GROWTH PATTERNS AND HYDROGEN SULFIDE PRODUCTION BY MIXED CULTURES IN A CELLULOSE-BASED MICROCOSM

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Studies of the trophic relationships in microbial reduction of sulfate established the need for three physiological types of organisms: cellulose-digesters (*Cellulomonas*, *Cytophaga* or *Micromonospora*), a lactic acid fermenter (*Enterobacter*), and a sulfate reducer (*Desulfovibrio*). Microcosms based on cellulose mineral salts medium were inoculated with varying combinations of mixed cultures and incubated at 30°C for 18 days. The extent of sulfate reduction varied in the microcosms. A 5-member culture which contained the three cellulolytic organisms reduced the most sulfate (52%). *Cellulomonas* was the most efficient cellulolytic partner. Unless all three physiological types were present, sulfate was not reduced.

In nature, the formation of sulfides through sulfate reduction is enhanced by low redox potential (*Eh*), availability of organic matter and a suitable temperature (*I*). When sulfate is reduced, the hydrogen sulfide (H₂S) evolved may combine with ferrous salts to form black sulfide deposits which appear in the profiles of boggy soils and aquatic muds. Sulfate-reducing bacteria may use organic substrates such as lactate (2), choline and acetate (3, 4) as electron donors for sulfate reduction. In some strains, a reductive pathway through hydrogen oxidation has been observed (5, 6). Species of *Desulfovibrio* have limited substrate range so trophic interactions with other organisms is necessary to release nutrients from decomposing organic matter and to produce a reducing microenvironment for sulfate reduction. The degradation of plant detritus is through the activities of cellulolytic organisms. It is therefore clear that the generation and maintenance of various nutrient cycles in nature necessitate complex syntrophic relationships among microbial species. TEZUKA et al. (7) in an ecological study of the Sumida River

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Valley, noted that a carbohydrate-like substrate provided nutrients for bacterial sulfate reduction. TUTTLE et al. (8) had similarly observed the roles of specialized microbial groups in the successive biotransformation pathways leading to sulfate reduction in acid mine water. In a subsequent study, TUTTLE et al. (8) used laboratory models to evaluate trophic relationships in an acid mine ecosystem, with an undefined mixture of organisms as inoculum. To our knowledge, no studies on trophic interactions have used standardized microcosms with defined inoculum to evaluate the niche and response of each biotic component in a simulated ecosystem. This report defines the minimal number, the generic and physiological identity, and the responses of organisms that were required for necessary substrate transformations, starting with cellulose as the only organic substrate and culminating in the production of H_2S following sulfate reduction. Such relationships may be envisaged for natural ecosystems in which a mixture of specialized microbial groups create suitable microenvironments and release metabolites used as electron donors in the sulfate reduction pathway.

MATERIALS AND METHODS

Organisms. Three physiological types of organisms were used: cellulose digesters (*Cellulomonas* sp., *Micromonospora* sp. and *Cytophaga* sp.; designated C, M, and S respectively), a lactic acid fermenter (*Enterobacter cloacae*, E), and a sulfate-reducer (*Desulfovibrio desulfuricans*, D). Cellulose-digesters were isolated from soil or aquatic muds, the lactic acid fermenter from aquatic habitats and the sulfate-reducer from aquatic mud.

Media for cellulolytic organisms. Except for *Cytophaga* sp. which was isolated according to WARKE and DHALA (9), other cellulose digesters were isolated after enrichment in the filter paper mineral broth (FMB) medium of DUBOS (10), which was also used for their maintenance. A modification of the mineral medium (10), designated CMB and used for preparing microcosms contained (% w/v): 0.025, $(NH_4)_2SO_4$; 0.05, K_2HPO_4 ; 0.025, $MgSO_4 \cdot 7H_2O$; 0.005, $CaCl_2 \cdot 5H_2O$; 0.01, $FeSO_4 \cdot 7H_2O$; 1.0, Microcrystalline Cellulose (Sigma Cell Type 20, Sigma Chemical Co., St. Louis, Mo.), pH 7.2. Cellulolytic colonies were purified on CMA streak plates consisting of CMB medium and agar (1%). Viable counts were on CMA pour plates prepared with 3 ml of inoculated CMA soft agar (0.7%) overlaid on CMA base.

Media for lactic acid fermenter. *E. cloacae* was purified on cellobiose mineral agar (CbMA) prepared from mineral medium (10) and supplemented with yeast extract (1%), D(+)-cellobiose (1%) (Sigma Chemical Co., St. Louis, Mo.) and solidified with agar (1%). Viable counts of E were done on sucrose bromothymol-blue agar (SBA) spread plates consisting of mineral medium (10), supplemented with sucrose (1%), bromothymol blue (0.003%), yeast extract (1%) and agr (1%).

Media for sulfate-reducer. The two media used, MBC and MBA, were modifications of medium C of BUTLIN et al. (11). MBC used for the isolation and

maintenance of D contained (% w/v): 0.05, K_2HPO_4 ; 0.1, NH_4Cl ; 0.1, $CaCl_2 \cdot 2H_2O$; 0.2, $MgSO_4 \cdot 7H_2O$; 0.1, Na_2SO_4 ; 0.35, lithium lactate; 0.1, yeast extract; 0.02, ascorbic acid; 0.001, $FeSO_4 \cdot 7H_2O$ (pH 7.6). Cultures were maintained at 4°C with weekly sub-cultures. The viable count of D was by the three-tube Most Probable Number (MPN) method (12), using MBA deep tubes prepared from MBC medium solidified with agar (1%).

Microcosms. The microcosms consisted of 7 ml of CMB in screw cap tubes (150 × 16 mm). For inoculation, cultures grown individually in maintenance broths were diluted in sterile distilled water and pooled to prepare a mixed inoculum. Microcosms were inoculated with 0.1 ml of composite inoculum to give the following final concentrations; Strain C, 5.2×10^4 /ml or 1.3×10^5 /ml; S, 4.1×10^3 /ml; M, 1.6×10^3 /ml; E, 6.4×10^3 /ml and D at 6.4×10^2 /ml or 5.3×10^3 /ml. Variations of mixed inocula were used in different microcosms and designated as TXB (consisting of C, S, M, E, and D); CXB (C, E, and D), SXB (S, E, and D), MXB (M, E, and D). Control microcosms were solitary cultures and various combinations of 2-member cultures. Lead acetate-impregnated filter paper strips suspended in microcosm tubes were retained with loosened screw caps. All incubations were stationary in air at 30°C for 18 days. Analyses of replicate tubes were done at 3-day intervals thereafter.

Analyses. Viable organisms (C, S, M, E, and D) from microcosms were counted with the media as already described. Serial dilutions of D, were prepared with 0.1 M phosphate buffer (pH 7.3) containing 0.02% ascorbic acid. Lactic acid produced by E in stationary CbMB was determined according to BARKER and SUMMERSON (13).

Soluble carbohydrate was determined by the phenol-sulfuric acid method (14) after filtration (0.45 μ m filter, Millipore Corp., Bedford, Mass.) of mixed replicate microcosm tubes. Products of cellulose hydrolysis by C, S, and M in CMB were identified by thin layer chromatography (Chromatogram Type 6061, Eastman Kodak Co., Rochester, N.Y.) in isopropanol:butanol:water (140:40:20) solvent. A methanol:chloroform (1:1) extract of the culture filtrate (10 μ l) was spotted. Plates dipped in aniline (1%), diphenylamine (1%), acetone:85% phosphoric acid (10:1) were heated at 100°C for 10 min. D(+)-Glucose and D(+)-cellobiose were used as controls. Sulfate concentrations were determined by the turbidimetric method (Hach Chemicals Co., Loveland, CO.). The amount of H_2S produced in each microcosm was estimated semi-quantitatively (on a 4-point scale) by visual assessment of sulfide precipitate and the blackness of lead acetate papers.

RESULTS

Characterization of isolates

Identification of organisms and characteristics important for this investigation are given in Table 1. Strain C, *Cellulomonas* sp. was identified according to Bergey's Manual (15). S was a *Cytophaga* sp. identified according to Bergey's Manual (15)

Table 1. A summary of the characteristics of the isolates.

Strain	Identification	Identification reference	Source	Relationship to oxygen	Metabolic activity	Substrate transformation
C	<i>Cellulomonas</i> sp.	15	Soil	Facultative anaerobe	Cellulose digestion	Cellulose to cellobiose
S	<i>Cytophaga</i> sp.	15, 16	Soil	Facultative anaerobe	Cellulose digestion	Cellulose to glucose
M	<i>Micromonospora</i> sp.	17	Aquatic mud	Aerobe	Cellulose digestion	Cellulose to glucose
E	<i>Enterobacter cloacae</i>	15	Aquatic habitat	Facultative anaerobe	Lactic acid fermentation	Cellobiose/glucose to lactic acid
D	<i>Desulfovibrio desulfuricans</i>	18	Aquatic mud	Obligate anaerobe	Sulfate reduction	Lactate to acetate, sulfate to sulfide (H ₂ S)

Table 2. Production of H₂S in microcrystalline culture medium by pure cultures and various mixtures of organisms.

Organisms	Strain	Control cultures											Test cultures			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
<i>Cellulomonas</i>	S	S	O	O	O	O	S	S	O	O	O	O	S	S	O	O
<i>Cytophaga</i>	C	O ^a	C	O	O	O	O	O	C	C	O	O	C	O	C	O
<i>Micromonospora</i>	M	O	O	M	O	O	O	O	O	O	M	M	M	O	O	M
<i>Enterobacter</i>	E	O	O	O	E	O	E	O	E	O	E	O	E	E	E	E
<i>Desulfovibrio</i>	D	O	O	O	O	D	O	D	O	D	O	D	D	D	D	D
H ₂ S production		—	—	—	—	—	—	—	—	—	—	—	+	+	+	+

^a O = Organisms not present in culture.

and STANIER (16). M, an actinomycete, was identified as *Micromonospora* sp. (17). E was identified as *Enterobacter cloacae* (15). Lactic acid (125 µg/ml) was detected in 24 stationary CbMB cultures of E (data not shown). D was identified as *Desulfovibrio desulfuricans* (18).

Detection of H₂S production in mixed cultures

Table 2 shows the pattern of H₂S production by mixed cultures. No hydrogen sulfide was produced either by solitary cultures (rows 1–5) or by any 2-member cultures (rows 6–11). Only 5-member (rows 12) and 3-member cultures (rows 13–15) produced H₂S.

Fig. 1. Growth of mixed cultures of *Cellulomonas* ○, *Cytophaga* □, *Micromonospora* ◇, *Enterobacter* ○, and *Desulfovibrio* △, and changes in the concentrations of sulfate ●, soluble carbohydrate ■, and sulfide¹ ▲ in TXB.

¹ Sulfide grade schale:

- 4+ =cellulose precipitate completely black, ≥15 mm of lead acetate paper black.
- 3+ =cellulose precipitate partly black, >10–15 mm of lead acetate paper black.
- 2+ =cellulose precipitate white, >6–10 mm of lead acetate paper black.
- 1+ =cellulose precipitate white, >1–5 mm of lead acetate paper black.
- 0 =cellulose precipitate white, lead acetate paper white.

Fig. 2. Growth of mixed cultures of *Cellulomonas* ○, *Enterobacter* ○, and *Desulfovibrio* △, and changes in the concentration of sulfate ●, soluble carbohydrate ■, and sulfide ▲ in CXB.

Growth of mixed populations

Microbial growth patterns in the microcosms are shown in Figs. 1–4. Of the 3 cellulose digesters in TXB, *Cellulomonas* grew the most in contrast with the growth suppression of *Cytophaga* and *Micromonospora*. In CXB (Fig. 2), the only cellulolytic partner was *Cellulomonas*. Growth increases of this organism in TXB and CXB were comparable (Figs. 1 and 2). *Cytophaga* was the only cellulose

Fig. 3. Growth of *Cytophaga* □, *Enterobacter* ○, and *Desulfovibrio* △, and changes in the concentration of sulfate ●, soluble carbohydrate ■, and sulfide ▲ in SXB.

Fig. 4. Growth of *Micromonospora* ◇, *Enterobacter* ○, *Desulfovibrio* △, and changes in the concentration of sulfate ●, soluble carbohydrate ■, and sulfide in MXB.

digester in SXB (Fig. 3) where its growth was more than that detected in TXB. In MXB (Fig. 4), the increases in the *Micromonospora* population, the only cellulolytic partner, were marginal, but represented more growth relative to its suppression in the 5-member TXB system. Growths in the order of 4-log increases by day 3 were characteristic of *E. cloacae* in all microcosms (Figs. 1–4). For *Desulfovibrio*, growth in TXB and CXB (Figs. 1 and 2) were remarkable, similar and generally more than it was in SXB and MXB (Figs. 2 and 4).

Changes in soluble carbohydrates, sulfate concentration and sulfide production

Figures 1–4 show the changes in the concentration of soluble carbohydrates, sulfates, and the amount of sulfides. Except for *Cellulomonas* which produced cellobiose, all of the organisms produced glucose by cellulose hydrolysis (Table 1).

In all microcosms, there was an initial concentration of glucose of 26 $\mu\text{g}/\text{ml}$. During incubation, changes in the concentration of the soluble carbohydrate varied in each microcosm (Figs. 1–4).

Of the 650 μg of sulfate initially present in the microcosms, the highest reductions of 32 and 52% occurred in TXB and CXB respectively (Figs. 1–2), where there was marked blackness of cellulose precipitate and lead acetate papers. Less reductions (15%) in SXB and MXB were only manifested as blackness of lead acetate papers (Figs. 3 and 4).

DISCUSSION

Repeated attempts to isolate anaerobic cellulose digesters from aquatic muds were unsuccessful (unpublished data). For aerobic cellulose digesters we have shown that their metabolic products could not serve as electron donors for *Desulfovibrio* (Table 2, cultures 7, 9, 11). These organisms produced mono- and disaccharides (Table 1). Therefore an organism able to ferment these saccharides to lactic acid was needed. *E. cloacae* fulfilled this requirement (Table 1). Tested on this trophic relationship, sulfate reduction by *Desulfovibrio* became possible (Table 2, cultures 12–15). Sulfide production was not improved by the simultaneous presence of three different cellulose digesters (Fig. 1), as compared to only *Cellulomonas* (Fig. 2). Therefore the simultaneous presence of one representative of each of the three physiological types (cellulose digester, fermenter, sulfate reducer) is the sufficient and necessary condition for H_2S production from cellulose in mineral media. The marked growth of *Cellulomonas* in TXB and CXB contrasted with the reduced growth of *Cytophaga* and *Micromonospora* in TXB. But *Cytophaga* and *Micromonospora* were not inhibited in SXB and MXB respectively. The importance of nutritional adaptation and sulfide tolerance on growth quality in these microcosms is not clear. The toxicity of H_2S for microorganisms is well known (1, 2, 19, 20). Presumably H_2S toxicity (and or lack of oxygen) was a factor in the suppression of growths of *Cytophaga* and *Micromonospora* in TXB. Adaptation to competition for nutrients in an oxygen gradient can determine the quality of submerged growth, especially on an insoluble substrate such as cellulose which has a tactile influence on cellulolytic organisms. However, we were cautious in the interpretation of growth of *Micromonospora* since, as a mycelial organism, its growth may not be assessed reliably by colony counts which rely on the changes in the numbers of individual cells. The growth of *Desulfovibrio* and the sulfate reduction which occurred only in 5-member and 3-member systems suggested that suitable reducing micro-environments prevailed in these microcosms. The redox potential of -140 to -120 mV are the observed optima for *Desulfovibrio* (10, 21). We did not determine changes in Eh and pH values in our microcosms. However observations from preliminary studies on microcosms incorporating resazurin indicated active reduction of sulfate below fluid columns of high oxygen tension.

We cannot explain why 12% sulfate reductions in SXB and 15% in MXB

manifested themselves only as blackness of lead acetate papers (2+) while similar or lesser reductions in TXB and CXB were marked by blackness of both cellulose precipitate and lead acetate papers (3+, 4+ respectively). It is possible for such discrepancy to be due to error inherent in the subjective method used for sulfide assessment. Furthermore, it seems clear that the non-dissimilatory loss of sulfate in SXB and MXB may be due to the small amounts assimilated by organisms for biosynthesis. The increases in *Desulfovibrio* populations in SXB and MXB with low overall reduction of sulfate cannot be explained from this study.

Very interesting was the stability achieved with the 3-member cultures which appeared to be such that other bacterial species such as the methanogenic bacteria which require acetate or *Beggiatoa* which uses H₂S and acetate for mixotrophic metabolism may be introduced into these co-cultures. The system reported in this study has been found to support mixotrophic growth of *Beggiatoa* in co-culture (Utsalo and Maier, unpublished data). It is therefore a refinement of the enrichment of FAUST and WOLFE (22) for *Beggiatoa* cultures and has the advantage of standardized inocula and growth conditions.

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