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NOTES

End Products of Anaerobic Chitin Degradation by Salt Marsh Bacteria as Substrates for Dissimilatory Sulfate Reduction and Methanogenesis†

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The anaerobic pathway of chitin decomposition by chitinoclastic bacteria was examined with an emphasis on end product coupling to other salt marsh bacteria. Actively growing chitinoclastic bacterial isolates produced primarily acetate, H_2, and CO_2 in broth culture. No sulfate-reducing or methanogenic isolates grew on chitin as sole carbon source or produced any measurable degradation products. Mixed cultures of chitin degraders with sulfate reducers resulted in positive sulfide production. Mixed cultures of chitin-degrading isolates with methanogens resulted in the production of CH_4 with reductions in headspace CO_2 and H_2. The combination of all three metabolic types resulted in the simultaneous production of methane and sulfide, with more methane being produced in mixed cultures containing CO_2-reducing methanogens and acetoclastic sulfate reducers because of less interspecific H_2 competition.

Chitin and cellulose are the major structural polymers in invertebrates and plants, respectively. Both are polysaccharides composed of β{1-4}-linked monomers of *N*-acetyl-D-glucosamine in chitin or glucose in cellulose. The biosynthesis of chitin and cellulose proceeds via parallel enzymatic pathways (13), as does aerobic degradation by bacteria (4, 12). Billions of tons of chitin are produced annually in the oceans (5). It is not known how much of this production is incorporated into anaerobic habitats, but the amount could conceivably be substantial because its particulate nature favors rapid sedimentation and it occurs in many benthic organisms (15).

The occurrence of anaerobic chitin degradation has been largely ignored in the literature. A study by Alshina (1) showed that the amendment of chitin to anoxic sediments stimulated sulfate reduction, a result which led the author to conclude that sulfate-reducing bacteria were responsible for anaerobic chitin cycling. Billy (3) and Timmis et al. (14) have shown that certain clostridia can colonize and use chitin as a carbon source. In contrast, the amount of information concerning anaerobic cellulolysis is immense, mostly from work with the rumen system (9).

Considering the similar polymeric structures of chitin and cellulose, it was hypothesized that under anoxic conditions (i) chitin would be broken down by anaerobic sediment bacteria with an enzyme system similar to that of cellulolytic organisms, (ii) the products of chitin fermentation would be comparable to those of cellulolysis, and (iii) the fermentation products could be coupled via interspecies transfer to both sulfate reduction and methanogenesis.

Sediment samples from the Great Sippiwissett Salt Marsh, Mass., were taken with core tubes (5-cm inner diameter). The anoxic portions of the cores (5 to 10 cm below the surface) were extruded into Whirl-Pac bags (American Scientific Products, McGaw Park, Ill.). Air was expressed from the bags before they were sealed. Upon return, the cores were pooled and homogenized by being mixed in a covered beaker under a constant stream of N_2. Samples were streaked onto bottle plates (6) containing prereduced medium selective for the isolation of anaerobic chitinolytic, sulfate-reducing, and methanogenic bacteria. Medium components used for the selective isolation of salt marsh bacteria are listed in Table 1. Chitin degrader medium consisted of a heterotrophic marine agar base with an agar overlay of finely ground reprecipitated chitin (Calbiochem-Behring, San Diego, Calif.) as sole carbon source (8). Sulfate-reducing bacteria were isolated on a minimal salts medium containing sodium sulfate with acetate instead of lactate used as the carbon source (10) under an atmosphere of 80% H_2–20% CO_2. Ferrous iron was incorporated into the medium as a sulfide indicator. Methanogen medium was modified from that of Balch et al. (2) with acetate and CO_2 as carbon sources. Resazurin was incorporated into all media as an E_h indicator.

Plates were inoculated at 30°C for 5 days. Zones of clearing around colonies on chitin agar indicated the presence of extracellular chitinase. Formation of black colonies caused by precipitation of FeS on minimal salts medium was considered positive sulfate reduction. Methane production in the headspace was determined by a Carle gas chromatograph equipped with a thermal conductivity detector with a Porapak column (Supelco, Bellefonte, Pa.). Anaerobic transfers were performed until pure cultures were obtained as confirmed by light microscopy of gram-stained slides.

Axenic cultures of the bacterial types were inoculated into Balch tubes (2) containing chitin coculture medium, a mineral salts broth with reprecipitated chitin as sole carbon source and kept under N_2 (Table 1). The purpose was to ascertain whether sulfate reducers or methanogens could degrade chitin. Growth was defined as the presence of CO_2

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TABLE 1. Medium composition

Medium component	Amt (per liter of distilled water)				
	Chitin degrader		Sulfate reducer	Methanogen	Chitin coculture
	Base	Overlay			
K_2HPO_4	0.25 g		0.5 g		
NH_4Cl			1.0 g		
$CaCl_2 \cdot 2H_2O$			0.1 g		
$Mg_2SO_4 \cdot 6H_2O$			2.0 g	3.0 g	
NaCl	30.0 g	18.0 g	30.0 g	25.0 g	18.0 g
$NaSO_4$			1.0 g		
$NaHCO_3$		3.0 g	3.0 g	2.0 g	
$Fe(NH_4)_2(SO_4)_2 \cdot 7H_2O$	0.04 g	0.04 g		0.04 g	0.04 g
$FeSO_4$			0.1 g		0.1 g
1 N HCl				9.0 g	
Mineral 2[a]				40.0 ml	
Mineral 3[b]		500.0 ml			500.0 ml
Trace minerals[c]		10.0 ml	10.0 ml	10.0 ml	10.0 ml
Trace vitamins[d]		10.0 ml	10.0 ml	10.0 ml	10.0 ml
Yeast extract[e]	0.5 g	0.2 g	0.2 g	0.2 g	
Peptone[f]	1.0 g				
Sodium glycerophosphate	0.1 g				
Acetate			2.0 g	2.0 g	
Chitin		4.0 g			4.0 g
Cysteine-sodium sulfate (1:1)	0.5 g	0.5 g		0.5 g	
Ascorbic acid			0.02 g		0.02 g
Sodium thioglycolate			0.02 g		0.02 g
0.1% Resazurin	1.0 ml	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Agar	15.0 g	10.0 g	15.0 g	15.0 g	
N_2	+[g]	+			+
80% H_2–20% CO_2			+	+	

[a] Grams per liter of distilled water: KH_2PO_4, 6; $(NH_4)_2SO_4$, 6; NaCl, 12; $MgSO_4 \cdot 7H_2O$, 2.6; $CaCl_2 \cdot 2H_2O$, 0.16.

[b] Grams per liter of distilled water: KCl, 0.67; $MgCl_2 \cdot 2H_2O$, 5.5; $MgSO_4 \cdot 7H_2O$, 6.9; NH_4Cl, 0.5; $CaCl_2 \cdot 2H_2O$, 0.28; K_2HPO_4, 0.28.

[c] Grams per liter of distilled water: nitriloacetic acid, 1.5; $MgSO_4 \cdot 7H_2O$, 3.0; $MnSO_4 \cdot 2H_2O$, 0.5; NaCl, 1.0; $FeSO_4 \cdot 7H_2O$, 0.1; $CoSO_4$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.1; $ZnSO_4$, 0.1; $CuSO_4 \cdot 5H_2O$, 0.01; $AlK(SO_4)_2$, 0.01; H_3BO_3, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.01 (pH to 7.0 with KOH).

[d] Grams per liter of distilled water: biotin, 0.002; folic acid, 0.002; pyridoxine hydrochloride, 0.01; thiamine hydrochloride, 0.005; riboflavin, 0.005; nicotinic acid, 0.005; DL-calcium pantothenate, 0.005; vitamin B_{12}, 0.0001; *p*-aminobenzoic acid, 0.005; lipoic acid, 0.005.

[e] Difco Laboratories, Detroit, Mich.

[f] BBL Microbiology Systems, Cockeysville, Md.

[g] +, Present.

or CH_4 in the headspace, blackening of the medium, or formation of volatile fatty acids, particularly acetate. Volatile fatty acids were extracted from the medium with ether (7) and separated on a Resoflex column (Burrell Corp., Pittsburgh, Pa.) by a Carle gas chromatograph equipped with a flame ionization detector.

A final experiment was performed to evaluate whether sulfate reducers or methanogens could grow on the fermentative products of chitin degradation. Various combinations of culture types were inoculated into chitin coculture medium, and growth was ascertained as previously described.

A total of 17 anaerobic chitinoclastic colony types were observed (Table 2). Eight types were obligate anaerobes, and nine types were facultative. Five of the obligately anaerobic chitinoclastic isolates were gram-positive motile rods, while the other three were gram-negative motile rods. Of the nine facultative isolates, all were motile and gram negative. One of the three slender rods with gliding motility was pigmented. The other six were motile by flagella. Morphologically, these ranged from coccoid to curved and straight rods. Colonies of coccoid cells were luminescent when grown aerobically. All isolates produced some CO_2, H_2, and various types and amounts of volatile fatty acids.

Five distinct colonies of sulfate reducers were isolated on acetate. All colonies were made up of obligately anaerobic, motile rods that reduced Fe^{3+} to FeS. The four methanogenic isolates were distinctly different in their morphology, motility, and gram-staining characteristics, but all grew on acetate under H_2-CO_2 and produced methane. None of the sulfate-reducing or methanogenic isolates grew on chitin as sole carbon source or produced any measurable degradation products.

Results of mixed culture experiments with various combinations of chitinoclasts, sulfate reducers, and methanogens were similar. The most metabolically active cocultures (Table 3) are representative of the entire data set. As previously mentioned, the chitinoclastic bacteria degraded chitin and produced CO_2, H_2, and acetate. In some cases, a series of volatile fatty acids was produced, including propionate and butyrate.

Combinations of chitinoclasts with sulfate reducers showed two distinct pathways of sulfate reduction, i.e., an acetoclastic route, by which acetate was oxidized to CO_2 and sulfide was produced, and an H_2-utilizing route, by which sulfate was directly reduced. These results are consistent with the findings of others (11). Because sulfide was only qualitatively determined, the relative magnitude of sulfate reduction by each isolate was unknown. Cocultures of methanogens and chitinoclasts resulted in the production of CH_4, with decreases in H_2 and CO_2. Unfortunately, none of the methanogenic isolates could ferment acetate to CH_4.

Cocultures of chitin degraders with both sulfate reducers and methanogens showed the ability of methanogens to

TABLE 2. Sediment isolates on agar plates

Type and name of isolate	Characteristic of isolate					
	Obligate anaerobe	Gram stain	Motility	Morphology	Pigment	Luminescence
Chitin degrading						
CD1	+	+	+	Rod	–	–
CD2	+	+	+	Rod	–	–
CD3	–	–	+	Curved rod	–	–
CD4	+	+	+	Rod	–	–
CD5	–	–	Gliding	Rod	–	–
CD6	–	–	+	Coccoid	–	+
CD7	+	–	+	Rod	–	–
CD8	+	+	+	Rod	–	–
CD9	+	–	+	Rod	–	–
CD10	–	–	Gliding	Rod	Yellow	–
CD11	–	–	+	Curved rod	–	–
CD12	+	+	+	Rod	–	–
CD13	–	–	+	Rod	–	–
CD14	–	–	+	Coccoid	–	+
CD15	–	–	Gliding	Rod	–	–
CD16	+	–	+	Rod	–	–
CD17	–	–	+	Curved rod	–	–
Sulfate reducing						
SR1	+	–	+	Curved rod	–	NA[a]
SR2	+	–	+	Curved rod	–	NA
SR3	+	+	+	Rod	–	NA
SR4	+	–	+	Rod	–	NA
SR5	+	–	+	Curved rod	–	NA
Methanogenic						
M1	+	+	+	Coccoid	–	NA
M2	+	+	–	Coccoid	–	NA
M3	+	+	+	Rod	–	NA
M4	+	–	+	Rod	–	NA

[a] NA, Not applicable.

compete for the limiting resource, hydrogen. An H_2-utilizing methanogen in coculture with an acetoclastic sulfate reducer produced more methane than the same methanogen cultured with an H_2-utilizing sulfate reducer. Because no acetoclastic methanogens were isolated, observations on interspecific competition between acetoclastic sulfate reducers and methanogens were not possible.

It must be emphasized that this study was not designed to taxonomically characterize the bacteria involved in anaerobic chitin decomposition, but to determine which metabolic types are important. Clearly, there exists in anoxic salt marsh sediments a consortium of chitin-fermenting bacteria whose end products are coupled to both sulfate reduction and methanogenesis in pure culture. The relative contribution of these metabolic types to the total flux of carbon from chitin in anoxic sediments awaits further study.

TABLE 3. Coculture experimental results

Product	Amt produced (mmol/100 mmol of chitin)					
	CD3	CD3 cocultured with:				
		SR4	SR1	M2	M2 + SR4	M2 + SR1
Acetate	47.9	35.2	44.6	51.3	31.4	46.1
H_2	10.9	9.1	ND[a]	ND	ND	ND
CO_2	11.7	27.2	ND	ND	12.4	ND
CH_4	ND	ND	ND	20.6	15.4	2.1
S^{2-}	–[b]	+	+	–	+	+

[a] ND, Not detectable.
[b] –, Not present; +, present.

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ADDENDUM IN PROOF

After this paper had been accepted, I became aware of a recent publication by Roel Pel and Jan C. Gottschal, FEMS Microb. Ecol. **38**:39–49, 1986. This article describes cultivation and characterization of eight strains of obligately anaerobic chitinolytic bacteria and the fermentation products resulting from their growth: acetate, ethanol, formate, hydrogen, carbon dioxide, and ammonium.

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