Competition and Coexistence of Sulfate-Reducing and Methanogenic Populations in Anaerobic Biofilms

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The microbial population structure and function of natural anaerobic communities maintained in laboratory fixed-bed biofilm reactors were tracked before and after a major perturbation, which involved the addition of sulfate to the influent of a reactor that had previously been fed only glucose (methanogenic), while sulfate was withheld from a reactor that had been fed both glucose and sulfate (sulfidogenic). The population structure, determined by using phylogenetically based oligonucleotide probes for methanogens and sulfate-reducing bacteria, was linked to the functional performance of the biofilm reactors. Before the perturbation, the methanogenic reactor contained up to 25% methanogens as well as 15% sulfate-reducing bacteria, even though sulfate was not present in the influent of this reactor. *Methanobacteriales* and *Desulfovibrio* spp. were the most abundant methanogens and sulfate-reducing bacteria, respectively. The presence of sulfate-reducing bacteria (primarily *Desulfovibrio* spp. and *Desulfobacterium* spp.) in the absence of sulfate may be explained by their ability to function as proton-reducing acetogens and/or fermenters. Sulfate reduction began immediately following the addition of sulfate consistent with the presence of significant levels of sulfate-reducing bacteria in the methanogenic reactor, and levels of sulfate-reducing bacteria increased to a new steady-state level of 30 to 40%; coincidentally, effluent acetate concentrations decreased. Notably, some sulfate-reducing bacteria (*Desulfococcus/Desulfosarcina/Desulfobotulus* group) were more competitive without sulfate. Methane production decreased immediately following the addition of sulfate; this was later followed by a decrease in the relative concentration of methanogens, which reached a new steady-state level of approximately 8%. The changeover to sulfate-free medium in the sulfidogenic reactor did not cause a rapid shift to methanogenesis. Methane production and a substantial increase in the levels of methanogens were observed only after approximately 50 days following the perturbation.

The anaerobic degradation of organic material is a complex process carried out by multiple microbial populations interacting in a food web. Although anaerobic microbial communities have been studied widely, our understanding of natural and engineered anaerobic systems is still limited. The identification and quantification of all contributing populations in such systems, as well as an in-depth description of the link between population structure and function (i.e., metabolic activity), have not yet been achieved. This is, at least partially, due to the limitations of traditional microbiological techniques, including selective enrichments, pure culture isolations, most probable number estimates, and phenotypic characterizations. Molecular biology techniques are now being developed and used to address these limitations. For example, molecular tools allow for identification and quantification at the level of populations and even single cells (for recent reviews see references 3, 38, 54). The combination of culture-based techniques, microscopy, chemical analyses, and molecular techniques should serve to better link microbial structure and function.

We have used this integrated approach to link the population structure and function of anaerobic biofilms in a long-term study of four fixed-bed biofilm reactors fed glucose or glucose and sulfate. We previously presented results from this study in which we emphasized the combined use of whole-cell hybridizations and hybridizations after RNA extraction with a collection of phylogenetically defined rRNA probes (36). We also used results from these biofilm reactor systems to illustrate the potential of combining PCR, cloning, sequencing, and whole-cell hybridizations to identify, visualize, and isolate biofilm community members (4, 25) and to determine the in situ growth rate of specific biofilm populations (34). This study presents a detailed analysis of the microbial population dynamics in the biofilm reactors before and after a major change in sulfate availability. We focus on the relationships between and among sulfate-reducing bacteria (SRB) and methanogens and discuss their competitive and cooperative interactions. Methanogens and SRB catalyze the terminal stages of the anaerobic mineralization of organic compounds and have a relatively limited substrate spectrum. They depend on other microorganisms to convert complex organics to simpler compounds, which in turn can serve as their substrates. Nevertheless, the role of methanogens and SRB is critical in the anaerobic food web, and the final steps catalyzed by these microorganisms are often rate limiting for the overall conversion process.

In the presence of nonlimiting levels of sulfate, methanogens are generally poor competitors with SRB in natural environments (e.g., marine sediments [55] and human large intestine [17]) and in engineered systems (e.g., anaerobic digesters [23]). In contrast, methanogens tend to be the dominant scavengers of hydrogen and acetate in low-sulfate environments (the contribution of homoacetogens to the process of hydrogen consumption has been resolved only for a few environments [8, 61]). SRB are believed to outcompete methanogens in the presence of nonlimiting sulfate concentrations because they compete better for common substrates (hydrogen and acetate)

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TABLE 1. Overview of influent glucose and sulfate concentrations for the four biofilm reactors

Time (days)	Influent glucose concentration, mg/liter (mM)				Influent sulfate concentration, mg/liter (mM)			
	MA	MB	SA	SB	MA	MB	SA	SB
−527 to −166	10 (0.056)		10 (0.056)		0 (0)		9.7 (0.102)	
−331 to −166		10 (0.056)		10 (0.056)		0 (0)		9.7 (0.102)
−165 to −148	20 (0.111)	20 (0.111)	20 (0.111)	20 (0.111)	0 (0)	0 (0)	19.5 (0.203)	19.5 (0.203)
−147 to −132	40 (0.222)	40 (0.222)	40 (0.222)	40 (0.222)	0 (0)	0 (0)	19.5 (0.203)	19.5 (0.203)
−131 to −64	20 (0.111)	20 (0.111)	20 (0.111)	20 (0.111)	0 (0)	0 (0)	19.5 (0.203)	19.5 (0.203)
−63 to −1	30 (0.167)	30 (0.167)	30 (0.167)	30 (0.167)	0 (0)	0 (0)	30 (0.313)	30 (0.313)
0 to 450	30 (0.167)	30 (0.167)	30 (0.167)	30 (0.167)	0 (0)	30 (0.313)	30 (0.313)	0 (0)

due to kinetic and thermodynamic advantages (55, 57). This classical paradigm of SRB and methanogen predominance in high-sulfate and low-sulfate environments, respectively, has been established through the accumulation of results from numerous studies since the mid 1970s (for reviews, see references 44, 55, 57). More recently, the coexistence of methanogens and SRB (or even the outcompetition of SRB by methanogens) has been observed in the presence of nonlimiting sulfate concentrations (6, 23, 30, 31, 33, 64). These observations have been attributed to mass transfer limitations (31), differences in microbial colonization and adhesion properties (23, 64), or variable sulfide toxicities (21, 33). In addition, recent studies have found large populations of SRB in sulfate-depleted environments (28, 48, 49, 63). These observations may be explained since some SRB can grow syntrophically with hydrogen- or formate-consuming methanogens on lactate, ethanol, propionate, fumarate, and pyruvate in defined cocultures, eliminating their need to reduce sulfate (5, 9, 18, 24, 47, 51, 63).

The established paradigm of mutual exclusion of SRB and methanogens, as well as more recent observations which indicate that interactions between SRB and methanogens may be more complex, has been derived from culture-based and/or physiologically based (e.g., measurement of metabolic activities) studies. Here, we evaluate those findings by characterizing the population structure in anaerobic biofilm reactors by using small subunit (SSU) rRNA-directed oligonucleotide hybridization probes specific for various populations of SRB and methanogens (13, 40). Concentrations of substrates, intermediates, and final products were measured to link the community function to its structure.

MATERIALS AND METHODS

Laboratory biofilm reactors. Four anaerobic, completely mixed, fixed-bed biofilm reactors consisting of glass columns (2.54 by 11 cm) filled with glass beads (3-mm diameter) were operated at 25 ± 5°C with glucose as the only added carbon source and electron donor. Two of the reactors (denoted SA and SB) were fed sulfate, while the other two reactors (denoted MA and MB) were operated without sulfate addition. The inoculum was taken from similar methanogenic and sulfidogenic reactors, which had been seeded by an inoculum from a groundwater sample concentrated by filtration (62). After continuous operation for approximately 17 months (reactors MA and SA) and 11 months (reactors MB and SB), sulfate was added to reactor MB and withheld from reactor SB. The feed solutions for reactors MA and SA were not altered. Table 1 gives an overview of the different glucose and sulfate concentrations in the feed media during various operating periods. The composition of the feed media was as follows (concentration in mg/liter): $C_6H_{12}O_6$ (10, 20, 30, or 40); NH_4Cl (174.6); KH_2PO_4 (181.5); $CaCl_2 \cdot 2H_2O$ (2.0); $MnCl_2 \cdot 2H_2O$ (0.2); $FeCl_2 \cdot 4H_2O$ (6.25 × 10^{-4}); $NaHCO_3$ (25.2); $MgCl_2 \cdot 6H_2O$ (63.4 or 0.0); $MgSO_4 \cdot 7H_2O$ (0, 25, 50, or 70); and resazurin (0.09). A stock vitamin solution (1.25 ml/liter) and stock mineral solution (0.1 ml/liter) were added to the media. The stock vitamin solution contained the following compounds (stock concentration in mg/liter): vitamin B12 (0.1); biotin and folic acid (2); riboflavin, thiamine, nicotinic acid, pantothenic acid, p-aminobenzoic acid, and thioctic acid (5); and pyridoxin-HCl (10). The stock mineral solution was composed as follows (stock concentration in mg/liter): $CuCl_2 \cdot 2H_2O$ and $Na_2SeO_3 \cdot 5H_2O$ (5); $Na_2MoO_4 \cdot 2H_2O$ (10); $Na_2WO_4 \cdot 2H_2O$ (30); $NiCl_2 \cdot 6H_2O$ (50); H_3BO_3 (60); $ZnCl_2$ (70); and $CoCl_2 \cdot 6H_2O$ (100). Dissolved oxygen was removed from autoclaved media by extensively bubbling the hot media with oxygen-free nitrogen. Subsequently, the media were kept under an oxygen-free atmosphere by maintaining a constant pressure of approximately 0.3 atm of nitrogen. In addition, $Na_2S \cdot 9H_2O$ (9.4 mg/liter, final concentration) was added, after autoclaving, to reduce the media. These media were fed to the reactors at a flow rate of approximately 1 liter/day, and the effluent was recycled at a recycle ratio of 72:1. This high recycle ratio was required to ensure a nearly completely mixed environment within the reactor, promoting uniform biofilm growth throughout the reactor.

Chemical analyses. (i) VFA. Volatile fatty acids (VFA) concentrations were measured with a gas chromatograph (GC) or a high-performance liquid chromatograph (HPLC). For the GC analyses, samples were filtered through 0.2-μm-pore-size filters, the pH of 10 ml of filtrate was raised with 150 μl of 6 N NaOH before complete evaporation at 95°C, and 2 ml of 25% H_3PO_4 was added to redissolve the VFA. A Hewlett-Packard 5730A GC, equipped with a flame ionization detector and a glass column (910 mm by 6.4 mm [outside diameter] by 4 mm [inside diameter]) packed with 60/80 Carbopack C–0.3% Carbowax 20 M–0.1% H_3PO_4, was used for the analyses. The GC was operated isothermally at 130°C (column) and 200°C (inlet and detector), and 1-μl injections were made. Standard curves were prepared for acetate, propionate, and butyrate. For the HPLC analyses, samples were filtered through 0.2-μm-pore-size filters, acidified with H_2SO_4, and analyzed as previously described (41). Standard curves were prepared for succinate, lactate, formate, acetate, propionate, and butyrate.

(ii) Total carbohydrates. Samples were filtered through 0.2-μm-pore-size filters, acidified with H_3PO_4, and, when necessary, diluted to obtain a total carbohydrate concentration of less than 20 mg/liter. Total carbohydrate concentrations were determined by using the phenol reaction described by Daniels et al. (10). To increase the detectability, long-path spectrophotometer cells (10 cm) were used.

(iii) Glucose. Samples were filtered through 0.2-μm-pore-size filters and, when necessary, diluted to obtain a glucose concentration of less than 10 mg/liter. Glucose concentrations were determined by using an enzymatic (glucose oxidase) assay (Sigma Diagnostics, procedure no. 510). To increase the detectability, the enzyme solution was prepared at 10 times the standard concentration, and 0.5 ml of a combined enzyme-color reagent solution was added to 5-ml samples.

(iv) Sulfate. While being purged with oxygen-free nitrogen to strip traces of H_2S, 20-ml samples were combined with 0.1 ml of 2 N zinc acetate and 0.05 ml of 6 N NaOH. Subsequently, the samples were centrifuged for 15 min at 1,000 × g to separate the zinc sulfide precipitate. Sulfate concentrations in the supernatant were determined with a Dionex AI-450 Model II ion chromatograph, equipped with a Dionex automated sampler (Sunnyvale, Calif.). The eluant flow rate was 2 ml/min, and a temperature compensation of 1.7 was used. The system set-up was as follows: separator column, HPIC-AS4A; guard column, HPIC-AG4A; regenerant, 25 mN H_2SO_4; regenerant flow rate, 3 ml/min; eluant, 8.5 mM $NaHCO_3$ plus 9.0 mM Na_2CO_3; sample loop volume, 50 μl; background conductivity, 15 to 20 μS; and suppressor, Anion Micro Membrane (AMMS).

(v) Methane. Methane concentrations in the effluent were determined by a modification of earlier described methods (42, 59). Samples (15 ml) were collected in a 30-ml syringe previously flushed with oxygen-free nitrogen gas, 15 ml of nitrogen gas was added to the syringe, the needle tip was inserted into a rubber stopper to provide a temporary seal, and dissolved methane was stripped into the nitrogen gas phase by vigorously shaking the syringe for 30 s. Then, the gas phase in the syringe was injected into a 9-ml serum vial, which was sealed with a neoprene serum stopper and completely filled with a saturated solution of Na_2SO_4 containing 100 mg of $HgCl_2$ per ml, while the liquid in the vial was displaced through another needle. When the vial was completely filled with the gas phase, the exit needle and syringe were removed simultaneously. Due to the low solubility of methane in water, approximately 97% of the dissolved methane should be recovered in the gas phase at equilibrium. To check if equilibrium was reached, the remaining gas phase was removed from the syringe, and the stripping procedure was repeated.

A Hewlett-Packard 5830A GC equipped with a flame ionization detector and a stainless steel column (6 ft. by $\frac{1}{8}$ in. [outer diameter]) packed with Porapak Q

TABLE 2. Oligonucleotide probes

Probe name[a]	Target group	Reference organism	Original reference	Probe name in original reference
Universal probe				
S-∗-Univ-1392-a-A-15	Virtually all organisms		32, 46	Universal
Probes for domains				
S-D-Arch-0344-a-A-20	Virtually all *Archaea*	*Methanosarcina* sp. strain WH2	40	ARC344
S-D-Arch-0915-a-A-20	Virtually all *Archaea*	*Methanosarcina* sp. strain WH2	2	Arch915
S-D-Bact-0338-a-A-18	Virtually all *Bacteria*	*Desulfobacterium vacuolatum* (DSM 3385ᵀ)	2	Eubacterial
S-D-Euca-0502-a-A-16	Virtually all *Eucarya*	*Dictyostelium discoideum*	2	Eucaryotic
Probes for methanogens				
S-F-Mcoc-1109-a-A-20	*Methanococcaceae*	*Methanococcus voltae* PS (DSM 1537ᵀ)	40	MC1109
S-F-Mbac-0310-a-A-22	*Methanobacteriaceae*	*Methanobacterium bryantii* M.o.H.G. (DSM 862)	40	MB310
S-O-Mmic-1200-a-A-21	*Methanomicrobiales*	*Methanogenium cariaci* JR1 (DSM 1497ᵀ)	40	MG1200
S-O-Msar-0860-a-A-21	*Methanosarcinales*	*Methanosarcina* sp. strain WH2	40	MSMX860
S-G-Msar-0821-a-A-24	*Methanosarcina*	*Methanosarcina* sp. strain WH2	40	MS821
S-F-Msae-0825-a-A-23	*Methanosaetaceae*[b]	*Methanosaeta concillii* FE (DSM 3013)	40	MX825
Probes for sulfate-reducing bacteria				
S-F-Dsv-0687-a-A-16[c]	*Desulfovibrionaceae*	*Desulfovibrio desulfuricans*	13	687
S-G-Dsbm-0221-a-A-20	*Desulfobacterium*	*Desulfobacterium vacuolatum* (DSM 3385ᵀ)	13	221
S-G-Dsb-0129-a-A-18	*Desulfobacter*	*Desulfobacter latus*	13	129
S-∗-Dscoc-0814-a-A-18	*Desulfococcus* group	*Desulfococcus multivorans*	13	814
S-G-Dsbb-0660-a-A-20	*Desulfobulbus*	*Desulfobulbus propionicus*	13	660

[a] Probe names have been standardized as described by Alm et al. (1).
[b] *Methanosaeta* is the only genus that has been defined so far within the family *Methanosaetaceae* (7).
[c] Probe S-F-Dsv-0687-a-A-16 was designed for the family *Desulfovibrionaceae* but also hybridizes to a few organisms in the *Pelobacter/Geobacter/Desulfuromonas* lineage. Although this may compromise our interpretation in this study to some extent, functional results (rapid onset of sulfidogenesis upon addition of sulfate to a methanogenic system) and evidence of *Desulfovibrio* strain PT2 presence in both methanogenic and sulfidogenic reactors (4, 25, 34) support that probe S-F-Dsv-0687-a-A-16 was targeting SRB (*Desulfovibrio* spp.). We are continuing to evaluate *Desulfovibrionaceae* representation in our biofilm reactors with more specific probes.

80/100 was operated isothermally at 50°C (column) and 150°C (inlet and detector), and injections of 20 μl were made.

Oligonucleotide probe synthesis and labeling. Six oligonucleotide probes specific for different methanogenic populations, five probes specific for different groups of SRB, two *Archaea*-specific probes, a *Bacteria*-specific probe, a *Eucarya*-specific probe, and a universal probe were used in this study. These probes are listed in Table 2, along with their target groups. All the oligonucleotide probes are complementary to regions of the SSU rRNA. The probes were synthesized on a DNA synthesizer (Applied Biosystems, Foster City, Calif.) at the University of Illinois Biotechnology Center Genetic Engineering Facility, purified by HPLC, and 5′-end labeled with ^{32}P by using polynucleotide kinase and 5′-[γ-^{32}P]ATP (40).

Biofilm sampling, nucleic acid extraction, slot-blot hybridization, and quantification. For each nucleic acid extraction, 20 glass beads with attached biomass (biofilm) were removed from the biofilm reactors, transferred into 2.2-ml screw-cap polypropylene vials (Sarstedt, Inc.), and stored at −20°C until extracted. The nucleic acid extraction procedure, described by Stahl et al. (46), was slightly modified. Sodium dodecyl sulfate (50 μl of a 20% solution) and 0.7 ml of phenol equilibrated with a pH 5.1 buffer (50 mM sodium acetate, 10 mM EDTA) were added to the sample vials. Nucleic acids were recovered by mechanical disruption for 3 min on a reciprocating shaker (Mini-bead beater; Biospec Products, Bartlesville, Okla.). After two additional extractions with an equal volume of buffer-equilibrated phenol-chloroform-isoamyl alcohol (100:24:1, vol/vol/vol) and one extraction with an equal volume of chloroform, total nucleic acids were precipitated with ethanol (46) or isopropanol (39), washed with ethanol, and resuspended in double-distilled (dd) H$_2$O (39). Nucleic acids of reference organisms (Table 2) were extracted as previously described by Stahl et al. (46). The amount of specific and total SSU rRNA were determined by slot-blot hybridizations with Magna Charge membranes (Micron Separation Inc., Westboro, Mass.), and bound probe signals were quantified with Storage Phosphor Screens and a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) (38–40).

RESULTS AND DISCUSSION

This study evaluates the relationship between the population structure and performance of four anaerobic, fixed-bed biofilm reactors before and after a major perturbation. The results for reactors MA, MB, SA, and SB are depicted in Fig. 1, 2, 3, and 4, respectively. For each reactor, panels A and B of each figure show changes in the population structure with time. These results are presented as abundances of populations targeted by specific probes and are expressed as a percentage of total SSU rRNA. Detailed statistical analyses were performed to evaluate the precision of the values obtained for relative population abundances (38). This is important because of the large variability in local properties of membranes used for nucleic acid immobilization. Membrane variability is the principal source of experimental error in the quantitative hybridization method employed in this and similar studies (37, 38). To estimate the variability in each experiment, RNA samples and standards (reference RNA) were applied in triplicate; the overall coefficient of variation for the relative concentration of a specific population was determined to be between 5 and 20% in most cases.

Panels A present changes in methanogen population abundances for each reactor. These results were obtained by using two order-specific and two family-specific probes (Table 2), which circumscribe most of the currently known methanogen diversity for mesophilic environments. The two family-specific probes target the families *Methanococcaceae* (S-F-Mcoc-1109-a-A-20) and *Methanobacteriaceae* (S-F-Mbac-0310-a-A-22), which comprise mesophilic methanogens, but do not target the thermophilic members of the respective orders (*Methanococcales* and *Methanobacteriales*). Therefore, these probes can be considered order-specific probes when used in mesophilic environments (41). In addition, panels A provide two measures of the total methanogenic population. The first measure was arrived at by calculating the average of the percent SSU rRNA obtained by using two general *Archaea* probes (S-D-Arch-0344-a-A-20 and S-D-Arch-0915-a-A-20). For a long time, methanogens were believed to be the only *Archaea* that were

not restricted to extreme environments (56, 60). On the basis of this assumption, all *Archaea* present in our biofilm reactors should have been methanogens, and the archaeal domain probes should have detected methanogens only. However, several recent publications suggest the presence of a wide diversity of undescribed *Archaea* in a number of environments (e.g., coastal [11] and subsurface marine waters [14, 15] and frigid marine surface waters [12]). Therefore, the presence of undescribed nonmethanogenic *Archaea* in our biofilm reactors is a possibility. The total methanogenic population was also determined by summation of the percent SSU rRNA obtained by using the four methanogenic probes that circumscribe all known mesophilic methanogens. The difference between these two independent measures of the total methanogenic population in the biofilm reactors is an indication either of the experimental error associated with this hybridization method or of the presence of unidentified (or unsequenced) species not detected by either the general or specific probes. Overall, good agreement between these two measures was observed. Panels B show results obtained by using five SRB probes, which circumscribe most known mesophilic, gram-negative sulfate-reducing species (13) (Table 2).

In addition to the results reported in panels A and B, data were collected by using domain-specific probes for *Bacteria* (S-D-Bact-0338-a-A-18) and *Eucarya* (S-D-Euca-0502-a-A-16). These data are not reported in the figures, because they varied little with time. In all four reactors, *Bacteria* were most abundant (SSU rRNA levels ranged from approximately 75 to 95%), while *Eucarya* were present in very small amounts (percent SSU rRNA was always less than 1% and often undetectable).

Panels C show the functional (chemical) changes with time for the four biofilm reactors. Sulfate concentrations in the influent and effluent, and acetate and dissolved methane concentrations in the effluent, are reported. In addition to these compounds, influent and effluent samples were analyzed for total carbohydrates or glucose, and effluent samples were analyzed for succinate, lactate, formate, propionate, and butyrate (data not reported in the figures). During the time period for which data are reported, glucose was added to the media (before autoclaving) at a concentration of 30 mg/liter (0.167 mM). Total carbohydrates and glucose analyses of influent samples resulted in concentrations of 30.2 ± 2.9 mg/liter (expressed as glucose) and 20.0 ± 7.7 mg/liter, respectively. The glucose levels were lower and more variable than anticipated. Apparently, since glucose was added before autoclaving, heating the media during autoclaving resulted in the conversion of a portion of the glucose to other carbohydrates (total carbohydrate analyses gave the anticipated result of 30 mg/liter). Effluent total carbohydrates and glucose levels were very low, always below 1 mg/liter (0.006 mM) and often below 0.5 mg/liter (0.003 mM), which was the detection limit for total carbohydrate and glucose assays. Propionate was the only VFA, besides acetate, that was consistently present at measurable concentrations in the effluent of all four biofilm reactors, but concentrations usually remained below 1 mg/liter (0.014 mM). The concentrations of other organic acids (succinate, lactate, formate, and butyrate) were usually below the detection limit of 0.5 mg/liter.

Comparing Fig. 1 with Fig. 2 and Fig. 3 with Fig. 4 shows that, before the sulfate perturbation (day −50 to day 0), the community structure and function of replicate reactors (MA and MB, SA and SB) were very similar, even though replicate reactors had been operated independently for approximately 1 year. Transient changes were observed in the control reactors for some populations following day 0. We attribute this to

perturbation resulting from more frequent sampling during this period. However, their average representation was relatively constant throughout the experimental period. The most notable transient was observed for the *Desulfococcus* group in the sulfidogenic control reactor (Fig. 3). Recent studies have shown that members of this group may be adapted to environments experiencing periodic exposure to oxygen (41a). Thus, the transient increase of this assemblage during the period of most frequent sampling, and therefore greatest opportunity for exposure of the reactor system to oxygen, is consistent with greater tolerance to, or possible use of, oxygen. Thus, differences between reactors MA and MB and between SA and SB after the first perturbation reflected the effect of the alteration of the feed media.

Effects of sulfate addition to the methanogenic reactor. Before the changeover, the replicate methanogenic reactors (MA and MB) were significantly enriched in methanogens: up to approximately 25% of the total SSU rRNA was methanogenic (Fig. 1A and 2A). The order *Methanobacteriales* accounted for the largest fraction of methanogens (average percentages were 12.7% ± 0.9%). Methanogens of the other three orders—*Methanococcales, Methanomicrobiales*, and *Methanosarcinales*—were also present but in smaller proportions.

Prior to sulfate addition, SRB also comprised a significant fraction of the methanogenic reactors. Especially SRB belonging to the *Desulfovibrio* and *Desulfobacterium* genera were present at high proportions (average percentages were 16.2% ± 0.8% and 2.8% ± 0.4%, respectively) (Fig. 1B and 2B). These SRB had a small amount of sulfate present in the feed media at their disposal (generally less than 0.02 mM) (Fig. 1C and 2C), likely due to chemical oxidation of the sulfide added to the feed media as a reducing agent. Generally, the effluent sulfate concentration was only slightly lower than the influent sulfate concentration (average influent and effluent concentrations were 0.014 mM and 0.009 mM, respectively), indicating that the influent sulfate concentration was close to the minimum threshold for the SRB in these reactors. The limited amount of sulfate reduction was insufficient to account for the high levels of SRB, if SRB were solely dependent upon sulfate reduction for their growth. A mathematical model for the bioreactors predicted that the limited amount of sulfate reduction by itself could support the growth of SRB only at levels below 1% of the total community (35).

Several SRB have been shown to grow syntrophically on lactate, ethanol, propionate, fumarate, and pyruvate (5, 9, 18, 24, 47, 51, 63), and syntrophy with hydrogen- or formate-consuming methanogens eliminates the need for SRB to reduce sulfate. SRB also can compete with fermenting bacteria for degradation products that can be fermented further (such as lactate, ethanol, fumarate, and pyruvate) in the absence of sulfate (55, 57). In addition, fructose can be fermented by some SRB in the absence of sulfate (*Desulfovibrio fructosovorans*, *Desulfotomaculum nigrificans*, and *Desulfotomaculum geothermicum* [19]). Widdel (57) describes that an observation of glucose utilization by certain SRB with autoclaved medium was not reproducible when the medium was filter sterilized, indicating that the autoclaving of glucose may produce fructose or other fermentable substrates. The autoclaving of our medium caused the glucose concentration to decrease from ~30 mg/liter to ~20 mg/liter, while total carbohydrates remained approximately constant (~30 mg/liter). Thus, the available data suggest that proton-reducing acetogenic and/or fermentation pathways were the likely mechanisms resulting in the high relative levels of *Desulfovibrio* spp., as well as the presence of other SRB, in the absence of sulfate. Neither possibility conforms to generally accepted paradigms.

FIG. 1. Microbial structural and functional dynamics for the methanogenic control reactor (reactor MA). Feed characteristics are provided in Table 1. (**A**) Methanogenic composition. The *Archaea* result was determined by averaging the results obtained with probes S-D-Arch-0344-a-A-20 and S-D-Arch-0915-a-A-20; *Methanosarcinales, Methanococcales, Methanobacteriales*, and *Methanomicrobiales* results were obtained by using probes S-O-Msar-0860-a-A-21, S-F-Mcoc-1109-a-A-20, S-F-Mbac-0310-a-A-22, and S-O-Mmic-1200-a-A-21, respectively; and the results represented by *SUM* were determined by summation of the results obtained with the four methanogen probes. (**B**) Sulfidogenic composition. *Desulfobulbus, Desulfobacter, Desulfobacterium, Desulfococcus* group (*Desulfosarcina variabilis, Desulfococcus multivorans*, and *Desulfobotulus sapovorans*), and *Desulfovibrionaceae* results were obtained by using probes S-G-Dsbb-0660-a-A-20, S-G-Dsb-0129-a-A-18, S-G-Dsbm-0221-a-A-20, S-*-Dscoc-0814-a-A-18, and S-F-Dsv-0687-a-A-16, respectively. (**C**) Effluent acetate and methane concentrations and influent and effluent sulfate concentrations.

Following the addition of sulfate to reactor MB, sulfate reduction started within 6 h (first sample point) and reached levels comparable to those in the sulfidogenic control reactor (SA) within a few days (Fig. 2C and 3C). The rapid onset of sulfate reduction may be attributed to the prior presence of relatively large *Desulfovibrio* and *Desulfobacterium* populations and a low, but significant, fraction of other SRB. Even though these SRB were not dependent upon sulfate reduction before the changeover, some or all SRB converted to using sulfate as

their electron acceptor as soon as it became available. The relative numbers of *Desulfovibrio* and *Desulfobacterium* spp. sharply increased (to maximum levels of 26.3 and 7.7%, respectively), subsequently decreased (to minimum levels of 20.5 and 3.5%, respectively), and, finally, gradually increased to levels of approximately 35 and 4.5%, respectively (Fig. 2B). The relative concentrations of other SRB groups also increased significantly soon after the changeover. However, these were only temporary increases. Levels of *Desulfobulbus*
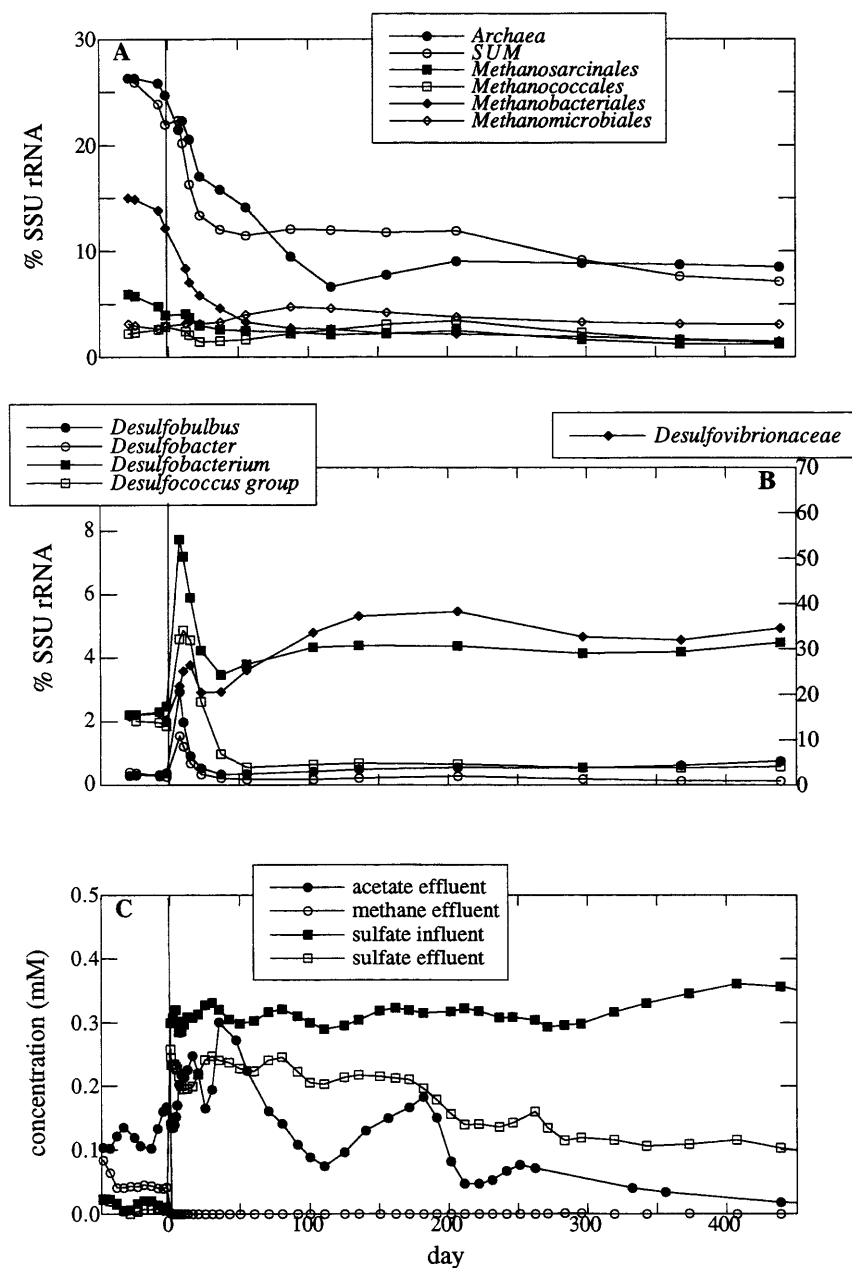
FIG. 2. Microbial structural and functional dynamics for reactor MB (sulfate was added following day 0). Detailed feed characteristics are provided in Table 1. Panels A, B, and C correspond to those discussed in the legend to Fig. 1.

and *Desulfobacter* spp. subsequently decreased to values comparable to those before the changeover (Fig. 2B). The level of the species quantified by probe S-*-Dscoc-0814-a-A-18 (*Desulfococcus, Desulfosarcina*, and *Desulfobotulus* spp.) fell below the value observed before the changeover. Thus, contrary to expectations, this latter group appeared to compete more effectively in the absence of sulfate.

Over time, sulfate reduction increased, while effluent acetate concentrations decreased, although two periods with higher effluent acetate concentrations were observed (Fig. 2C). During the same period, a similar increase in sulfate reduction and decrease in effluent acetate concentrations occurred in the sulfidogenic control reactor (SA) (Fig. 3C). It is possible that the communities were becoming more efficient in reducing

sulfate, which appeared to be linked to acetate consumption, because of a population shift. However, the levels of *Desulfobacter* spp., which are generally believed to be the most important acetate-oxidizing SRB (57), did not increase. Furthermore, no significant increase in the levels of any of the other SRB populations was observed. We note that shifts within the phylogenetic groups defined by the probes, as well as shifts of populations that were not encompassed by our probes, would not have been observed. The sulfidogenic control reactor, whose operating conditions had not been changed for almost a year and apparently had reached some sort of steady-state, became much more efficient in sulfate reduction over a time period of approximately 150 days (days 150 to 300). Even though the rate of change in sulfate-reducing efficiency was low
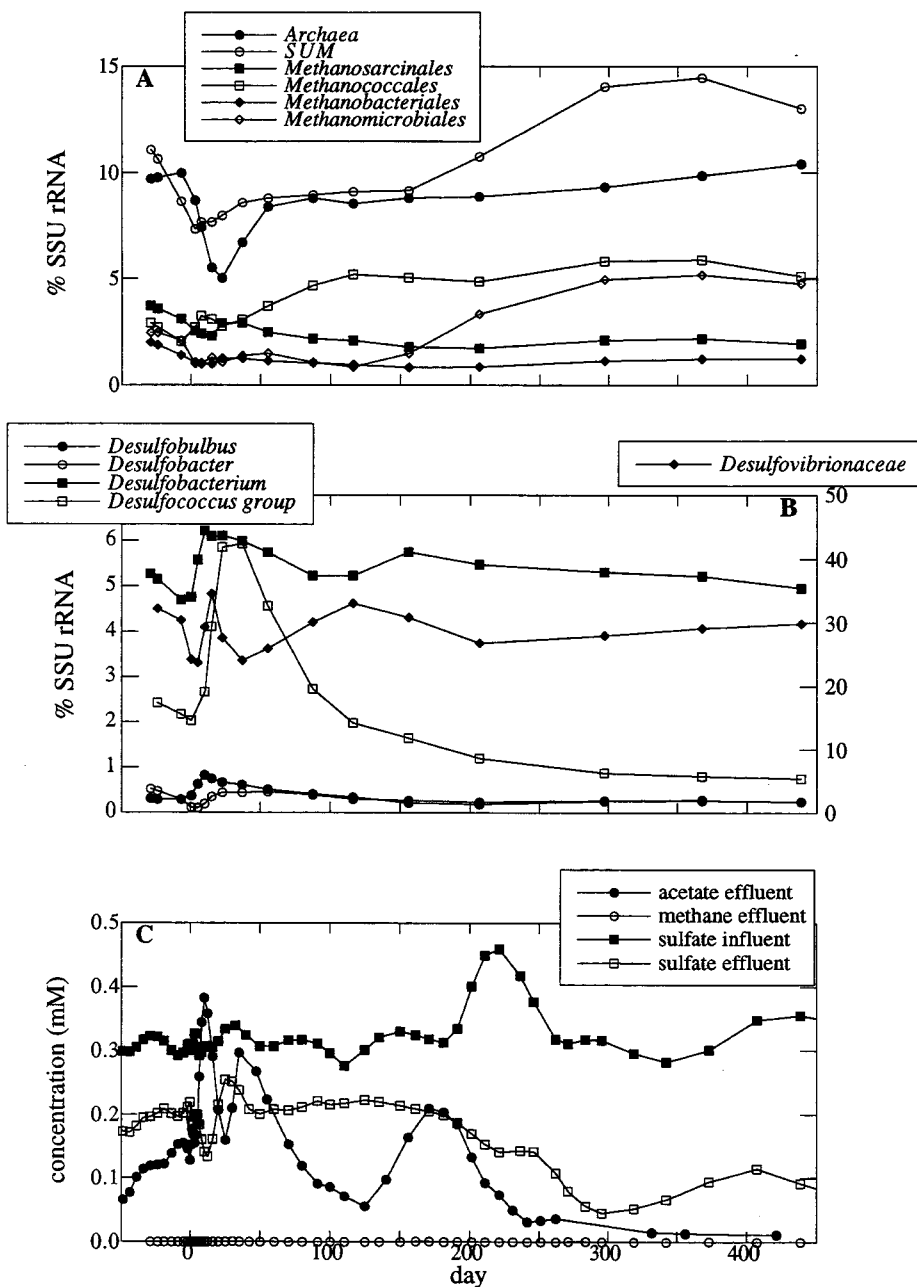
FIG. 3. Microbial structural and functional dynamics for the sulfidogenic control reactor (reactor SA). Feed characteristics are provided in Table 1. Panels A, B, and C correspond to those discussed in the legend to Fig. 1.

compared with the rate of change after the induced perturbation in reactor MB, these observations underscore the need for a careful evaluation of the concept of steady-state condition in anaerobic reactor studies.

After the addition of sulfate, methane production decreased immediately in reactor MB, and after 24 h, the methane concentration in the effluent fell below the detection limit (Fig. 2C). This rapid conversion from methanogenesis to sulfate reduction was subsequently followed by a slow but significant decrease in the levels of methanogens (Fig. 2A). The SRB, by converting from a proton-reducing acetogenic and/or fermenting metabolism in the absence of sulfate to sulfate reduction in the presence of sulfate, were able to compete immediately with

methanogens for common substrates. The fact that SRB are often better competitors (on the basis of kinetic and thermodynamic considerations) for common substrates than methanogens was reflected in the immediate decrease in dissolved methane in the effluent and the subsequent slow decrease in methanogen levels. It appears that the methanogens were able to maintain their ribosome content for a long time period despite the inactivity of their primary metabolic pathways. The level of methanogens reached a new steady-state at approximately 8% after several months of operation at sulfidogenic conditions.

The coexistence of methanogens and SRB in the presence of nonlimiting sulfate concentrations has also been reported in

the literature (6, 23, 30, 31, 64). Most of these studies were performed with attached-growth reactors or reactors with granules, i.e., systems in which factors such as mass transfer limitations and microbial colonization and adhesion are important phenomena that may affect the competition between SRB and methanogens. The fact that methanogens seem to be able to compete more effectively with SRB in attached-growth systems compared with dispersed-growth systems, in which kinetic and thermodynamic considerations are the dominant mechanisms regulating the competition, agrees with the observed coexistence of methanogens and SRB in our sulfidogenic biofilm reactors.

The decrease in the concentration of methanogens was most obvious for the *Methanobacteriales* but also was significant for the *Methanosarcinales* (Fig. 2A). Their levels decreased from approximately 16 to 2% and from 5 to between 1 and 2%, respectively. The concentration of *Methanococcales* and *Methanomicrobiales* remained relatively constant (average levels were approximately 2.5 and 3%, respectively). These results suggest that, within the group of hydrogen- (or formate-) utilizing methanogens, *Methanobacteriales* have a competitive advantage over *Methanococcales* and *Methanomicrobiales* when sulfate reduction is not taking place. In contrast, when sulfate reduction is taking place and SRB are competing with methanogens for hydrogen (or formate), the *Methanomicrobiales* and *Methanococcales* become the most competitive methanogens. A possible cause for the different predominance patterns is a difference in hydrogen- (or formate-) utilization kinetics for the different methanogenic groups. In addition to kinetics, mass transfer limitations, as yet unrecognized metabolic properties, and differences in microbial colonization properties may be important in explaining these competition patterns.

Before the changeover, aceticlastic methanogens (*Methanosarcinales*) were present at relatively low levels in reactors MA and MB (5% or less) (Fig. 1A and 2A). Using a family-specific and a genus-specific probe, it was determined that both *Methanosaeta* and *Methanosarcina* spp. were present at approximately equal levels (data not reported). In natural anaerobic consortia, the competition between *Methanosarcina* and *Methanosaeta* spp. may be very complex, because *Methanosarcina* spp. can, in addition to acetate, also use methanol, methylamines, and hydrogen (except for *Methanosarcina acetivorans*). Several studies have indicated that these other substrates are often used preferentially by several *Methanosarcina* spp. (reference 20 and references therein). Assuming that methanol and methylamines were not present in our reactors, *Methanosarcina* spp. can produce methane only from H_2-CO_2 and acetate. Harper and Pohland (20) calculated that, at hydrogen concentrations commonly found in complex anaerobic systems, aceticlastic methanogenesis becomes the preferred pathway for *Methanosarcina* spp., suggesting that the outcome of the competition between *Methanosarcina* and *Methanosaeta* spp. may be predicted on the basis of their respective kinetic parameters for acetate.

Kinetic parameters (μ_{max}, K, μ_{max}/K, q_{max}, and minimum thresholds) for cultures of *Methanosarcina* and *Methanosaeta* spp. reported in the literature (16, 22, 26, 27, 29, 43, 45, 50, 53, 65–67) show considerable variation, although, in general, *Methanosarcina* spp. have higher μ_{max} values than *Methanosaeta* spp., and *Methanosaeta* spp. have lower K values and minimum thresholds than *Methanosarcina* spp. The μ_{max}/K ratios are fairly similar, indicating that, when acetate concentrations are low, *Methanosarcina* and *Methanosaeta* spp. could coexist (57). However, the scarce available data do not allow predictive certainty of coexistence. Because of their lower K values and minimum thresholds, *Methanosaeta* spp. are capable of using acetate at concentrations below the minimum threshold value of *Methanosarcina* spp. This gives *Methanosaeta* spp. a competitive advantage over *Methanosarcina* spp. under such conditions. When acetate concentrations are higher, *Methanosarcina* spp. outcompete *Methanosaeta* spp. because of their higher μ_{max} values. Before the changeover, effluent acetate concentrations in the replicate methanogenic reactors (MA and MB) ranged from 0.10 to 0.18 mM, which is below the minimum threshold of most *Methanosarcina* spp. This suggests that *Methanosarcina* spp. could not survive on the low concentrations of acetate present in the reactors. However, the observed coexistence of *Methanosarcina* and *Methanosaeta* spp. indicates that the minimum thresholds for the *Methanosarcina* spp. in our reactors were different from the values reported in the literature or that effluent acetate concentrations were significantly different from acetate concentrations in the biofilm, indicating that mass transfer limitations may be important in our biofilm reactors.

After the addition of sulfate to reactor MB, the concentration of *Methanosarcina* spp. decreased, while the *Methanosaeta* levels increased to approximately 90% of the *Methanosarcinales* present in the reactors (data not reported). The competitive advantage of *Methanosaeta* over *Methanosarcina* spp. following sulfate addition can be explained by the generally lower effluent acetate concentrations after the changeover.

Effects of sulfate removal from the sulfidogenic reactor. The microbial community structure and performance of the replicate sulfidogenic reactors (SA and SB) before the changeover (day −50 to day 0) were comparable to each other and to those of reactor MB several hundred days after its perturbation (sulfate addition) (days 300 to 500). The changeover to sulfate-free medium in reactor SB did not induce a rapid shift to methanogenesis. Methane was detected for the first time approximately 50 days after the perturbation. Subsequently, its effluent concentration gradually rose and finally stabilized in the range of 0.06 to 0.10 mM (Fig. 4C). Coincidently with the rise in methane concentration, an increase in methanogen abundance in the community was observed (Fig. 4A). This increase in total methanogens can be attributed to an increase in the concentration of *Methanobacteriales* (Fig. 4A), which reached a level (approximately 15 to 18%) comparable to that in the methanogenic control reactor (MA). The relative abundances of the other methanogens remained fairly constant.

Following the cessation of the addition of sulfate to the influent of reactor SB, the concentrations of *Desulfovibrio* and *Desulfobacterium* spp. decreased (Fig. 4B). Three months of operation without sulfate resulted in levels similar to those in the methanogenic control reactor (MA). The levels of other SRB were low (below 1%) and did not change significantly following the perturbation.

In general, after several hundred days of operation without sulfate, the microbial community structure and function of reactor SB had become similar to those of the methanogenic control reactor (MA). One exception to this observation is that the *Desulfosarcina/Desulfococcus/Desulfobotulus* group failed to return to its concentration of approximately 1% observed in reactor MA. However, there is some indication (Fig. 3B) that this group is also being slowly excluded from the control reactor with time, again suggesting that the control reactor had yet to fully achieve steady state conditions. Nevertheless, these communities exhibit remarkable resilience.

Concluding remarks. In this study we linked microbial population shifts to functional changes in anaerobic biofilm reactors. This approach enabled us to reevaluate a number of established paradigms in anaerobic microbial ecology. As anticipated, we demonstrated that SRB were more competitive
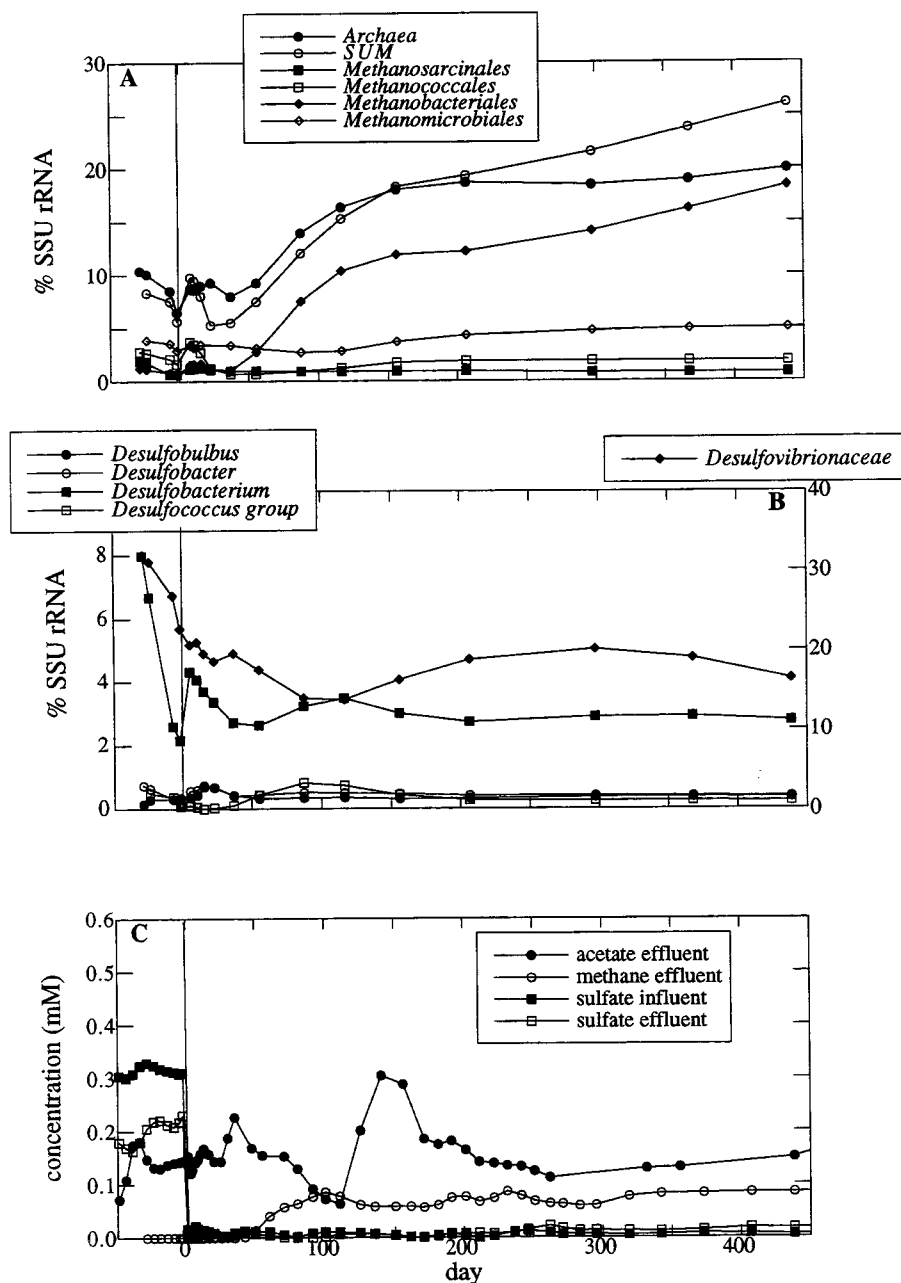
FIG. 4. Microbial structural and functional dynamics for reactor SB (sulfate was withheld following day 0). Detailed feed characteristics are provided in Table 1. Panels A, B, and C correspond to those discussed in the legend to Fig. 1.

than methanogens in high-sulfate environments. However, we also determined that the presence of SRB (especially *Desulfovibrio* spp. and *Desulfobacterium* spp.) was independent of the presence of sulfate. Moreover, we observed that some SRB (*Desulfococcus/Desulfosarcina/Desulfobotulus* group) were more competitive in environments without sulfate. Proton-reducing acetogenic and/or fermentation pathways are suggested as the most probable mechanisms allowing the high levels of these SRB in the absence of sulfate. *Methanobacteriales* were the major competitors for hydrogen in low-sulfate environments. In the presence of sulfate, *Methanomicrobiales* and *Methanococcales* became the most competitive methanogens.

Contrary to expectations, *Desulfobacter* spp. were not responsible for an increase in sulfate reduction, which was linked to increased acetate oxidation. Widdel (57) notes that *Desulfobacter* spp. grow most easily in media with high levels of sodium and magnesium chloride (corresponding to marine environments), although the physiological role of these salts for growth is not yet understood. The concentrations of these compounds in our reactors correspond to freshwater environments, which may explain the lack of an increase in the relative levels of *Desulfobacter* spp. during increased acetate oxidation in the sulfidogenic reactors (Fig. 2B and 3B) and following the addition and subsequent oxidation of acetate to reactor MB (data not reported). *Desulfobacterium, Desulfococcus*, and *De-*

sulfosarcina spp. have been shown to utilize acetate slowly in the presence of sulfate, while no *Desulfovibrio* spp. are known to use acetate as their growth substrate (57) (*Desulfovibrio baarsii*, which can also utilize acetate slowly, is not detected by probe S-F-Dsv-0687-a-A-16 and has recently been reclassified as *Desulfoarculus baarsii* [58]).

This study identified some unresolved issues related to the use of rRNA-targeted probes for studies of population dynamics. We observed that the SRB activity increased rapidly immediately after sulfate was supplied to a methanogenic reactor (Fig. 2B and unpublished results). The SRB appear to "overshoot", i.e., initially reach a higher relative activity or population level than can be maintained under steady-state conditions for the level of sulfate reduction taking place. It also appears that several populations are able to maintain a higher ribosome content for a longer time period than is necessary to match their metabolic activity level. This result was observed after the addition of sulfate to a methanogenic reactor (Fig. 2A and C). Although the dissolved methane concentration in the effluent dropped immediately, the methanogen levels decreased at a much lower rate. A similar observation has been made by Wagner et al. (52) analyzing samples from a wastewater treatment plant conducting nitrification. Oligonucleotide probes specific for ammonia oxidizers (*Nitrosomonas* spp.) were used to monitor changes in rRNA content after addition of a selective inhibitor for ammonia oxidizers. The inhibitor resulted in a complete and immediate shutdown of nitrification (measured by evaluating the changes in concentration of ammonium, nitrite, and nitrate). However, oligonucleotide probe-conferred signals did not change significantly compared with those in the uninhibited sample during the 2-h period of this experiment (no results were reported for a longer inhibition period). Wagner et al. concluded that it would be difficult to base an early-warning system for toxic inhibition of nitrification on hybridizations with rRNA-targeted probes. While it may be difficult to obtain an immediate response for decreased activity by using rRNA-based methods, our results indicate that increased activities of populations correlate well with increases in rRNA-based signals: the increase in methanogen rRNA levels after a changeover to sulfate-free medium in a sulfidogenic reactor corresponded well with an increase in effluent methane concentration (Fig. 4A and C). This observation indicates that early-warning systems that monitor increases in the levels of microorganisms that have beneficial or detrimental effects on system performance could benefit from rRNA-based methods, in particular if those microorganisms are difficult to identify or quantify by using more traditional techniques.

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ADDENDUM IN PROOF

Recent studies prompt us to add a note of qualification to these studies. This concerns improvements in probe design and hybridization conditions. Probe S-*-Univ-1392-a-A-15 has been commonly used to normalize specific probe results (this study and references 11, 17a, 31a, 39, and 41a). We recently observed that the use of this probe may result in an overestimation of relative archaeal representation in environmental

samples (65a). Consequently, the methanogen representation observed in the biofilm reactors may be slightly overestimated. However, the interpretation of our results and subsequent discussions remain true since the observed trends in methanogen population dynamics and perturbations are valid.

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