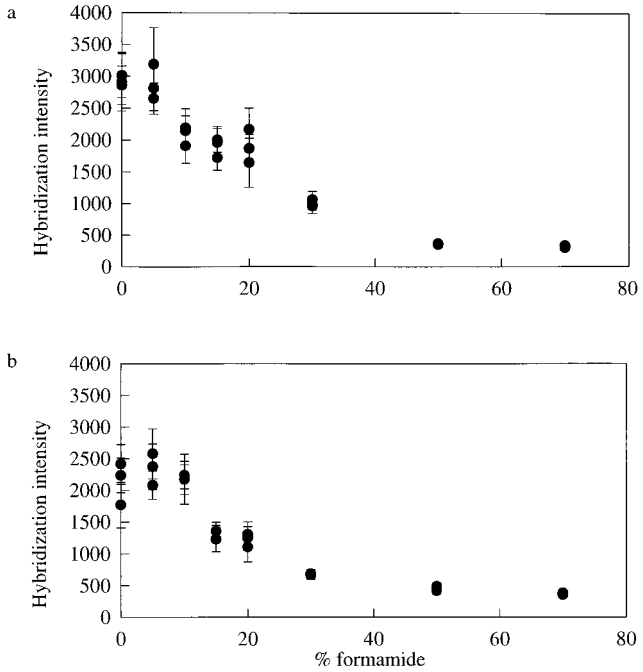

FIG. 2. Melting curves of probe SARCI551 (a) and SARCI645 (b) obtained by whole-cell hybridization with single cells of *Methanosarcina mazeii* S-6. Each spot represents the average intensity measured from one image. The error bars show the SDs of each point. The intensities measured without probe were 263 (SD = 12; n = 20) and 272 (SD = 29; n = 20), respectively.

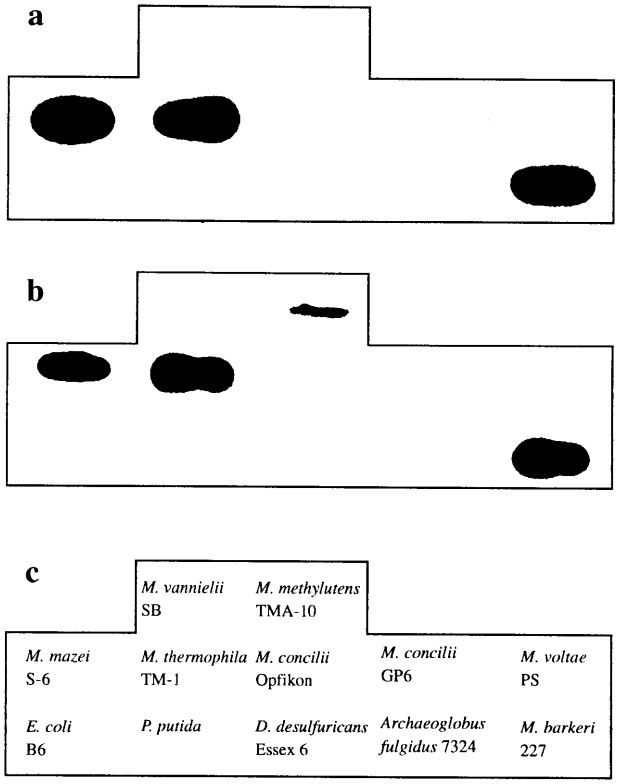

FIG. 3. Autoradiographs showing hybridizations of probe SARCI551 (a) and SARCI645 (b) to slot blotted RNA samples. The positions of the RNA and the outline of the filters are shown (c).

Methanosarcina rRNAs but also showed a weak hybridization with rRNA from *Methanococcoides methylutens.*

The test of specificity performed with the Ribosomal Database Project sequences showed the following. (i) The sequence of probe SARCI551 matched all the *Methanosarcina* sequences perfectly. The strain designations of the database are *Methanosarcina* sp. strain WH1, *Methanosarcina frisia* C16, *Methanosarcina frisius*, *Methanolobus siciliae* T4/M, *Methanosarcina acetivorans* C2A, *Methanosarcina barkeri* 227, and *Methanosarcina thermophila* TM-1. (Recently, *Methanolobus siciliae* was transferred to the genus *Methanosarcina* [29]. *Methanosarcina frisia* is a subjective synonym of *Methanosarcina mazeii* [24]). No strains with one mismatch to the probe were found. Only one strain with two mismatches was found, namely, *Methanohalobium evestigatus* Z-7303, an extremely halophilic flat polygon-shaped methanogen belonging to the *Methanosarcinaceae* family (53). (ii) The sequence of probe SARCI645 was found to perfectly match the sequences of six of the *Methanosarcina* strains. The probe had one mismatch to the seventh strain, *Methanosarcina* sp. strain WH1, and two mismatches to a number of strains belonging to other genera of the *Methanosarcinaceae* (including *Methanococcoides methylutens*) and to *Methanoculleus marisnigri* JR1 (of family *Methanomicrobiaceae*) and *Methanococcus voltaei* PS (of family *Methanococcaceae*).

DISCUSSION

An important step in developing in situ hybridization protocols is to ensure that probes have access to the rRNA (8, 14, 16). The results of the whole-cell hybridizations clearly show

TABLE 2. Specificities found by whole-cell hybridization

Strain	Hybridization (max pixel intensities)[a]		
	With SARCI551 plus 20% formamide	With SARCI645 plus 15% formamide	Without probe
Escherichia coli B6	202 (12)	200 (18)	166 (6)
Desulfobulbus propionicus	400 (42)	503 (76)	315 (29)
Methanosaeta concilii GP6	196 (13)	234 (19)	212 (21)
"*Methanobacterium*" sp. strain Marburg	178 (11)	177 (7)	172 (7)
"*Methanobacterium*" sp. strain CB12	189 (7)	185 (14)	200 (15)
Methanosarcina mazeii S-6	894 (93)	1,071 (197)	238 (32)
Methanosarcina thermophila TM-1	1,473 (197)	715 (70)	240 (29)
Methanococcoides methylutens TMA-10	244 (17)	269 (32)	152 (5)
Methanococcus vannielii SB	387 (33)	307 (28)	167 (9)
Methanococcus voltaei PS	414 (53)	322 (26)	167 (6)

[a] The dark background between the cells had a pixel intensity of around 150. This value is not subtracted. Values in parentheses are standard deviations (n = 10).

that the new *Methanosarcina* probes enter the cells and hybridize to the ribosomes. The penetration of fluorochrome-labelled probe through the protein cell wall of *Methanosarcina* cells (17) has not been shown before. Difficulties with penetration of fluorochrome-labelled probes were shown by Hahn et al. (16) with gram-positive cells of a *Frankia* sp. requiring a lysozyme treatment to permeabilize the cells. Likewise, Wagner et al. (46) showed that some *Microthrix parvicella* cells in activated sludge did not hybridize to fluorescent-oligonucleotide probes, probably because of impermeability of the cell surface.

The finding that Citifluor AF1 led to melting of the probe from the rRNA in whole cells is a matter of concern with hybridizations including the fluorochrome fluorescein. This fluorochrome is widely used and is well suited for staining protocols in combination with rhodamine due to differences in excitation and emission wavelengths of the two fluorochromes. Fluorescein, however, is very sensitive to fading and is preferably used in combination with a nonfading agent (19). Citifluor AF1 is excellent for this purpose and is used extensively (5, 31, 33). Besides, it strongly enhances the fluorescence intensity of fluorescein. The results presented point to the fact that the use of fluorescein labelling of the two *Methanosarcina* probes will not be feasible.

The change in shape upon hybridization of cells of strain S-6 fixed as described by Raskin et al. (33) was probably due to lysis of the cells after adhesion to the glass surface. Single cells of *Methanosarcina* have previously been reported to be osmotically fragile (3, 17, 51). The hybridization buffer found to be the best suited was the formamide-containing buffer without EDTA described by Poulsen et al. (31). Formamide is added to enhance the stringency of the hybridization but is also reported in some cases to enhance the hybridization intensity significantly (8, 32).

The quantification of the hybridization intensities should ideally be performed by integrating the total signal from each cell and relating this to cell area or cell volume (30). The software used was, however, not able to detect the very weak fluorescence of the edges of the cells with low hybridization intensities. Therefore, only the brightest centers of the cells were measured and included in the intensity measurement performed by the software (for a discussion of this phenomenon, see the work of Viles and Sieracki [45]). Together with the variable size and shape of the cells, this problem also hindered manual circumscription of the cell perimeter. To avoid this, only the peak intensities of the cells were measured. The Cellstat program presented by Møller et al. (26) was also examined for quantification of the hybridization signal but was found to be unsatisfactory for detection of the highly variable and odd-shaped *Methanosarcina* cells.

The high variation in hybridization signals encountered with the *Methanosarcina* probes and reference cells of the *Methanosarcina* strains used could be a consequence of variable cell size or activity (i.e., ribosome number [12]), variable permeability of the cells, or occasional lysis of cells during the hybridization and staining procedure as described above. In the present work, only hybridization intensities of the brightest cells were quantified, and the reported brightness values are thus not representative of the cell sample as such but can be used as a comparable indication of the labelling intensity at different hybridization conditions.

The high variation of the hybridization intensities may also be the reason why we could not demonstrate any effect of dual probing. The lack of significant enhancement of the signal by dual probing is in contrast to the results of Amann et al. (7), who obtained a twofold increase in hybridization intensity by dual probing with ^{32}P-labelled probes, and of Amann et al. (6), who quantified the fluorescence of hybridized cells by flow cytometry and found highly increased intensities after combining two or three probes.

The finding that there was no detectable unspecific probe binding in the biomass sample (Fig. 1) is in accordance with the work of Hahn et al. (15), who tested the applicability of in situ hybridization with tetramethylrhodamine isothiocyanate-labelled probes to soil samples and found a high autofluorescence of the organic material present in the samples but no nonspecific binding of the probes. Likewise, the autofluorescence of our biomass sample was intense but could be lowered by application of specific narrow-band filters.

In their work on probes for methanogens, Raskin et al. (33, 36) described the use of a *Methanosarcina* oligonucleotide probe, S-G-Msar0821a-A-21, for detection of this genus in reactor samples by hybridization with membrane-bound RNA. Generally, they found only low amounts of *Methanosarcina* 16S rRNA in their samples with this probe, which was consistent with the relatively low acetate concentration found in these reactors. A low acetate concentration will tend to favor *Methanosaeta* instead of *Methanosarcina* (54). The probe was also labelled with rhodamine and used for in situ hybridizations of samples. Hereby, it could detect the presence of both aggregated and single cells of *Methanosarcina*. The performance of the whole-cell hybridizations was, however, not discussed in detail.

The probes SARCI551 and SARCI645 exhibited the specificities expected. The weak hybridization found upon whole-cell hybridization with cells of *Methanococcoides methylutens*, *Methanococcus vannielii*, and *Methanococcus voltaei* was also found after hybridization with the NON probe and must be unspecific binding of the probe to these particular cells. No hybridization of the SARCI645 probe to cells of *Methanococcoides methylutens*, corresponding to the low cross-reaction found with purified rRNA (see below), could be found. The *Methanosarcina mazeii* S-6 cells did not bind the NON probe. Amann et al. (6) and Hahn et al. (16) encountered unspecific binding of tetramethylrhodamine isothiocyanate-labelled probes to cells. In accordance with this, Poulsen et al. (31) found that hydrophobic fluorochromes such as tetramethylrhodamine isothiocyanate and, to some extent, lissamine rhodamine bound unspecifically to hydrophobic structures in their specimens. These authors suggested the use of more-hydrophilic fluorochromes like CY3. If problems of high unspecific binding of the *Methanosarcina* probes to non-*Methanosarcina* cells are encountered, as is probably also the case with *Desulfobulbus propionicus* (Table 2), the use of less-hydrophobic fluorochromes could be a solution.

Methanosarcina thermophila TM-1 hybridized with different intensities with the two *Methanosarcina* probes. A similar finding was reported for *Pseudomonas cepacia*, which showed higher intensities upon hybridization with the S-D-Bact0338a-A-18 probe than with a specific probe, Pce54a (52). These authors suggested that the difference was caused by different accessibilities of the target sites of the rRNA due to the higher-order structure of the ribosomes. This may also be the explanation for the different intensities found for *Methanosarcina thermophila* TM-1.

The low cross-reaction of probe SARCI645 with purified rRNA of *Methanococcoides methylutens* could be correlated with the fact that this strain had only two mismatches to the probe. The absence of cross-reaction with *Methanococcus voltaei* RNA, which also had two mismatches to the probe, shows that the correlation between cross-reaction and the number of mismatches, however, is not unambiguous.

The specificity control test of the probes against the se-

quences of the Ribosomal Database Project verified the specificities of the probes for *Methanosarcina* cells. *Methanosarcina* sp. strain WH1, which has one mismatch with probe SARCI645 according to the Ribosomal Database Project, was isolated from a marine environment (37) and may not be relevant in anaerobic digesters.

The oligonucleotide probes described by Raskin et al. (33–36) are an important acquisition for further work to provide probes encompassing all known methanogens relevant for work on anaerobic reactors. The present work, however, revealed a number of problems and solutions related to the specific morphology and composition of *Methanosarcina* cells when detected by in situ whole-cell hybridization. Thereby, it illustrates the need for a careful inspection of the performance of the probes for new test designs. These results will be included in our future work on microbial characterization of samples from biogas reactors.

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