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Detection and Localization of Syntrophic Propionate-Oxidizing Bacteria in Granular Sludge by In Situ Hybridization Using 16S rRNA-Based Oligonucleotide Probes

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In situ hybridization with fluorescent oligonucleotides was used to detect and localize microorganisms in the granules of two lab-scale upflow anaerobic sludge blanket reactors that had been fed for several months with either sucrose or a mixture of volatile fatty acids. Sections of the granules were hybridized with 16S rRNA-targeted oligonucleotide probes for *Bacteria*, *Archaea*, specific phylogenetic groups of methanogens, and two syntrophic propionate-oxidizing strains, MPOB and KOPROP1. Cells of the syntrophic strain KOPROP1 were not detected in either type of sludge granules. Hybridizations of the sucrose-fed granules showed an outer layer of mainly bacterial microcolonies with different morphologies. More inwards of these granules, a layer of different methanogenic microcolonies mixed with large colonies of the syntrophic strain MPOB could be detected. The MPOB colonies were intertwined with hydrogen- or formate-consuming methanogens, indicating their syntrophic growth. The granules fed with volatile fatty acids showed an outer layer of mainly bacteria and then a thick layer of *Methanosaeta*-like methanogens mixed with a few bacteria and a layer of methanogens mixed with syntrophic MPOB microcolonies. The centers of both sludge types consisted of large cavities and methanogenic microcolonies. These results indicate a juxtapositioning of syntrophic bacteria and methanogens and provide additional evidence for a layered microbial architecture of anaerobic granular sludge.

Anaerobic degradation of organic matter leads to intermediate formation of alcohols and fatty acids. The oxidation of these products under methanogenic conditions is coupled to proton reduction and can only proceed at low hydrogen partial pressures and low formate concentrations. This is achieved by interspecies transfer of hydrogen or formate between syntrophic consortia of bacteria. Acetogens convert propionate into acetate, hydrogen or formate, and carbon dioxide, while methanogens use these products in the production of methane (4, 17). A specific spatial orientation of acetogens and methanogens has been suggested because the distance between these microorganisms should be sufficiently small to obtain a low hydrogen partial pressure (27). The architecture of syntrophic consortia has been studied in methanogenic granular sludge, in which microorganisms are immobilized in densely packed granules and high conversion rates of alcohols and fatty acids are reached (6, 15, 32). Immunogold and fluorescent labeling techniques with antibodies against methanogens and acetogens suggested a juxtaposition of syntrophic propionate-oxidizing bacteria and the hydrogen- or formate-utilizing methanogens in microcolonies throughout the entire granule, although the antibodies used were not sufficiently specific to detect the acetogens (6, 14, 20). Additionally, a layered structure of the granule, in which a central core of aceticlastic methanogens is surrounded by a layer of hydrogen- or formate-producing acetogens and hydrogen- or formate-consuming methanogens, was proposed (7, 12, 15). The outer layer of the granule consists most likely of bacteria that hydrolyze and acidify complex organic matter.

Various immunological techniques have been used successfully to study the spatial distribution of methanogens in methanogenic granular sludge (14, 30). However, the distribution of acetogenic bacteria in this sludge could not be examined due to the absence of identification methods that allowed differentiation between the groups of bacteria. Modern molecular biological techniques provide tools to investigate these issues in anaerobic ecosystems in situ (2, 21, 26). Consequently, in situ hybridization with fluorescently labeled oligonucleotide probes based on rRNA sequences has proven to be a powerful tool to identify and localize microorganisms in their natural habitats (31).

Recently, we described the phylogenetic characterization of three syntrophic bacteria, *Syntrophobacter wolinii* and strains MPOB and KOPROP1 (8, 9). Analysis of their 16S rRNA sequences revealed that these syntrophic bacteria are closely related and belong to the delta subclass of the *Proteobacteria*. These bacteria were also closely related to *Desulforhabdus amnigenus*, a sulfate-reducing bacterium isolated from sulfidogenic granular sludge which is not able to grow syntrophically on propionate (19). Specific oligonucleotide 16S rRNA-based probes were designed to detect these bacteria (8). The use of the fluorescently labeled probes to detect and localize MPOB and KOPROP1 in sludge from upflow anaerobic sludge blanket (UASB) reactors is described here. These probes were used in combination with fluorescently labeled 16S rRNA-based probes for *Bacteria* and *Archaea*. This approach allowed analysis of the spatial distribution of microorganisms in metha-

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nogenic granular sludge, especially that of syntrophic propionate-oxidizing bacteria.

MATERIALS AND METHODS

Origin of granular sludge samples. Sludge samples were taken from two 10-liter UASB reactors which had been inoculated with sludge from a UASB plant treating industrial sugar beet wastewater (CSM, Breda, The Netherlands). The reactors had been fed with a mineral medium described before (29), except sulfate was added as 170 mg of $(NH_4)_2SO_4$ per liter. One reactor was fed with sucrose with a chemical oxygen demand load of 5 kg/m^3/day, corresponding to approximately 8 mM sucrose in the influent. The other reactor was fed with a mixture of volatile fatty acids (VFA)-butyrate-propionate-acetate (42:32:24) with a chemical oxygen demand load of 10 kg/m^3/day. This corresponded to an approximate concentration of 13 mM butyrate, 16 mM propionate, and 19 mM acetate in the influent. The chemical oxygen demand removal was more than 90% in both cases. The samples were taken 6 months after inoculation from the lower part of the sludge blankets.

Fixation, embedding, and sectioning of granules. Fresh granule samples (3 ml) were gently washed in tap water (10 ml) and allowed to settle. Subsequently, the granules were fixed in freshly made 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, and left overnight at 4°C. The fixative was removed by washing the granules twice with PBS. The granules were dehydrated by resuspension in 50% (vol/vol) ethanol in water for 2 h followed by treatment with 70% ethanol overnight at 4°C. The water-ethanol was replaced with tertiary butyl alcohol (TBA) by washing the granules consecutively for 1 h (each) at 32°C with ethanol-water-TBA of the following proportions (by volume): 50:30:20, 50:20:30, 50:10:40, 50:0:50, and 25:0:75. Subsequently, the granules were transferred to a 1.5-ml tube which was incubated overnight in 100% TBA. The TBA was replaced by Paraplast (Sherwood Medical Co., St. Louis, Mo.) at 62°C by adding five pellets of Paraplast to the tubes, one pellet every 12 h. The tubes were opened for 2 h to allow the evaporation of TBA. The granules were then transferred with a small spatula to a 1-cm^3 cup with melted Paraplast. The cube was allowed to cool down and was then used for sectioning.

The granule cubes were cut with a conventional microtome in sections of 5 to 10 μm. Sections thinner than 5 μm showed a loss of structure and the sections broke easily. Sections thicker then 10 μm resulted in high background, especially with conventional epifluorescence microscopy, because of an excess of biomass. The sections (ribbons) were stretched in 50°C water and transferred to slides coated with Vectabond (Vector Laboratories, Inc., Burlingame, Calif.). The slides were dried overnight at 42°C. The sections on the slides were deparaffinated in xylol for 30 min, and the xylol was removed by rinsing the slides consecutively for 2 min each in xylol-TBA-ethanol of the following composition (by volume): 100:0:0, 75:25:0, 25:75:0, 0:50:50, 0:25:75, and 0:0:100. The sections were stored in a dry box for up to 6 months at 4°C.

In situ hybridization and transmission electron microscopy. The following oligonucleotide probes complementary to specific regions of 16S rRNA were used: (i) EUB338, specific for the domain *Bacteria* (1); (ii) ARC915, specific for the domain *Archaea* (25); (iii) MPOB1, specific for strain MPOB (8) (see Table 1); (iv) KOP1, specific for strain KOPROP1 (8); (v) MX825, specific for the genus *Methanosaeta* (22); (vi) MG1200, group specific for the order *Methanomicrobiales* (22); and (vii) MB310, group specific for the family *Methanobacteriaceae* (22). All oligonucleotides were synthesized and 5′ labeled with FLUOS (a fluorescein derivative) or rhodamine, using an amino linker, and subsequently purified by acrylamide gel electrophoresis (Eurogentec, Seraing, Belgium). Sections of several different granules were hybridized in 20 μl of hybridization buffer (0.9 M NaCl, 20 mM Tris HCl [pH 7.2], 0.01% sodium dodecyl sulfate) containing 100 ng of labeled oligonucleotide probe. These sections were incubated in a moist chamber (25) for 3 h at 45°C, and excess probe was removed by washing in hybridization buffer at 48°C for 30 min. The sections were rinsed in water, air dried, and subsequently viewed with a Nikon epifluorescence microscope or a Bio-Rad MRC-600 confocal laser scanning microscope equipped with a krypton-argon laser. Transmission electron microscopy was done on a Jeol JEM 100 CX II microscope by a modification of methods described previously (24).

RESULTS

Fluorescent oligonucleotide probing. The microbial composition and spatial orientation of the microorganisms in sections of granular sludge were investigated by fluorescent oligonucleotide probing. Two different sludge samples from lab-scale UASB reactors were analyzed; one had been fed with sucrose and another had been fed with a mixture of the VFAs acetate, propionate, and butyrate. The hybridizations shown in the figures are representative of several independent hybridizations of several granules.

Hybridization with fluorescein-labeled 16S rRNA-based universal probes for *Bacteria* (EUB338) and *Archaea* (ARC915) to detect methanogens was combined with hybridization with rhodamine-labeled specific probes for the syntrophic propionate-oxidizing strains MPOB and KOPROP1 (MPOB1 and KOP1). The specificity of probe MPOB1 is shown in an alignment of part of the 16S rRNA sequences of some reference organisms (Table 1). The MPOB1 probe has only one mismatch with the syntrophic propionate-oxidizing strain KOPR OP1 or the sulfate-reducing bacterium *D. amnigenus* and hybridizes with the rRNA of these organisms even when washed at 1 degree below the dissociation temperature (8). Probe KOP1 hybridized only to the 16S rRNA of the syntrophic strain KOPROP1, and this probe had at least three mismatches with all other 16S rRNA sequences currently known (8).

TABLE 1. Alignment of 16S rRNA sequences of the target site of probe MPOB1[a]

Reference organism	rRNA sequence
MPOB	5′ UUCGGGGAUGGGCCUGCGU 3′
KOPROP1	**UA**CGGGGAUGGGCCUGCGU
Desulforhabdus amnigenus	UUCGGGGAUG**A**GCCUGCGU
Syntrophobacter wolinii	**CA**CGGGGAUG**AGU**CUGCGU
Desulfomonile tiedjei	**CCAAA**GGAUG**GGC**U**C**GCG**G**
Desulfobulbus propionicus	**CNUGAA**GA**G**GGGUCUGCGU
Desulfosarcina variablis	UU**UGAA**GAUGGGCC**C**GCGU
Desulfovibrio desulfuricans	**CGUAA**GGAUG**AGUCC**GCGU
Desulfovibrio gigas	**CAAUGA**GAUG**AGUCC**GCGU
Myxococcus xanthus	U**AUUCA**GAUG**AGUCC**GCGU
Escherichia coli	**CCAUC**GGAUG**UGCCCAGA**U
Bacillus subtilis	**CUUACA**GAUGG**ACCC**GCG**G**
Methanococcus vannielii	**CC**CG**A**GGAU**AGGAC**UGCG**C**
Methanosaeta soehngenii	**CCUAA**GGAUGGG**U**CUGCG**G**

[a] The nucleotides corresponding to *Escherichia coli* positions 222 to 240 of several species of the delta subclass of the *Proteobacteria* and some reference organisms are aligned. The data were taken from the Ribosomal Database Project (13). Mismatches with the MPOB target site are marked in boldface.

Autofluorescence. Autofluorescence during epifluorescence microscopy is often a problem when in situ hybridization is performed on microbial cells present in complex environments. In the case of the granule sections, a considerable amount of autofluorescence was seen at 520 and 580 nm. However, we were able to distinguish the autofluorescent signal from that resulting from fluorescence of the labeled probes, since the autofluorescence gave a bright yellow signal when viewed at 520 nm while the fluorescence of the fluorescein-labeled probes was green. Usually, fibrous or granular structures showed fluorescence, probably caused by inorganic precipitates. At 580 nm, these structures were as red as the signal from the rhodamine-labeled probes, but viewing the sections at 520 nm afterwards showed that they were not of prokaryotic origin (marked by X in Fig. 1 through 4).

Microbial localization and architecture of sucrose-fed sludge. Sucrose-fed granules were hybridized with the fluorescein-labeled bacterial probe EUB338 in order to visualize all bacteria (Fig. 1A). Bright microcolonies and individual bacteria at the edge of the granule showed fluorescence. More inwards of the granule, large microcolonies of 2-μm-long, thick rods could be visualized. Most bacteria in this area have this morphology. Hybridization of the same sucrose-fed granule with the rhodamine-labeled probe MPOB1 showed only fluorescence of the large microcolonies located more inwards of the granule (Fig. 1B). The morphology of the cells in these microcolonies resembled that of the MPOB cells in the pure culture. The sucrose-fed granules were also hybridized with probe KOP1, specific for strain KOPROP1. This probe did not

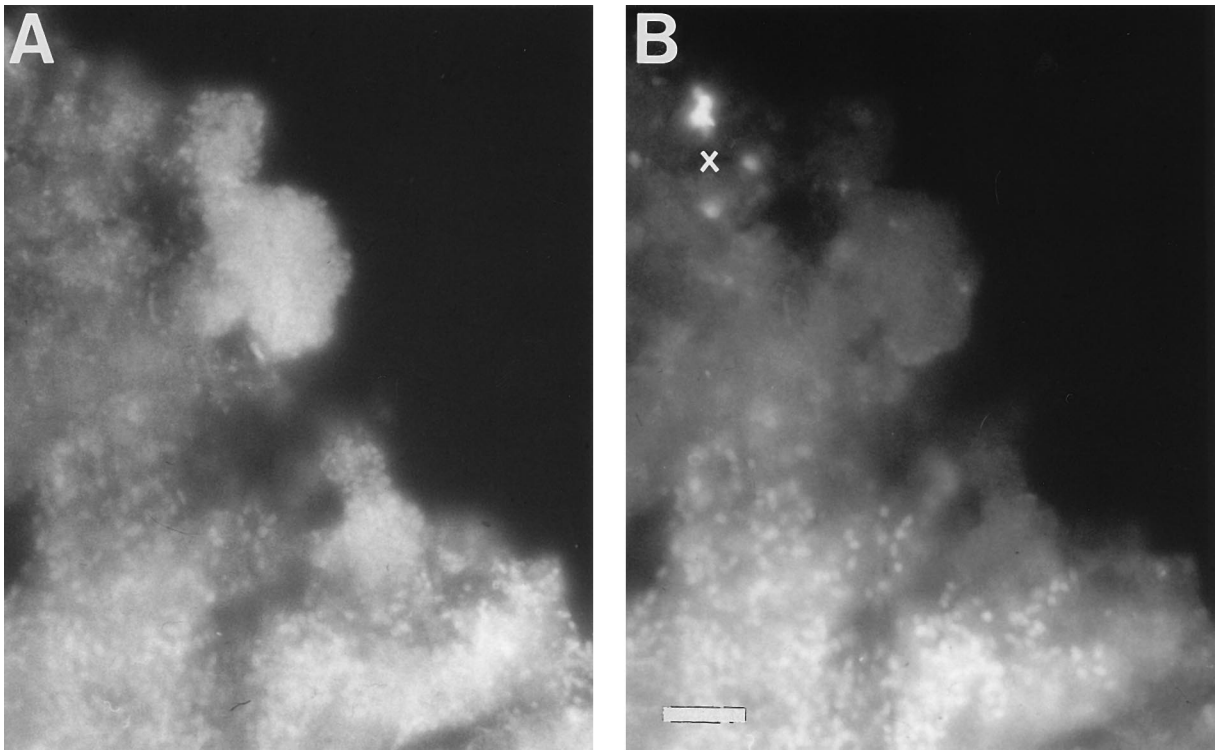

FIG. 1. In situ hybridization of sections of sucrose-fed anaerobic granular sludge. The sections were simultaneously hybridized with a fluorescein-labeled oligonucleotide probe universal for bacteria, EUB338, and a rhodamine-labeled specific probe, MPOB1, and viewed by epifluorescence microscopy with a fluorescein-specific (A) and rhodamine-specific (B) filter set. The photomicrographs were taken at the outer layers of the granule. Various morphotypes of rods and cocci hybridize with the bacterial probe, but only the short rods present in the microcolony in the left corner of the micrographs are visualized by the MPOB1 probe. X, autofluorescence. Bar, 10 μm.

hybridize to any of the cells (results not shown), indicating that the cells hybridizing with the MPOB1 probe were not KOPR OP1 cells but MPOB-like cells.

At a lower magnification, hybridization of sucrose-fed granules with probes EUB338 (Fig. 2A) and MPOB1 (Fig. 2B) showed hardly any individual cells. However, the overall structure of the granule and the localization of the bacteria were clearer. Both probes hybridized with the rRNA of microcolonies located more inwards of the granule, identifying them as MPOB-like bacteria. The microcolonies at the surface of the granule, only visualized by the EUB338 probe, showed a brighter fluorescent signal than the MPOB-like microcolonies (Fig. 2A). Additionally, there were individual cells more inwards of the granule that were only visualized by the EUB338 probe.

To visualize all methanogens present in the granule, the fluorescein-labeled archaeal probe ARC915 was used in hybridization (Fig. 3A). The ARC915 probe hybridized with the rRNA of several microcolonies more inwards of the granule, with different intensities of the fluorescent signal. Microcolonies with a bright fluorescent signal are adjacent to those that show a less pronounced signal (Fig. 3A). Although the green fluorescent signal of the latter microcolonies was low, it could clearly be distinguished from the brownish background and yellow autofluorescence. Hybridization of the same section with the MPOB1 probe showed large microcolonies at positions corresponding to those of the methanogenic microcolonies that showed a low fluorescent signal with the ARC915 probe (Fig. 3A and B; indicated by S for syntrophic microcolony). This overlap was observed in all cases; moreover, the MPOB-like microcolonies showed no overlap with the bright methanogenic microcolonies (marked Me in Fig. 3A).

To study the architecture of a possible syntrophic microcolony that hybridized with the ARC915 probe and the MPOB1 probe in more detail, magnifications were viewed with a confocal laser scanning microscope (Fig. 3C and D). This showed that the methanogens in the microcolonies with a low fluorescence (Fig. 3C) were juxtaposed to the MPOB-like cells (Fig. 3D). Group-specific probes for the order *Methanomicrobiales* or the family *Methanobacteriaceae* (22) were used to identify the hydrogen- or formate-utilizing methanogens growing in these syntrophic microcolonies. Both probes hybridized weakly to individual cells and microcolonies throughout the granules (results not shown). However, only the probe for *Methanobacteriaceae* showed fluorescence at the positions at which the MPOB-like microcolonies were located (results not shown). To confirm the juxtaposition of the methanogens and the syntrophs, sections of the granules were viewed by transmission electron microscopy, which showed syntrophic microcolonies containing cells of *Methanobacteriaceae*, which resembled *Methanobrevibacter* cells (Mb), next to cells hybridizing with the MPOB probe. As expected, *Methanosaeta*-like cells were found adjacent to cells of the syntrophic microcolony (Fig. 3E).

Microbial localization and architecture of VFA-fed sludge. Granules fed with VFA were hybridized with probes EUB338 and MPOB1 in order to show the effect of different substrates on the architecture of the granules (Fig. 4). Probe EUB338 hybridized with the rRNA of bacteria present on the surface of the granule, located more inwards, and present around the

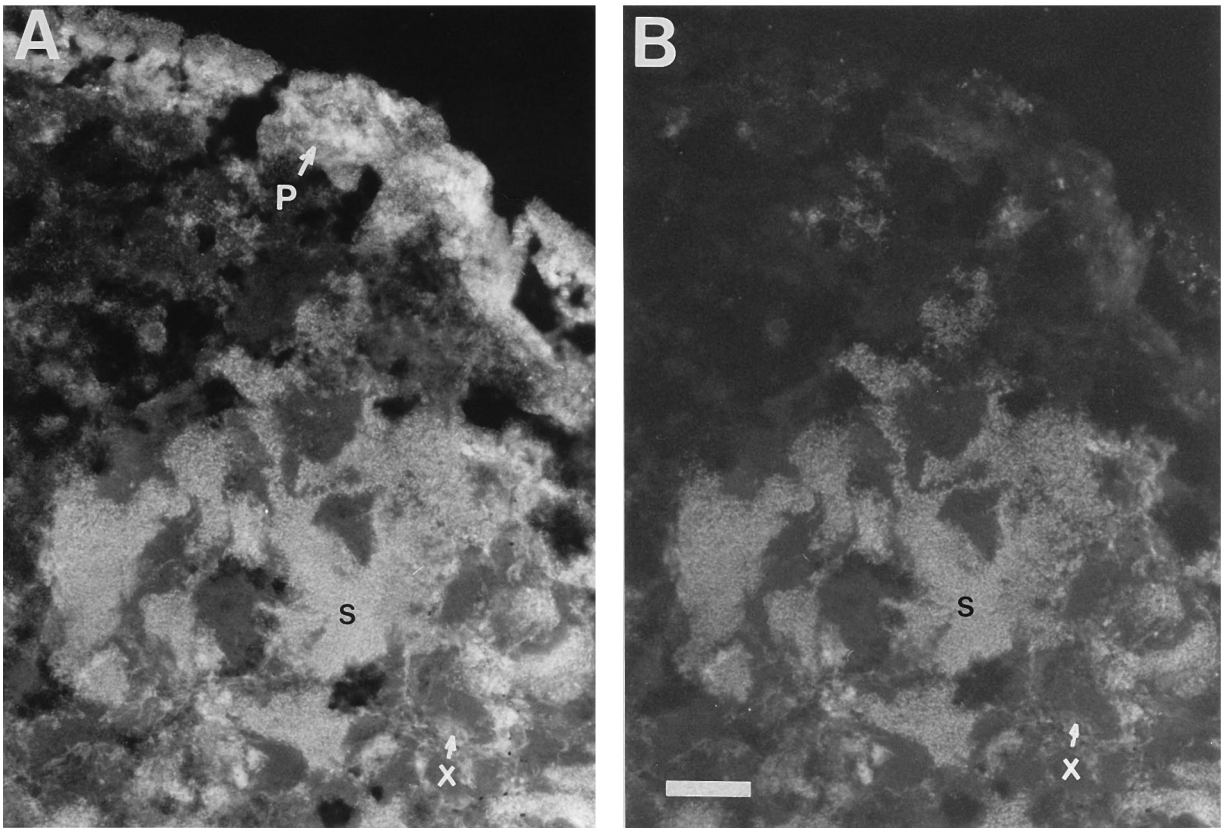

FIG. 2. In situ hybridization of a sucrose-fed sludge granule viewed by epifluorescence microscopy at a 200-fold magnification. (A) The fluorescein-labeled bacterial probe EUB338 detects bacterial cells mainly located in the outer layer of the granule (P, peripheric location) and the syntrophic microcolonies more inwards of the granule (S, syntrophic microcolony). (B) The rhodamine-labeled probe MPOB1 detects only the microcolonies inwards of the granule. X, autofluorescence. Bar, 50 μm.

center of the granule (Fig. 4A). The structures seemed to be organized differently from those in the sucrose-grown granules. The MPOB1 probe visualized almost all bacterial microcolonies located near the center (Fig. 4B). The hybridization of a VFA-fed granule with probes ARC915 and MPOB1 is shown in Fig. 5. The methanogens are located throughout the granule (Fig. 5A). The brightest microcolonies are located radially in the outer layers. Most of these microcolonies reacted with a rhodamine-labeled oligonucleotide probe specific for the aceticlastic genus *Methanosaeta* (22) (data not shown). Furthermore, typical microcolonies with a *Methanosarcina*-like morphology were detected in this layer. More inwards, less bright microcolonies are located, next to microcolonies that show a diffuse fluorescence of low intensity. The MPOB microcolonies are located outside the center of the granule at positions corresponding to those of the low fluorescing methanogenic microcolonies (Fig. 5B). These are again surrounded by the brighter fluorescing methanogenic microcolonies.

DISCUSSION

The microbial diversity of granular sludge has previously been studied by using traditional methods such as most-probable-number counting, immunolabeling techniques, and electron microscopy (6, 10, 14, 15, 30). However, the syntrophic bacteria could not always be identified unambiguously. In situ hybridization of microbes with fluorescent oligonucleotide probes has become a new approach to study microbial organization and its function in complex ecosystems (2). The application of this technique to sections of granular sludge provides additional information on the spatial orientation of acetogens and methanogens in syntrophic consortia and identifies syntrophic propionate-oxidizing bacteria.

The hybridizations described have shown that sucrose-fed sludge consists of three layers: an exterior layer composed mainly of bacteria; a second layer in which syntrophic microcolonies are mixed with microcolonies of *Methanosaeta* sp.; and a third layer which is the central part, with large cavities, anorganic material, and some methanogenic microcolonies. The VFA-fed sludge had one extra layer under the outer layer. This thick layer, which consisted of large quantities of *Methanosaeta* sp., changed gradually in the syntrophic layer. Similar layered structures were proposed previously for anaerobic aggregates from anaerobic digesters (7, 12, 14, 15, 30).

Hybridization with probe EUB338 was used to visualize all bacteria. However, it cannot be ruled out that gram-positive bacteria, such as the syntrophic butyrate-oxidizing bacterium *Syntrophospora bryantii*, and other bacteria with a rigid cell wall are not detected. The fixation methods used here might not be good enough to permeate all cell wall types for the probes (23). However, our methods are sufficiently adequate to detect members of the *Proteobacteria* and methanogens.

The isolation and enrichment of different strains of syntrophic propionate-oxidizing bacteria have been described previously (3, 5, 28, 33). However, there are several reasons to assume that the bacteria hybridizing to the MPOB1 probe are indeed MPOB cells. (i) The KOPROP1-specific probe KOP1 does not hybridize with the MPOB-like cells. (ii) The morphology of the hybridizing cells is similar to that of MPOB grown in

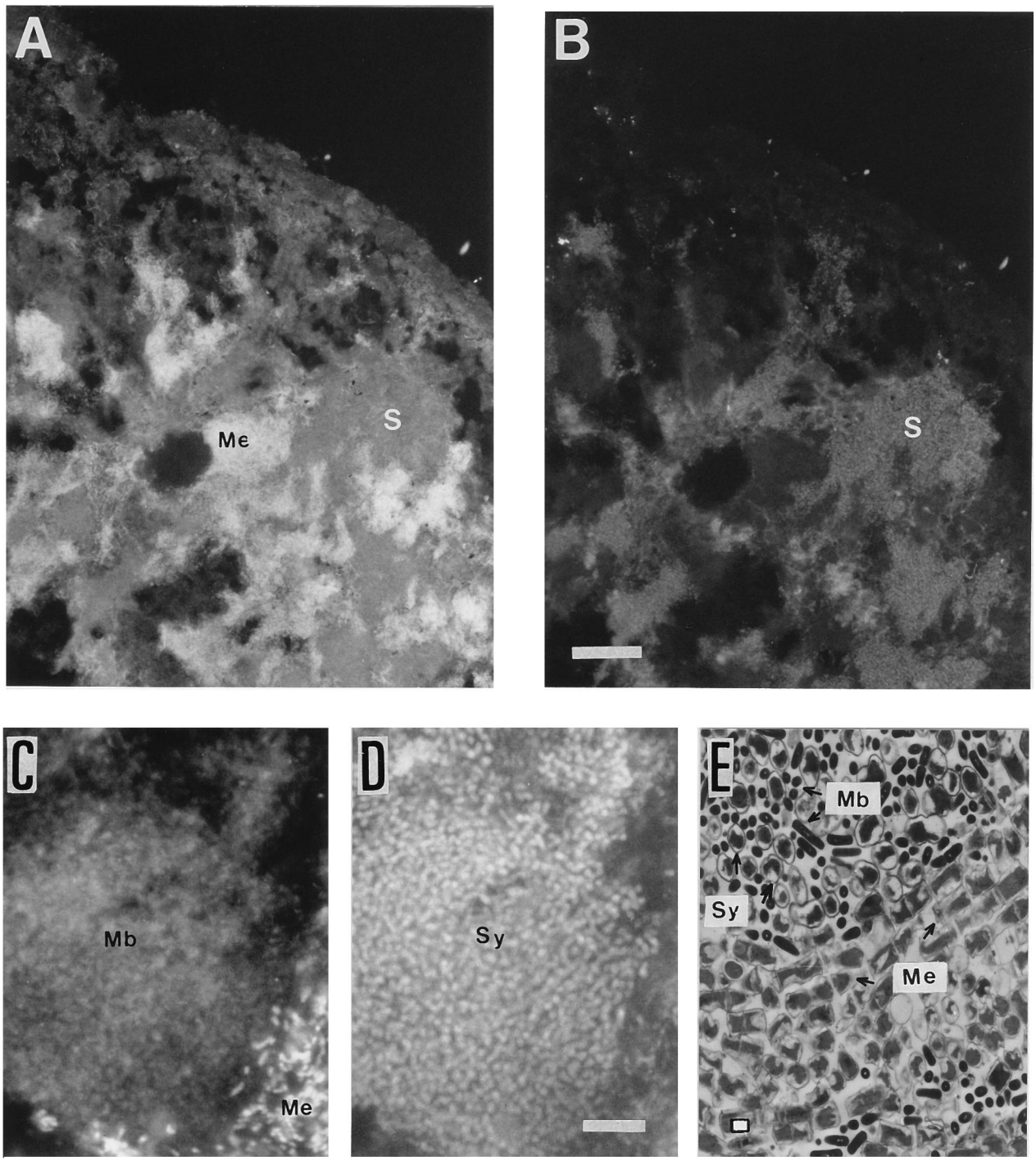

FIG. 3. In situ hybridization of a sucrose-fed granule with a fluorescein-labeled archaeal probe, ARC915 (A), and the rhodamine-labeled probe MPOB1 (B). Bar, 50 μm. A syntrophic microcolony hybridized with the ARC915 (C) and MPOB1 (D) probes is magnified by viewing with a confocal laser scanning microscope. Bar, 10 μm. A part of a syntrophic microcolony and the adjacent methanogenic microcolony is viewed by transmission electron microscopy (E). Bar, 0.5 μm. To illustrate the locations of the various relevant microbes, the presence of specific microcolonies (A and B) or cells (C to E) is indicated as follows: S, syntrophic microcolonies; Me, *Methanosaeta*-like cells or microcolonies; Mb, cells of *Methanobacteriaceae*; Sy, MPOB-like cells.

pure cultures and does not resemble the more lancet-shaped morphology of KOPROP1 cells (8). (iii) *D. amnigenus* can be detected by the MPOB1 probe but could not be detected in this sludge when a specific probe for this organism was used in dot blot hybridization experiments with rRNA extracted from sludge coming from the same reactors we used here (18). (iv) The MPOB culture has been enriched from sludge of the same industrial UASB reactor (28).

MPOB cells do not utilize butyrate or acetate under methanogenic conditions; therefore, the main substrate has to be propionate. Under these conditions, this bacterium can only oxidize propionate syntrophically. The MPOB-like cells do not

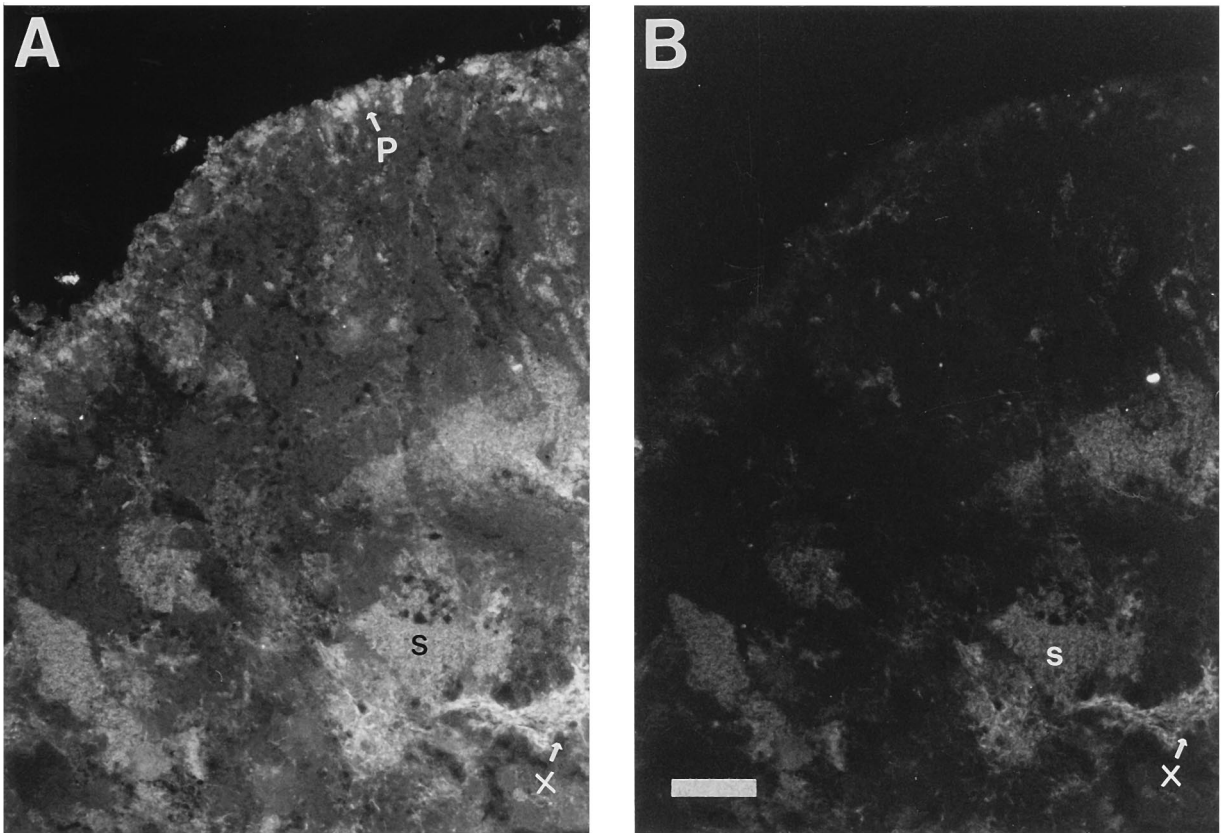

FIG. 4. In situ hybridization of a VFA-fed sludge granule with the fluorescein-labeled bacterial probe EUB338 (A) and the rhodamine-labeled probe MPOB1 (B). The EUB338 probe visualizes mainly bacterial cells in the outer layer of the granule (P, peripheric location) and the microcolonies inwards of the granule (S, syntrophic microcolony). The MPOB1 probe visualizes only the microcolonies located inwards, marked S. X, autofluorescence. Bar, 50 μm.

grow on the surface of the granule, but rather more inwards. Moreover, the MPOB-like cells were surrounded by cells of methanogens. This indicates that the MPOB-like cells are indeed growing syntrophically and depend on the methanogens to remove the reducing equivalents. There are several observations supporting the conclusion that in syntrophic microcolonies MPOB-like cells are juxtaposed to methanogens that utilize hydrogen and/or formate. (i) MPOB-like colonies were located in areas that showed a less (but significant) fluorescent signal with probe ARC915 (Fig. 3). (ii) MPOB-like cells are never found at other locations. (iii) Confocal laser scanning microscopy analysis of the data revealed that cells showing a low fluorescence with the ARC915 probe were positioned next to those fluorescing with the MPOB probe. Additional support for this architecture was obtained from the analysis of sections with transmission electron microscopy, which illustrated the positions of the individual cells (Fig. 3A).

The brightly fluorescent microcolonies surrounding the MPOB-like microcolonies reacted with the group-specific probe for *Methanosaeta* sp., which are obligately aceticlastic. This strongly suggests that the methanogens next to the MPOB-like cells are methanogens that do not utilize acetate and are likely hydrogen- and/or formate-consuming methanogens since they do not show the characteristic *Methanosarcina*-like morphology. This was confirmed by the hybridization with a fluorescent probe specific for *Methanobacteriaceae* that showed fluorescence with the methanogens in the syntrophic microcolonies. Furthermore, the methanogens seen in the syntrophic microcolonies on the transmission electron microscope micrographs resemble the *Methanobrevibacter* cells, which belong to the *Methanobacteriaceae*, as seen previously in such syntrophic associations (6).

The bacteria at the surface of the sucrose-fed granule grow on either sucrose, glucose, or fructose and produce acetate, butyrate, or propionate. They seem to grow as a loosely bound layer on, rather than in, the granule. The bacteria at the surface of the VFA-fed granule grow more in the granule and, next to the MPOB-like cells, they are the most abundant microorganisms. Therefore, it is likely that they are syntrophic butyrate-oxidizing bacteria. Butyrate oxidation also requires a low hydrogen partial pressure, and butyrate oxidation just inside the granule would permit syntrophic growth with the hydrogen- or formate-consuming methanogens. Acetate will be degraded by the acetoclastic methanogens abundantly present in the two granule types. It is feasible that acetate, after diffusing into the VFA-fed granules, is first consumed in the thick layer underneath the surface of the VFA-fed granules, mostly by the abundantly present *Methanosaeta* cells, before the propionate is degraded in the next layer. This is supported by the presence of *Methanosarcina*-like microcolonies in this layer. *Methanosarcina* spp. have a lower affinity for acetate than *Methanosaeta* spp. and will only by present if the concentrations are sufficiently high (11). This is in contrast to an earlier proposed model which suggests that propionate is consumed in the same layer as low-affinity acetate consumption takes place and is followed by high-affinity acetate consumption (7). In the sucrose-fed granules, this layer is absent because acetate is only produced inside the granule. This is an example of how the

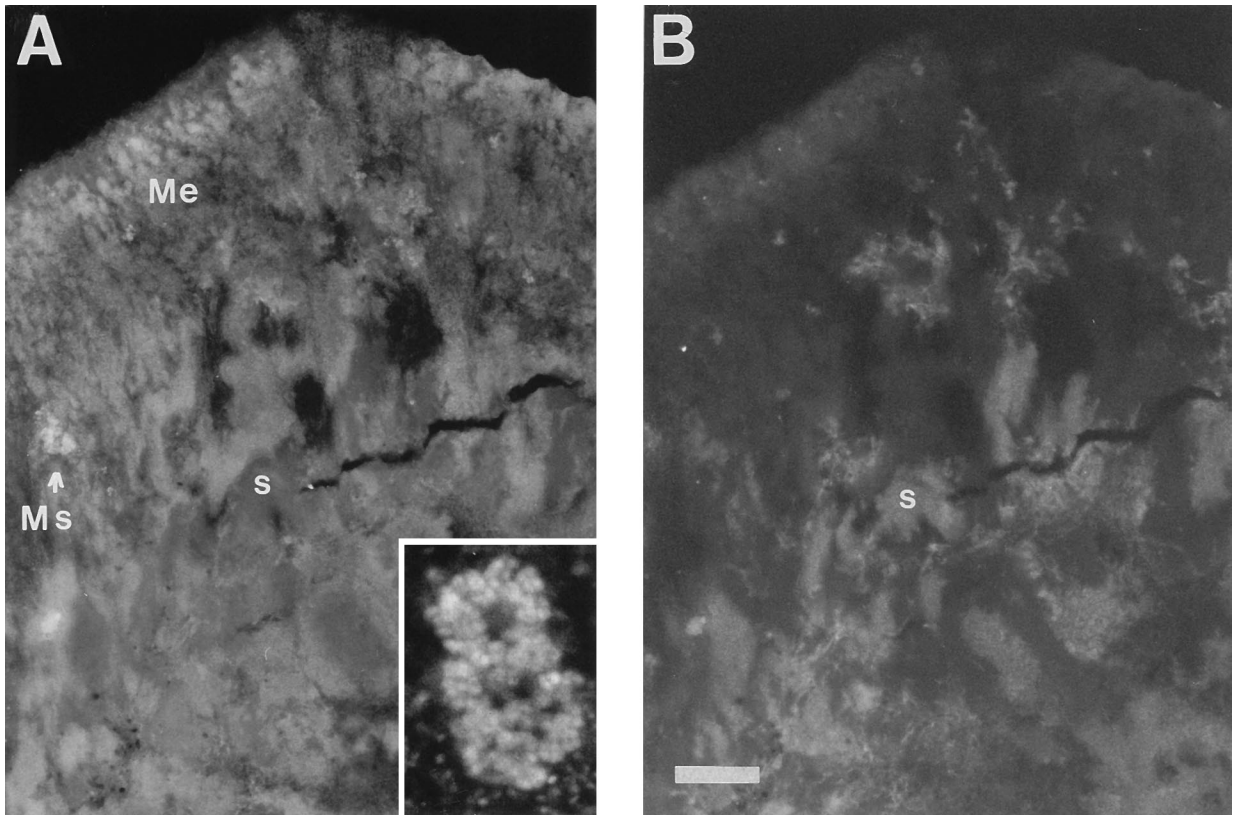

FIG. 5. In situ hybridization of a VFA-fed sludge granule with the fluorescein-labeled archaeal probe ARC915 (A) and the rhodamine-labeled probe MPOB1 (B), viewed at half the magnification as in Fig. 2 to 4. The ARC915 probe visualizes a thick layer of methanogens (Me), with some microcolonies with a *Methanosarcina*-like morphology (Ms). Furthermore, it hybridizes with a deeper layer of two types of microcolonies with bright and less fluorescence. The MPOB1 probe detects only the microcolonies on those sites which show little fluorescence with the ARC915 probe (S, syntrophic microcolonies). Bar, 100 μm. (Inset) Magnification of a *Methanosarcina*-like microcolony hybridized with the ARC915 probe viewed with a confocal laser scanning microscope. Bar, 10 μm.

architecture of the granule is changed by the substrates utilized by the granule. The structure of the two types of granules clearly relates to the function of the microorganisms in these man-made environments. The results shown in this paper indicate that hybridization of granular sludge with fluorescent probes is a powerful tool to study such structure-function relationships in situ. This approach can be further used to study the population dynamics of such ecosystems during changing nutrient and environmental conditions.

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