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Anaerobic Desulfonation of 4-Tolylsulfonate and 2-(4-Sulfophenyl) Butyrate by a *Clostridium* sp.

KARIN DENGER,[1] MICHAEL A. KERTESZ,[2] ESTHER H. VOCK,[2]† ROBERTO SCHÖN,[2]
ANDREAS MÄGLI,[2] AND ALASDAIR M. COOK[1,2]*

Faculty of Biology, University of Konstanz, D-78434 Konstanz, Germany,[1] and Institute of Microbiology, Swiss Federal Institute of Technology, ETH-Zentrum, CH-8092 Zürich, Switzerland[2]

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Alkyl- and arylsulfonates were tested as sole added sources of sulfur for the growth of enrichment cultures under strictly anaerobic denitrifying or fermentative conditions. Cultures that utilized taurine, ethylsulfonate, the dyestuffs orange II and acid red I, tolylsulfonate, 2-(4-sulfophenyl)butyrate (SPB), a dialkyltetralinesulfonate, and 1-(4-sulfophenyl)octane were readily obtained. We chose to work with the simple aromatic compounds and isolated a fermentative bacterium, strain EV4, which utilized SPB as the sole added source of sulfur in glucose-mineral medium. The organism was identified as a *Clostridium* sp. related to *Clostridium beijerinckii*. *Clostridium* sp. strain EV4 utilized seven of seven tested arylsulfonates quantitatively. The growth yield was about 3 kg of protein per mol of sulfur, whether sulfonate or sulfate was utilized. A major product specific to each sulfonate could be observed. Although no product was identified, the existence of anaerobic desulfonation has been established.

Sulfonated aromatic compounds are known to be biodegradable in pathways involving oxygenases (see, e.g., references 17 and 31). The idea that arylsulfonates could be degraded anaerobically has been reviewed (13, 24), but no reproducible evidence for the anaerobic cleavage of the C—S bond has been found. Direct determination of arylsulfonates in anaerobic environments supports the recalcitrance of these compounds under these conditions (14, 20). Aromatic sulfonates are, however, subject to anaerobic biotransformation, although not at the C—S bond (5, 21), so the compounds are bioavailable to anaerobic organisms, and a desulfonation reaction by methyl-coenzyme M reductase has indeed been recorded (16).

The cited work stresses degradation in the carbon cycle, as befits waste treatment. The possibility of using the other common constituent of organosulfonates, the sulfonate moiety, as a sulfur source for growth (8, 18, 27, 35) has been largely ignored (4, 7).

We now report anaerobic enrichment cultures able to utilize the sulfur moiety of several aromatic sulfonates for growth, and we describe a *Clostridium* sp. isolated to desulfonate, e.g., 2-(4-sulfophenyl)butyrate anaerobically to a defined product.

MATERIALS AND METHODS

Materials. The sulfonates studied (see Table 1) were a natural product (taurine [2-aminoethylsulfonate]), an alkylsulfonate, two dyestuffs, an optical brightener, a formulating agent in commercial surfactants (4-tolylsulfonate [TS]), a model surfactant [1-(4-sulfophenyl)octane (SPO)], a typical by-product from the synthesis of linear alkylbenzenesulfonate surfactant (1-methyl-4-butyl-6-sulfonotetraline [DATS]), and a typical intermediate in the (aerobic) degradation of these surfactants [2-(4-sulfophenyl)butyrate (SPB)] (12). The nine sulfonates were analyzed for contamination with sulfate by colorimetry (28) or for surfactants by ion chromatography (2). Two sulfonates, the optical brightener and orange II, were purified by high-pressure liquid chromatography (HPLC) because they contained >5 mol% sulfate. Other chemicals were of reagent grade or better and were obtained from Fluka, Buchs, Switzerland, or from Merck-Schuchardt, Munich, Germany. [*ring*-^{14}C-U]TS, sodium salt, was purchased from Sigma, St. Louis, Mo. The inocula for enrichment cultures were derived from (i) activated sludge from an industrial wastewater treatment plant (ARA Rhein, Schweizerhalle, Switzerland) and (ii) material from an anaerobic digestor for the treatment of communal waste (ARA Glattbrugg, Kloten, Switzerland).

Apparatus. The glove box in Zürich (Mecaplex GB 1011) was from Kleiner, Wohlen, Switzerland, and was routinely regassed with a mixture of N_2 and H_2 (92:8) prior to use. A Mecaplex glove box was available in Konstanz. The HPLC systems used were equipped with both UV and diode array detectors. The gas chromatograph-mass spectrometer (18) was described elsewhere.

Analytical methods. Aromatic sulfonates and their derivatives were determined after isocratic or gradient elution from reversed-phase HPLC columns (22, 35). Sulfate ion as an impurity in organosulfonates was determined spectrophotometrically as suspended barium sulfate (28). Sulfate ion in growth medium was determined by HPLC (26) because the carbonate-based medium interfered with ion chromatography (22). Protein in whole cells was measured by a Lowry-type method (9). ^{14}C in growth medium or in fractions of medium after separation by HPLC was quantified in a liquid scintillation counter (10). Solid-phase extraction of metabolites was carried out with 1 g of Extract-Clean C_{18} cartridges (Alltech, Lausanne, Switzerland), and compounds were eluted with methanol.

Growth media, enrichment cultures, and growth experiments. In Zürich, all growth media and their components were prepared in the anaerobic glove box under an atmosphere of N_2 and H_2 (92:8). The gas phase in culture vessels was changed, prior to incubation, to 20% CO_2 in N_2 at 2×10^5 Pa. Cultures (nominally 50 ml) in 125-ml screw-cap serum bottles with natural rubber stoppers were routinely grown in the dark at 30°C. Colonies from agar plates were picked into 5-ml cultures in 15-ml tubes with crimped natural-rubber stoppers. Agar plates were incubated in cylinders under an atmosphere of 20% CO_2 in N_2 at 2×10^5 Pa.

The sterile basal medium for enrichments under denitrifying conditions (20 mM potassium phosphate, 20 mM NH_4Cl, 5 mM $NaHCO_3$, 20 mM KNO_3 [pH 7.2]) was supplemented with presterilized solutions of glucose (to 5 mM), glycerol (to 10 mM), succinate (to 5 mM), $CaCl_2$ and $MgCl_2$ (each to 0.5 mM), vitamins (0.1%) (33), trace elements (1%) (29), and the sulfur source (to 30 μM), to which the inoculum (2 to 10% [vol/vol]) was added prior to introduction of the required gas phase.

The sterile basal medium for fermentative enrichment cultures (30 mM potassium phosphate, 20 mM NH_4Cl, 10 mM $NaHCO_3$ [pH 7.2]) was supplemented with presterilized solutions of glucose (to 6 mM), saccharose (to 6 mM), glycerol (to 6 mM), and succinate (to 6 mM), while $CaCl_2$, $MgCl_2$, vitamins, trace elements, the sulfur source, the inoculum, and the gas phase were as for enrichments under denitrifying conditions.

The cold, raw inoculum (350 ml) was centrifuged anaerobically (10,000 × *g* for 10 min at 4°C), and the supernatant fluid was discarded. The pellet was suspended in an anaerobic buffer (10 mM potassium phosphate, 25 mM NH_4Cl, 0.25 mM $MgCl_2$ [pH 7.3]), centrifuged, and then washed again. No sulfate was detected in the wash fluid. The pellet was suspended to its original volume in fresh buffer and used to inoculate enrichment cultures (5% [vol/vol]).

In Zürich, the routine medium for physiological experiments under fermentative conditions consisted of 50 mM potassium phosphate, 20 mM NH_4Cl, and

* Corresponding author. Mailing address: Fakultät für Biologie der Universität, Postfach 5560, D-78434 Konstanz, Germany. Phone: 49 7531 88 4247. Fax: 49 7531 88 29 66. Electronic mail address: Alasdair.Cook@uni-konstanz.de.

† Present address: Department of Toxicology, University of Würzburg, D-97078 Würzburg, Germany.

TABLE 1. Anaerobic enrichment cultures with limiting amounts of the named sulfonate as the sole added sulfur source

Sulfonate[a]	Success in enrichment[b]:		Disappearance of sulfonate
	Denitrification	Fermentation	
Taurine[c]	2 (2)	2 (2)	NA[d]
Ethylsulfonate[c]	1 (2)	1 (2)	NA
Orange II[c]	0 (2)	1 (2)	NA
Acid red I[c]	1 (2)	2 (2)	NA
Optical brightener[e]	0 (1)	0 (1)	
TS[c]	0 (2)	1 (2)	+
SPB[f]	0 (2)	1 (2)	+
DATS[g]	0 (1)	1 (1)	+
SPO[h]	0 (1)	1 (1)	−

[a] Trivial names not explained in the text: orange II, 2-hydroxynaphthalene-1-azo-4′-phenylsulfonate; acid red I, 5-acetamino-4-hydroxy-3-phenylazo-2,7-disulfonate; optical brightener, ionic 4,4′-bis(2-sulfostyryl)biphenyl.

[b] Enrichments were done under two conditions of energy generation, denitrification and fermentation. The number of positive enrichment cultures is indicated, as is the number of cultures set up (in parentheses).

[c] Fluka, Buchs, Switzerland.

[d] NA, no assay.

[e] Ciba-Geigy, Basel, Switzerland.

[f] Synthesis by Pius Kölbener, EMPA, St. Gallen, Switzerland.

[g] Vista, Austin, Tex.

[h] Aldrich, Steinheim, Germany.

10 mM $NaHCO_3$ (pH 7.2), supplemented with presterilized solutions of glucose (to 10 mM), $CaCl_2$ and $MgCl_2$ (each to 0.1 mM), vitamins, trace elements, and the sulfur source (as above), to which the inoculum (2% [vol/vol]) was added prior to introduction of the required gas phase. Cultures were plated on nutrient agar containing 5 mM L-cysteine and 4 μM resazurine. The plates were incubated at 30°C in the dark, and selected colonies were picked to selective minimal medium.

In Konstanz, strain EV4 was cultivated anaerobically in bicarbonate-buffered, freshwater mineral salts medium, which was prepared as described by Widdel and Pfennig (33). The mineral salts medium was buffered with 30 mM $NaHCO_3$ and supplemented with trace element solution SL 10 (32), a seven-vitamin solution (25), 2 μM resazurine, and, as the reductant, Ti(III) nitrilotriacetate (to about 0.5 mM). The sterile stock solution (pH 7.2) of the reductant contained about 100 mM Ti^{3+} chelated in about 150 mM nitrilotriacetate and about 600 mM NaCl, prepared by the method of Moench and Zeikus (23). The medium was further supplemented with 10 mM glucose as a carbon source, and the routine concentration of sulfur source was 60 μM. Cultures were incubated at 30°C under an atmosphere of N_2 plus CO_2 (90:10) in 50- or 100-ml infusion bottles sealed with butyl rubber septa.

Stock cultures were maintained at 4°C. The organism has been deposited in the Deutsche Sammlung von Mikroorganismen, Brunswick, Germany, as a *Clostridium* sp. with the accession number DSM 8245.

Growth experiments were done with 100-ml cultures inoculated (5% [vol/vol]) from a homologous preculture. Growth was assayed as the optical density at 500 nm. Samples were taken at intervals for the determination of protein and, after centrifugation, for analyses of substrates and products. The products were not sensitive to oxygen.

RESULTS

Enrichment cultures (36 cultures), each with a single added source of sulfur, were set up under two conditions of energy generation, denitrification, or fermentation (Table 1). Several carbon sources were added to each enrichment culture to widen the potential range of organisms able to grow. Two inocula were used. One was activated sludge from a plant treating industrial wastes, including sulfonates. The other was material from an anaerobic digestor. The inoculum was washed thoroughly to remove extraneous sources of sulfur; nonetheless, the first enrichment culture grew just as well in all the negative controls (no added sulfur) as in the positive controls (containing sulfate). Each culture was then used as the inoculum for homologous medium, and it became clear that very high background growth occurred in the negative control, such that little difference was visible between the negative and positive controls. The contamination with sulfur was traced in part to H_2S derived from reducing agents used in other media prepared in the glove box; the problem was reduced by replacing the gas phase in the glove box prior to use.

After four to six subcultures at 2-week intervals, some 14 enrichments were considered positive, estimated by turbidity; the rest were discarded. The positive enrichments were largely 10 fermentative cultures, mainly from the anaerobic digestor. When work on cultures containing the compounds that could not be assayed (taurine and ethylsulfonate) and on those in which disappearance was not necessarily coupled directly to desulfonation (azo dyestuffs [5]) was abandoned, four fermentative cultures remained, one of which failed to degrade its substrate (Table 1). Thus, three fermentative cultures, two of which (EV4SPB and EV3DATS) were from the anaerobic digestor and one (EV1TS) was from activated sludge, were retained.

The three mixed cultures grew overnight with 90 to 100% utilization of their respective sulfonate sulfur sources. The growth yield in each case was about 2 kg of protein per mol of sulfur, whether sulfonate or sulfate was utilized. There is thus mass balance for sulfur from sulfonate or sulfate recovered as protein presumed to contain methionine and cysteine. The protein synthesized in the controls without added sulfur represented about 10 μM sulfur, which we presumed to derive from the phosphate buffer (see Discussion).

The cultures were plated on nutrient agar, and apparently single colonies were picked to selective medium, in which growth dependent on substrate utilization was observed. When nutrient agar plates containing cysteine and resazurine were used, colonies grew on colorless agar, which confirmed that the organisms grew anaerobically. A pure culture of EV4SPB was obtained; however, we failed to obtain pure cultures of EV1TS and EV3DATS.

Strain EV4SPB was considered pure when it had undergone five cycles of microscopic purity in selective liquid medium and homogeneity on nonselective plates. The growth medium was simplified to contain solely glucose as the carbon source, and the fermentation products acetate and butyrate accounted for 60% of the glucose consumed (lactate and ethanol were also observed [15a]). The culture now required about 2 days to grow. It grew with complete utilization of SPB, and a growth yield of about 2 kg of protein per mol of sulfur was observed in phosphate-buffered medium (in Zürich), whether the sulfur was derived from sulfonate or sulfate.

Strain EV4SPB was a strictly anaerobic rod of variable length, which was very motile in the new medium used in Konstanz. The Gram reaction of young cultures was positive. The organism occurred singly, in pairs, and occasionally in chains and contained unidentified subcellular (usually polar) granules. Sporulation was observed at high frequency in Konstanz, and the subterminal spore caused a swelling of the vegetative cell. Vegetative cells were killed by standard heat treatment (80°C for 10 min), and spore suspensions were allowed to germinate and grow in selective medium. The culture morphology was unchanged, as was the ability to utilize SPB. The culture was tentatively attributed to the saccharolytic clostridia, and this conclusion has been supported by the German Collection of Microorganisms, where *Clostridium* sp. strain EV4 was given the accession number DSM 8245. The German Collection of Microorganisms used physiological and molecular methods (15b) to determine that strain EV4 had a close but nonidentical relationship to *C. beijerinckii* DSM 791^T and the atypical (34) *C. acetobutylicum* P262. *C. beijerinckii* and strain P262 both required complex additives, e.g., yeast extract, in the medium for growth; this supplement to the growth medium

provides so much sulfur that any desulfonation by these organisms is masked.

Clostridium sp. strain EV4 has a wide substrate range for aromatic sulfonates. In addition to SPB, the following six aromatic compounds were tested as sole sources of sulfur: phenylsulfonate, TS, SPO, DATS, *p*-sulfobenzoate, naphthalene-2,6-disulfonate, and acid red I (which was also decolorized). Each compound was completely utilized, as judged by HPLC, with growth yields in carbonate-buffered medium of 3.1 to 3.3 kg of protein per mol of substrate, the value also observed with sulfate. We presume that the disulfonates were subjected to only a single desulfonation. The new medium contained about 2 μM contaminative sulfur (estimated from growth yields), which meant that the negative controls were essentially nonturbid.

Strain EV4 could not utilize ethylsulfonate but grew with the sulfate ester 4-nitrocatecholsulfate. We presume that the organism expresses an arylsulfatase in response to sulfate starvation.

Strain EV4 experienced a lag phase and then grew exponentially with sulfate as the sole added source of sulfur for growth (Fig. 1a); sulfate utilization was quantitative (Fig. 1a, inset). The inset shows that about half the growth occurred in carbonate-buffered medium after apparent exhaustion of the supplied external sulfur source to give the same overall growth yield (about 3 kg of protein per mol of S) determined from end point assays (see above). Growth with TS was also exponential after a lag phase (Fig. 1b), and in this case, growth was concomitant with substrate utilization (Fig. 1b, inset) and with formation of a major product of much lower polarity than the substrate. The product was not detected in the corresponding control cultures utilizing sulfate or when other aromatic sulfonates were utilized; there was a major product specific to each substrate.

We attempted to identify the major product (see below) from growth with TS or SPB. The product was separated by HPLC on a semipreparative scale and analyzed by gas chromatography-mass spectrometry. No signal was obtained. We treated the separated material with diazomethane, but that sample and whole spent growth medium with and without methylation gave only compounds found in controls. Individual products were analyzed by liquid chromatography-mass spectrometry, but no signals were obtained, and we presume that the compounds were lost at the interface to the mass spectrometer or were not ionized.

To examine the stability of the product(s) further, we carried out an experiment with [^{14}C]TS as the source of sulfur (30 μM). Cell-free, spent growth medium, which contained effectively 100% of the added radioactivity, was chromatographed, and the location of the radioactivity in the eluted fractions was determined. Three major peaks of radioactivity were observed (75% of the radioactivity), one of which corresponded to the material we had previously examined. These three compounds were concentrated by solid-phase extraction and separated by HPLC. Although they were stable in solution, they proved to be extremely labile during isolation, even at 4°C, and rechromatography of the isolated products showed that each of them had decomposed to a mixture of compounds with increased retention times.

The pattern seen in Fig. 1b with TS is also representative of growth with SPB, for which one major and perhaps two minor products were observed which were also unstable in our hands during isolation. Growth with *p*-sulfobenzoate was also exponential, but the product was not stable in solution.

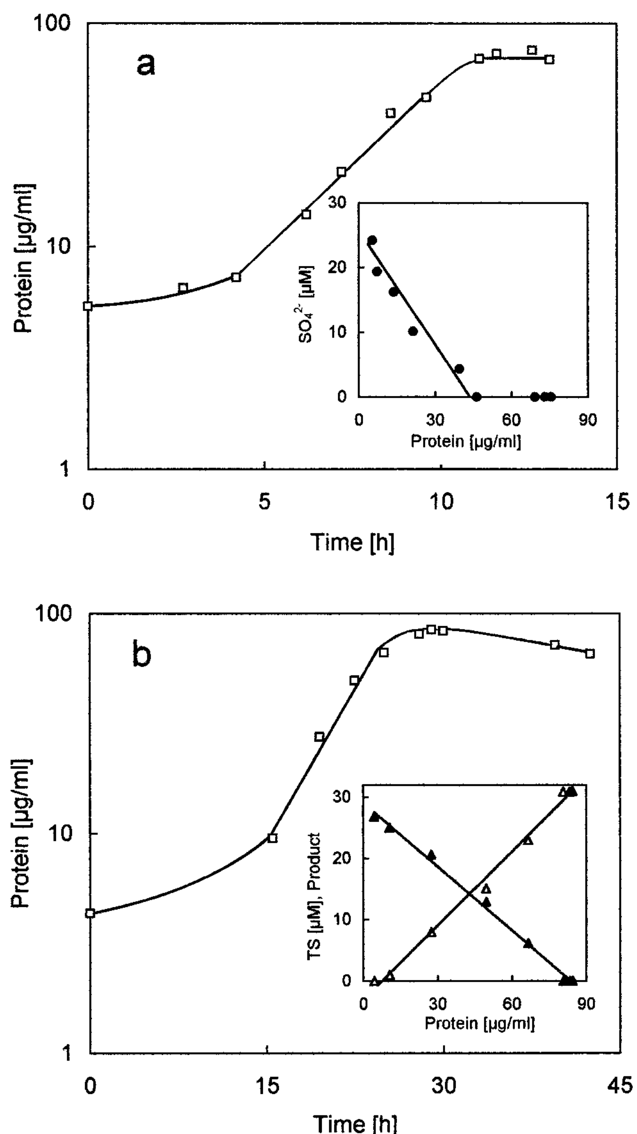

FIG. 1. Growth of *Clostridium* sp. strain EV4 with sulfate (a) or TS (b) as the sole added source of sulfur. The inset in each panel represents (sulfur) substrate concentration (and product formation, if appropriate) as a function of growth and illustrates two different patterns of assimilation of sulfur, as discussed in the text. These representative data derive from cultures inoculated from homologous medium. Symbols: □, protein; ●, sulfate; ▲, TS; △, main product from TS, plotted in arbitrary units.

DISCUSSION

Earlier claims of anaerobic desulfonation have been discounted because oxygen was obviously present in the systems studied (24). We used the strong, sulfur-free reductant Ti(III) nitrilotriacetate, coupled with the use of the indicator resazurine, to obtain highly reduced conditions throughout growth of *Clostridium* sp. strain EV4 with an arylsulfonate as the sole added source of sulfur. The arylsulfonate was stable in sterile, reduced medium. In the absence of added reductant, nonreduced conditions (indicator pink) at the start of incubation were occasionally observed; in these cases, strain EV4 generated reducing conditions in the medium (indicator colorless) before desulfonation occurred (9a). We are dealing with a strictly anaerobic reaction.

There are four lines of evidence that the aromatic $C—SO_3^-$ bond in at least two compounds can be cleaved anaerobically. Firstly, growth of a strictly anaerobic bacterium was observed (Table 1). Second, this growth was quantified and the molar growth yield (about 3 kg of protein per mol of S) indicated a mass balance for sulfur (6). Third, there was substrate utilization (Fig. 1). Finally, there was formation of one or more products (Fig. 1b, inset), which have not been identified. For five other substrates, we have observed complete substrate utilization, mass balance, but sometimes unstable products.

This is thus the first proof of the anaerobic desulfonation of an aromatic compound (7); our data thus tend to validate a preliminary report (4) in which anaerobiosis was not confirmed, substrate utilization and product formation were not determined, and different growth yields with sulfate and sulfonate were noted. It was previously believed that desulfonation of aromatic compounds always involves an oxygenation step (see Introduction). Desulfonation thus joins the other reactions which were once claimed to have an absolute requirement for oxygenation but for which nature has developed an alternative degradative strategy (11, 13).

Although only one defined arylsulfonate has been shown to occur naturally (3), we should perhaps not be too surprised that arylsulfonates are degraded anaerobically. Humus is a naturally occurring group of sulfonated aromatic compounds (30), and so the natural transformation of humus must involve desulfonation; it is logical to imagine a part of this process occurring anaerobically.

Whereas we have proven desulfonation for seven compounds, we have preliminary evidence (growth) for the desulfonation of taurine, ethylsulfonate, and another dyestuff. We thus suspect that a wider range of desulfonation exists than we have confirmed here. This idea stems in part from the observation that a wealth of anaerobic organisms could not grow under our conditions. Further, our strict selection in the enrichment cultures for extensive growth above a high background might have led us to eliminate slowly growing cultures. Another weakness was the use of a poorly buffered medium in the enrichments, so that organisms sensitive to changes in pH could have been lost. In addition to possible losses of organisms, the method is open to false-positive results (Table 1, SPO); therefore, strict attention to analytical chemical data is essential.

The choice of medium is also important for several reasons. The molar growth yield in Konstanz (about 3 kg of protein per mol of S) is the same as that under aerobic conditions (3 to 6 kg of protein per mol of S [6]) but much higher than that observed in Zürich (2 kg/mol). This is only one aspect of several (sporulation rate, motility) which indicated that the organism grew better in the new medium, which also allowed a higher substrate concentration to be added (60 μM) than could be used in Zürich (30 μM). The move to a carbonate buffer also reduced by 80% the level of sulfur contamination in the basal medium, so that negative controls are now essentially nonturbid. Most of the contamination in the salts medium was attributed to the phosphate buffer; in aerobic work, we changed the buffer to Tris for the same reason (19).

The substrate range of *Clostridium* sp. strain EV4 (seven substrates utilized of seven tested) is already wider than the ability of most organisms isolated (aerobically) to utilize sulfonoaromatics as carbon sources (29; cf. reference 15) and tends to approach the wide substrate ranges (e.g., 22 of 27 compounds tested) observed under aerobic sulfur-limited conditions (18, 35). We (1, 19) have evidence for broad-spectrum enzymes whose expression is regulated as part of a bacterial sulfate starvation-induced stimulon for the assimilation and distribution in the cell of sulfur from a wide variety of sulfur sources. Beil et al. (2) have examined the growth of *Pseudomonas putida* S-313 under conditions of limiting sulfate and interpreted the two aspects of exponential growth (cf. inset in Fig. 1a) to represent assimilation of sulfate and subsequent redistribution of organosulfur in the cell. We consider the data in the insert in Fig. 1a to be preliminary evidence for the occurrence of the sulfate starvation-induced stimulon in anaerobes, and we are continuing our efforts to confirm this hypothesis and to identify the products of desulfonation so as to establish the nature of the desulfonative reaction.

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