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Population Dynamics of Propionate-Oxidizing Bacteria under Methanogenic and Sulfidogenic Conditions in Anaerobic Granular Sludge

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Laboratory-scale upflow anaerobic sludge-bed reactors were inoculated with industrial granular sludge and fed with either propionate or propionate and sulfate. The population dynamics of the propionate-oxidizing bacteria *Desulfobulbus* sp. and the syntrophically growing strain SYN7 were studied in reactors by dot blot and in situ hybridization with 16S rRNA-based oligonucleotide probes.

Anaerobic granular sludge is the active heart of upflow anaerobic sludge-bed (UASB) reactors, in which microorganisms convert organic matter from wastewater into mainly methane and carbon dioxide (8). Degradation of the intermediate propionate is influenced by the low amounts of sulfate often present in wastewater (10). In the absence of sulfate, propionate conversion is thermodynamically possible only at a low partial hydrogen pressure and with a low formate concentration. These conditions are met in syntrophic consortia of acetogens and methanogens, where the acetogens convert propionate into acetate, carbon dioxide, and hydrogen and/or formate that are subsequently used by the methanogens (14). In the presence of sulfate, sulfate-reducing bacteria, such as *Desulfobulbus* spp., can convert propionate into acetate and hydrogen sulfide (17). However, recent studies revealed that syntrophic propionate-oxidizing bacteria such as *Syntrophobacter* spp. are themselves capable of oxidizing propionate by sulfate reduction (15, 16). It is not known if these syntrophic bacteria actually convert propionate in granular sludge in the presence of sulfate or if they are outcompeted by *Desulfobulbus* spp. Furthermore, little is known about the spatial distribution of the different propionate-oxidizing bacteria in the sludge.

Previously, we investigated the presence of syntrophic propionate-oxidizing bacteria in granular sludge originating from two different industrial UASB reactors. In one sludge type fed with beet-sugar wastewater, we identified strain MPOB as the dominant syntrophic bacterium by in situ hybridization (6). In the other sludge type fed with potato-processing wastewater, a new syntrophic strain, named SYN7, was enriched on propionate (6). 16S rDNA sequence analysis of SYN7, showed that it was not related to MPOB or other *Syntrophobacter*-like bacteria but belonged to a new species, related to the genus *Syntrophus*. Here we describe the effects of sulfate on the population dynamics and localization of propionate-oxidizing bacteria in the latter sludge.

Industrial granular sludge fed with potato-processing wastewater (Aviko, Steenderen, The Netherlands) was used to inoculate two 500-ml laboratory-scale UASB reactors (3) that

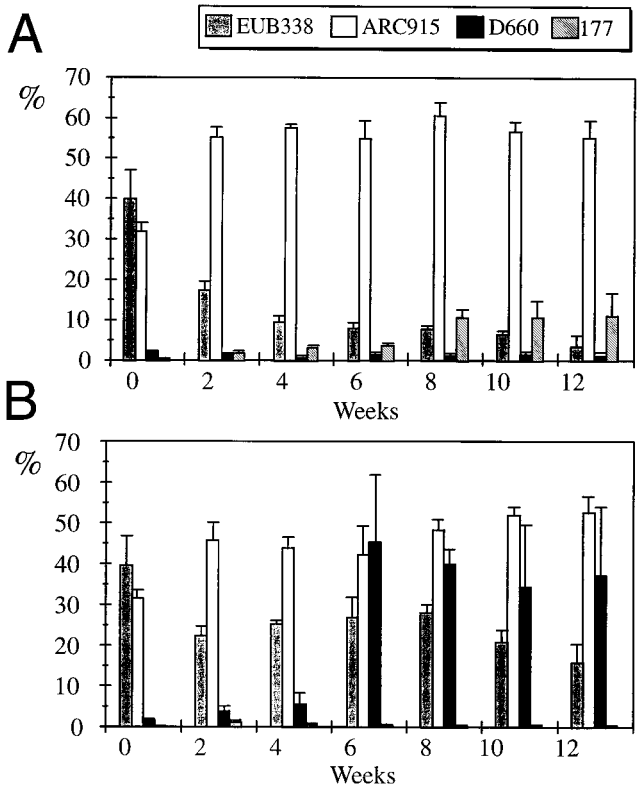

FIG. 1. Population dynamics of granular sludge fed with either propionate or propionate and sulfate. Relative amounts of the 16S rRNAs from domain *Bacteria* (probe EUB338), domain *Archaea* (probe ARC915), and propionate-oxidizing bacteria (probes D660 and 177), isolated from the granular sludge samples of the reactor fed with propionate (A) and the reactor fed with propionate and sulfate (B). The radioactive signals of the probes EUB338 and ARC915 are expressed as percentages of that obtained with the universal probe UNIV1392. The radioactive signals of the specific probes 177 and D660 are expressed as percentages of that obtained with the bacterial probe. Error bars indicate the standard deviations. The following reference organisms were used: *Desulfobulbus propionicus* (DSM 2505), *Syntrophobacter wolinii* (DSM 2805) in coculture with *Methanospirillum hungatei* (DSM 864), the syntrophic propionate-oxidizing strain MPOB (DSM 10017), and *Syntrophobacter pfennigii* (DSM 10092; kindly provided by B. Schink, Universität Konstanz, Konstanz, Germany), *Methanobacterium thermoautotrophicum* (DSM 1053), and pSYN7, a plasmid with a 1.5-kb insert of the 16S rDNA of SYN7 (4).

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TABLE 1. Summary of the oligonucleotide probes used in this study

Probe	Target group	Sequence	Reference
EUB338	*Bacteria*	5′GCTGCCTCCCGTAGGAGT	1
ARC915	*Archaea*	5′GTGCTCCCCCGCCAATTCCT	13
UNIV1392	Virtually all known organisms	5′ACGGGCGGTGTGT(G/A)C	9
177	SYN7	5′AGAAGTCATGCAGTATTATTCGG	4
S223	*Syntrophobacter wolinii*	5′ACGCAGACTCATCCCCGTG	4
MPOB1	MPOB	5′ACGCAGGCCCATCCCCGAA	5
KOP1	*Syntrophobacter pfeniggii*	5′TCAAGTCCCCAGTCTCTTCGA	5
D687	*Desulfovibrio* spp.	5′TACGGATTTCACTCCT	2
D660	*Desulfobulbus* spp.	5′GAATTCCACTTTCCCCTCTG	2
MX825	*Methanosaeta* spp.	5′TCGCACCGTGGCCGACACCTAGC	12
MG1200	*Methanomicrobiales*	5′CGGATAATTCGGGGCATGCTG	12
MB310	*Methanobacteriaceae*	5′CTTGTCTCAGGTTCCATCTCCG	12

were supplied with a basal mineral medium containing 20 mM propionate alone (methanogenic reactor) or additionally containing 15 mM sulfate (sulfidogenic reactor). These reactors were operated for 12 weeks with a liquid retention time of 6 h during which the sludge blankets retained their granular structure, had a constant volume, and utilized >95% of both propionate and the acetate formed. Methane was produced in both reactors, while the sulfidogenic reactor additionally produced hydrogen sulfide. Triplicate samples (0.25 ml) from the inoculum sludge and laboratory-scale reactors were used to isolate nucleic acids which were transferred to membranes with a slot blot apparatus and hybridized with radiolabeled oligo-

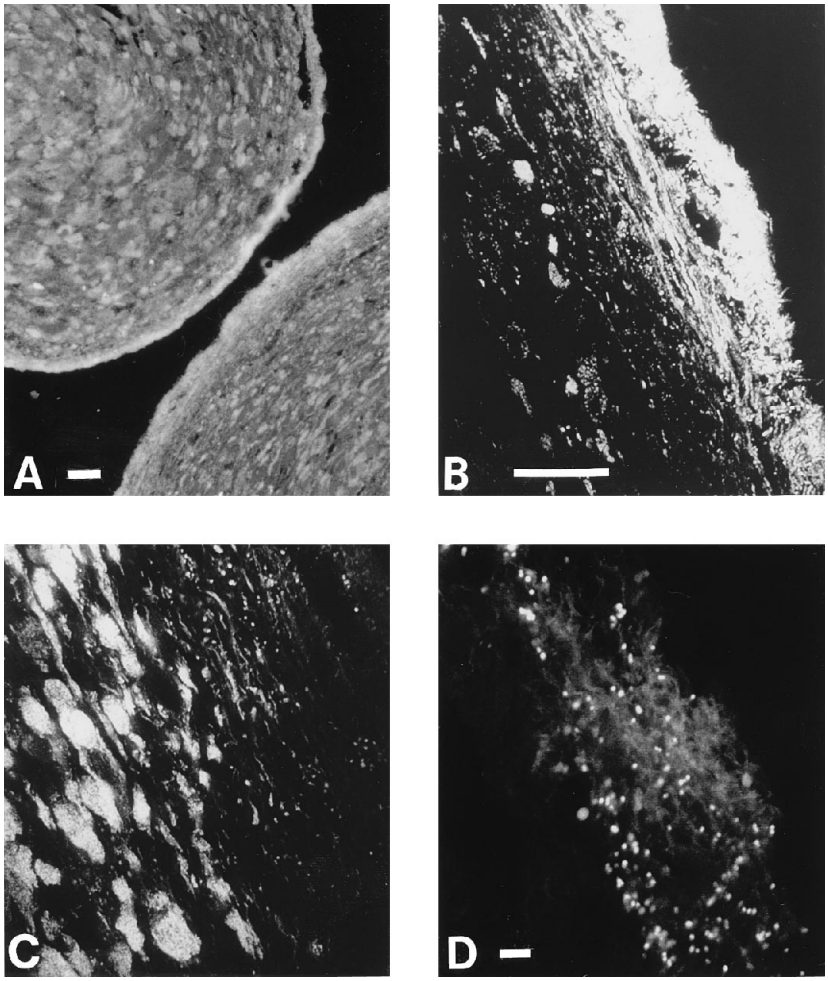

FIG. 2. Photomicrographs of in situ hybridization of sections of the industrial anaerobic granular sludge. Sections were hybridized in situ with fluorescein-labeled oligonucleotide probes universal for domain *Bacteria* (probe EUB338) (A and B), domain *Archaea* (probe ARC915) (C), and a fluorescein-labeled probe specific for the genus *Desulfobulbus* (probe D660) (D). Photomicrograph A was viewed by epifluorescence microscopy and shows a section of half the granule. Micrographs B to D were viewed with CLSM and show the outer layers of the granule. Bars, 50 μm (panels A to C) and 10 μm (panel D).

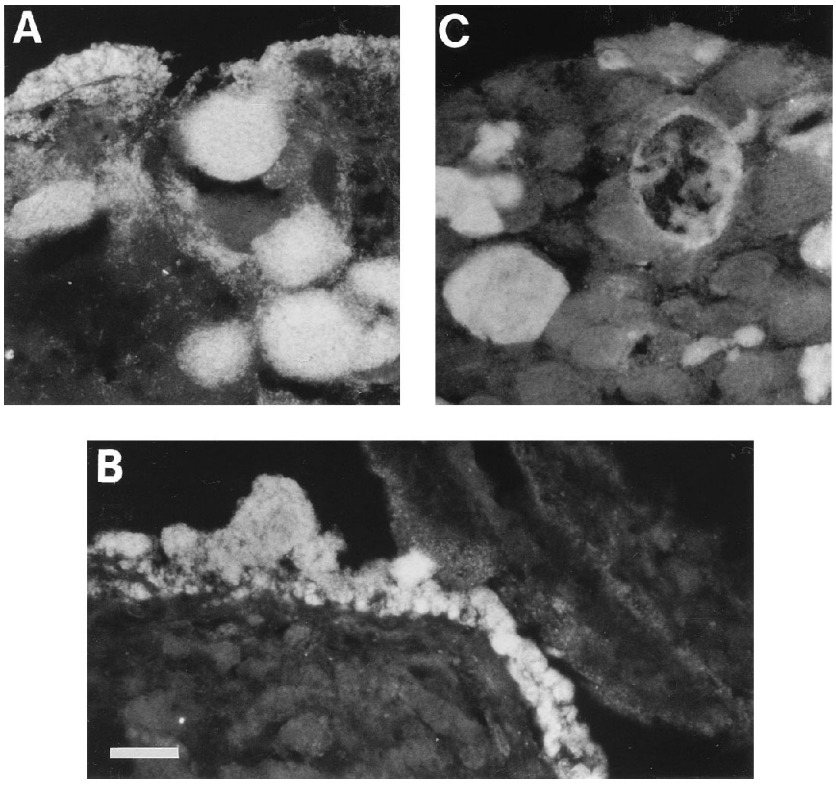

FIG. 3. Photomicrographs of in situ hybridizations of granules obtained from the sulfidogenic reactor. Sections showing the outer layers of the granules were hybridized with fluorescein-labeled probes specific for domain *Bacteria* (probe EUB338) (A), the genus *Desulfobulbus* (probe D660) (B), and domain *Archaea* (probe ARC915) (C). Bar, 50 μm.

nucleotide probes (Table 1) as described previously (5). The membranes were exposed to phosphor storage screens that were scanned with a PhosphorImager (Molecular Dynamics), and the digital signals were processed with ImageQuant software. Total 16S rRNA was quantified by hybridization of the nucleic acid samples and dilution series of the rRNAs of reference organisms, with the universal probe UNIV1392. The resulting standard curves were used to calculate the concentration of 16S rRNA for the different groups of microorganisms as described previously (11). The results of the slot blot hybridization showed marked differences in the dynamics of the microbial composition in the two reactors (Fig. 1). In the methanogenic reactor, the fraction of bacterial 16S rRNA decreased from 40% in the inoculum to 3% in the last sample, while that of the archaeal 16S rRNA, derived from the methanogens, increased slightly to about 55%. In the sulfidogenic reactor, the fraction of bacterial 16S rRNA decreased from 40 to 15%, while that of the methanogenic 16S rRNA increased from 30 to 50% in the last sample. The signals from the bacterial and archaeal (methanogen) probes added up to about 70% of that of the universal probe probably because of the relatively low specific activity of the UNIV1392 probe (set at 100%). Probes specific for the genus *Desulfobulbus* and strain SYN7 were used to quantify the rRNA fractions derived from the propionate-oxidizing bacteria relative to the bacterial rRNA (set at 100%). Probes specific for *Syntrophobacter wolinii* and related bacteria (probes MPOB1, KOP1, and S223 [Table 1]) and for the genus *Desulfovibrio* (probe D687 [Table 1]) (2) were also tested but did not hybridize to the nucleic acids isolated from the sludge. In the methanogenic reactor, the amount of 16S rRNA hybridizing with the *Desulfobulbus* probe D660 remained below 2%, while that hybridizing to the SYN7 probe 177 increased from 1 to 10%. In contrast, in the sulfidogenic reactor the amount of 16S rRNA from *Desulfobulbus* spp. increased from 2% to a maximum of 43% after 6 weeks and subsequently stayed rather constant, while the amount of SYN7 rRNA remained below 2% (Fig. 1).

In situ hybridizations of granular sludge samples (3 ml) taken from the inoculum sludge and laboratory-scale reactors 8 and 12 weeks after inoculum were performed as described previously (6) with oligonucleotides labeled at the 5′ end with the fluorescein derivative FLUOS (green) or, in the case of probe 177, at both ends with rhodamine (red) (Eurogentec, Seraing, Belgium). Hybridization of the industrial reactor sludge with the bacterial probe EUB338 showed a specific layered architecture with microcolonies ordered in concentric circles (Fig. 2A). There was a thick outer layer of bacteria with a large variety of morphologies (Fig. 2B). Hybridization of this sludge with the archaeal probe ARC915 confirmed this layered structure of the granules and showed that the outer layer was almost free of methanogens (Fig. 2C). Below the thick outer layer, there were other layers that contained two types of microcolonies. One type consisted only of methanogens, while other microcolonies contained bacteria intertwined with chains of methanogens. Hybridization of the sludge with probe 177 to detect SYN7 bacteria showed no significant signal with any of these microcolonies (results not shown). In contrast, hybridization with probe D660 identified individual cells of *Desulfobulbus* sp. growing in the thick outer layer of bacteria (Fig. 2D).

Hybridization of the sludge from the sulfidogenic reactor with the bacterial EUB338 probe showed a strong signal with an outer layer of bacteria with a characteristic morphology

FIG. 4 (top six panels). Photomicrographs of in situ hybridization of granules, obtained from the methanogenic reactor. Sections were simultaneously hybridized with the fluorescein-labeled bacterial probe EUB338 (A) and the rhodamine-labeled SYN7-specific probe 177 (B). The double exposures with both the fluorescein- and the rhodamine-specific filter sets show the hybridization of both probes (C). Sections of the methanogenic sludge were also simultaneously hybridized with the fluorescein-labeled archaeal probe ARC915 (D) and the rhodamine-labeled SYN7-specific probe 177 (E). The double exposures with both filter sets show the hybridization of both probes (F). Bars, 50 μm.

FIG. 5 (bottom two panels). In situ hybridization of a sludge granule obtained from the methanogenic reactor. Sections were simultaneously hybridized with the fluorescein-labeled archaeal probe ARC915 (green), the rhodamine-labeled bacterial probe EUB338 (red), and the rhodamine-labeled probe *Methanosaeta* sp. strain MX825 (red). The double (red and green) labeling of the *Methanosaeta* sp. results in yellow fluorescence (A). Simultaneous hybridization of the sections with the fluorescein-labeled probe MG1200 specific for *Methanomicrobiaceae* (green) and the rhodamine-labeled SYN7 probe 177 (red) identifies the microbes present in the syntrophic microcolonies (B). The CLSM micrographs include only signals from a single focus plane 0.7 μm thick, thus reducing the background. Digital signals obtained with the two specific filter sets for the fluorescent labels were merged into one figure and show details of sections containing syntrophic microcolonies. Bars, 20 μm.

(Fig. 3A). This layer was present in roughly half of the granules and covered other fluorescing bacterial microcolonies. Hybridization with probe D660 revealed that the bacteria in this outer layer belonged to the genus *Desulfobulbus* (Fig. 3B). This probe did not hybridize to other microcolonies in the granule. Hybridizations with the archaeal probe ARC915 showed that methanogens were present in large microcolonies below the surface (Fig. 3C). Probe 177, specific for the syntrophic SYN7 bacteria, did not hybridize with any of the microcolonies present in the granules (results not shown).

Hybridization of the methanogenic sludge with the EUB338 probe showed that the granules had changed and lacked the typical bacterial outer layer found in the sulfidogenic reactor (Fig. 4A). The new outer layer consisted of several bacterial microcolonies with a relatively low fluorescence signal. However, few microcolonies showed very bright fluorescence. The number of these microcolonies varied but never exceeded 10 microcolonies per section of the granule. Hybridization of these sections with both probe EUB338 (green) and probe 177 (red) revealed that the microcolonies, which showed bright fluorescence with the EUB338 probe, hybridized to the 177 probe, indicating that these are SYN7 microcolonies (Fig. 4B and C). Combined hybridization of these granules with the ARC915 probe (green) and the 177 probe (red) showed that the SYN7 microcolonies were intertwined with methanogens, illustrating their syntrophic character (Fig. 4D to F). Hybridization of the methanogenic granules with the *Desulfobulbus* probe D660 showed no significant fluorescence.

Confocal laser scanning microscopy (CLSM) with a Bio-Rad MRC-600 microscope was used to investigate in more detail the syntrophic character of microcolonies of SYN7 intertwined with methanogens in the methanogenic sludge (Fig. 5). This technique was used to view hybridization with three oligonucleotide probes, the EUB338 and ARC915 probes and probe MX825 (Table 1), which is specific for the aceticlastic genus *Methanosaeta* (12), to discriminate acetate-utilizing methanogens from those growing on hydrogen or formate (Fig. 5A). The syntrophic microcolonies showed red and green fluorescence because of the presence of acetogens and methanogens, reacting with the EUB338 (red) and ARC915 (green) probes, respectively. The *Methanosaeta* microcolonies were yellow in the merged image as a result of the red and green fluorescence and were always located outside the syntrophic microcolonies. To identify the methanogens present in the syntrophic consortia, the granules were hybridized with a combination of the probes 177 (red) and MG1200 (green) (Table 1). This latter probe is specific for the order *Methanomicrobiales* (12), including the genera *Methanogenium* and *Methanospirillum* that predominantly utilize hydrogen and/or formate. The results indicate that the large SYN7 microcolonies, which resembled *Methanospirillum* spp., were intertwined with chains of methanogens hybridizing with the MG1200 probe (Fig. 5B). This contrasts with the juxtapositioned *Methanobacteriaceae*-like methanogens found previously in syntrophic microcolonies (6). Hybridization of the methanogenic sludge sections with the probe MB310 (Table 1), specific for the family *Methanobacteriaceae* (12), showed fluorescence with some individual cells but not with cells in the syntrophic consortia (results not shown).

In conclusion, enrichment of industrial granular sludge on propionate or propionate and sulfate resulted in marked differences between the obtained granules. The most important difference was the enrichment of the newly discovered SYN7 bacteria (4) in the methanogenic reactor while in the sulfidogenic reactor *Desulfobulbus* sp. were enriched. The results clearly indicate that the *Desulfobulbus* sp. do not utilize propionate syntrophically in this sludge, in contrast to what has previously been suggested for other anaerobic sludge types (7, 18). Furthermore, it shows that SYN7, although it may be capable of growing on propionate by sulfate reduction, cannot outcompete *Desulfobulbus* spp. in the presence of sulfate.

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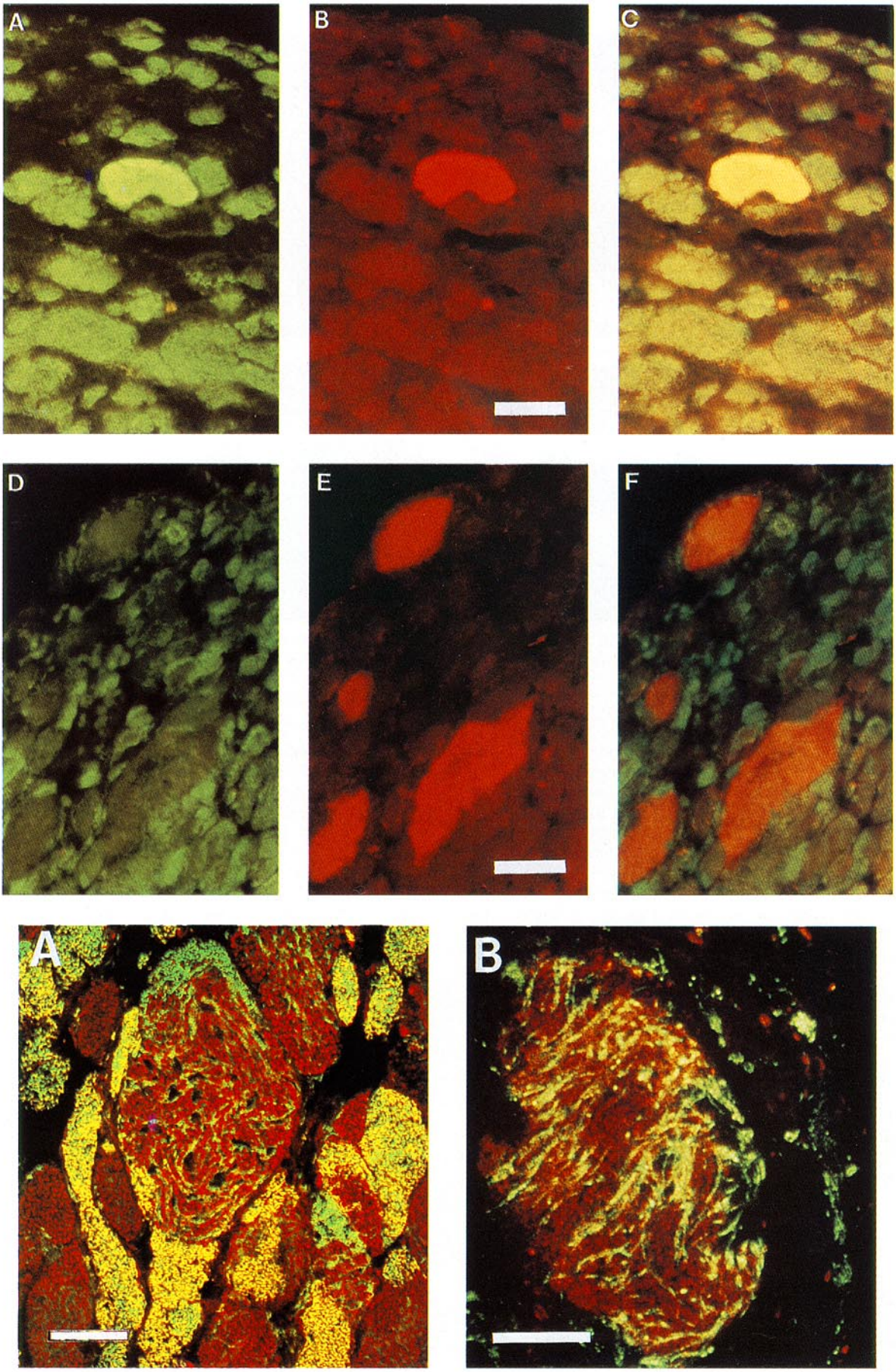
REFERENCES

1. **Amann, R. I., J. Stromley, R. Devereux, R. Key, and D. A. Stahl.** 1992. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. Appl. Environ. Microbiol. **58:**614–623.
2. **Devereux, R., M. D. Kane, J. Winfrey, and D. A. Stahl.** 1992. Genus- and group-specific hybridization probes for determinative and environmental studies of sulfate-reducing bacteria. Syst. Appl. Microbiol. **15:**601–609.
3. **Grotenhuis, J. T. C., J. C. Kissel, C. M. Plugge, A. J. M. Stams, and A. J. B. Zehnder.** 1991. Role of substrate concentration in particle size distribution of methanogenic granular sludge in UASB reactors. Water Res. **25:**21–27.
4. **Harmsen, H. J. M.** 1996. Detection, phylogeny and population dynamics of syntrophic propionate-oxidizing bacteria in anaerobic granular sludge. Ph.D. thesis. Wageningen Agricultural University, Wageningen, The Netherlands.
5. **Harmsen, H. J. M., H. M. P. Kengen, A. D. L. Akkermans, and A. J. M. Stams.** 1995. Phylogenetic analysis of two syntrophic propionate-oxidizing bacteria in enrichment cultures. Syst. Appl. Microbiol. **18:**67–73.
6. **Harmsen, H. J. M., H. M. P. Kengen, A. D. L. Akkermans, A. J. M. Stams, and W. M. de Vos.** 1996. Detection and localization of syntrophic propionate-oxidizing bacteria in granular sludge by in situ hybridization using 16S rRNA-based oligonucleotide probes. Appl. Environ. Microbiol. **62:**1656–1663.
7. **Heppner, B., G. Zellner, and H. Diekmann.** 1992. Start-up and operation of a propionate-degrading fluidized-bed reactor. Appl. Microbiol. Biotechnol. **36:**810–816.
8. **Lettinga, G.** 1995. Anaerobic digestion and wastewater treatment systems. Antonie Leeuwenhoek **67:**3–28.
9. **Olsen, G. J., D. J. Lane, S. J. Giovannoni, N. Pace, and D. A. Stahl.** 1986. Microbial ecology and evolution: a ribosomal RNA approach. Annu. Rev. Microbiol. **40:**337–365.
10. **Oude Elferink, S. J. W. H., A. Visser, L. W. Hulshoff Pol, and A. J. M. Stams.** 1994. Sulfate reduction in methanogenic bioreactors. FEMS Microbiol. Rev. **15:**119–136.
11. **Raskin, L., L. K. Poulsen, D. R. Noguera, B. E. Rittmann, and D. A. Stahl.** 1994. Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotide probe hybridization. Appl. Environ. Microbiol. **60:**1241–1248.
12. **Raskin, L., J. M. Stromley, B. E. Rittmann, and D. A. Stahl.** 1994. Group-

specific 16S rRNA hybridization probes to describe natural communities of methanogens. Appl. Environ. Microbiol. **60:**1232–1240.
13. **Stahl, D. A., and R. I. Amann.** 1991. Development and application of nucleic acid probes, p. 205–248. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Inc., New York.
14. **Stams, A. J. M.** 1994. Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie Leeuwenhoek **66:**271–294.
15. **van Kuijk, B. L. M., and A. J. M. Stams.** 1995. Sulfate reduction by a syntrophic propionate-oxidizing bacterium. Antonie Leeuwenhoek **68:**293–296.
16. **Wallrabenstein, C., E. Hauschild, and B. Schink.** 1995. *Syntrophobacter pfennigii* sp. nov., new syntrophically propionate-oxidizing anaerobe growing in pure culture with propionate and sulfate. Arch. Microbiol. **164:**346–352.
17. **Widdel, F., and N. Pfenning.** 1982. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. II. Incomplete oxidation of propionate by *Desulfobulbus propionicus* gen. nov., sp. nov. Arch. Microbiol. **131:**360–365.
18. **Wu, W.-M., R. F. Hickey, and J. G. Zeikus.** 1991. Characterization of metabolic performance of methanogenic granules treating brewery wastewater: role of sulfate-reducing bacteria. Appl. Environ. Microbiol. **57:**3438–3449.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