

Sulfide Production from Cysteine by *Desulfovibrio desulfuricans*

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Two rumen nitrate-reducing isolates of *Desulfovibrio desulfuricans* were found to hydrolyze cysteine with the production of sulfide and pyruvate. When cultured on agar medium containing yeast extract with nitrate as the primary electron acceptor and ferrous chloride as the indicator, blackening of colonies occurred. The blackening of colonies appeared sooner and was more intense when either cysteine or sulfate was added to the culture medium with nitrate present.

Desulfovibrio desulfuricans is a chemoorganotrophic bacterium that obtains energy either by anaerobic respiration, reducing sulfates or other reducible sulfur compounds to H₂S, or by dismutation of fumarate or malate (10). Recently, *D. desulfuricans* isolates capable of utilizing nitrate as the terminal electron acceptor in place of sulfate have been isolated from the rumen (2, 6; M. P. Bryant, personal communication; G. L. Dilworth and H. D. Peck, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, K137, p. 209; B. H. Howard, personal communication).

When the rumen isolates of *D. desulfuricans* were grown in a sulfate-deficient medium with nitrate as the terminal electron acceptor and cysteine as the reducing agent, a black FeS-like precipitate developed which probably resulted from the degradation of added cysteine. In this study, the cysteine-degrading activity of the *D. desulfuricans* isolates was investigated, and the effect of added cysteine on the blackening of colonies in roll tubes containing FeCl₂ as the indicator was evaluated.

The cultures of *D. desulfuricans*, ATCC 27774 and isolate BH, were generously provided by M. P. Bryant and B. H. Howard (6), respectively. The bacteria were grown in 9-ml volumes of medium of the following composition: KNO₃, 0.6 g; sodium lactate, 1.0 g; yeast extract, 0.3 g; Minerals 1 (3), 60 ml; Minerals 2, with the sulfate salts replaced by the corresponding chloride salts (3), 60 ml; water, 156 ml; resazurin (0.1%, wt/vol), 0.3 ml; dithiothreitol (2%, wt/vol), 4.5 ml; NaHCO₃ (5%, wt/vol), 21 ml. The medium was prepared by adding all components with the exception of the NaHCO₃ to a 500-ml round-bottom flask. It was heated to boiling while the surface was gassed with a mixture of N₂-CO₂ (80:20, vol/vol). After the medium was reduced, the flask was sealed with a rubber stopper and autoclaved. Sterile 5% (wt/vol) NaHCO₃ prepared

under the same gas mixture was added to the cool, sterile medium. A 5-ml portion of this medium gave a barely perceptible cloudiness with acidified BaCl₂. For solid medium, agar was added to a final concentration of 1.5% (wt/vol). When included, sterile solutions of cysteine hydrochloride, sodium sulfate, and ferrous chloride (all maintained under N₂) were added to the medium to give concentrations of 0.05%, 0.02%, and 0.02% (wt/vol), respectively.

To test for cysteine-degrading activity, the isolates were each grown in 600-ml volumes of culture medium (24 h) with and without cysteine hydrochloride. The cells were harvested, washed once in 0.05 M tris(hydroxymethyl)amino-methane (Tris) buffer (pH 8.0) containing 0.01 M MgCl₂ and 0.001 M dithiothreitol, and suspended in 7 ml of the same buffer under an N₂ atmosphere. The cells were disrupted in an N₂ atmosphere by sonication at maximum power using a Biosonic III ultrasonic cell disintegrator (Bronwill Scientific, Inc., Rochester, N.Y.) by four treatments of 30 s with 1.5-min cooling intervals at 4°C. The disrupted cells were centrifuged at 40,000 × g for 20 min at 4°C to sediment particulate components. The enzyme extract was assayed at 39°C, under an N₂ atmosphere in sealed tubes, using a 1.5-ml reaction mixture of the Tris-Mg²⁺-dithiothreitol buffer containing 2 mM cysteine and 10 μM pyridoxal phosphate. Sulfide produced was determined as described by Siegel (19), and total pyruvate (9) was measured using a dinitrophenyl-hydrazine reagent prepared as described by Friedemann and Haugen (4). A substrate concentration of 2 mM was selected as an appropriate compromise for optimizing color development and substrate concentration, since cysteine inhibits color development in the assay for sulfide (9). Sulfide was determined in duplicate for 0.05, 0.1, and 0.2 ml of enzyme extract after 0, 5, 10, and 15 min.

The zero-time reading for sulfide was subtracted from the subsequent readings. There was no increase in sulfide during incubation of the reaction mixture in the absence of cysteine. Pyruvate was determined after 0, 10, 20, and 40 min. Enzyme activities were calculated from the slope of the best-fit line through the duplicate values at each time interval. Product was produced in a linear fashion for the enzyme concentrations used, and no more than 6% of the substrate was utilized during the assay. A unit of enzyme activity is defined as that amount which gave a velocity of 1 nmol of product (sulfide or pyruvate) per min. Protein was assayed by using the method of Lowry et al. (11). Controls were run to correct for the interference by dithiothreitol, which was minimal.

When cells were grown in either the presence or absence of cysteine, cell extracts prepared from the two rumen isolates of *D. desulfuricans* produced sulfide from cysteine. The rate of sulfide production was somewhat higher when cells were grown in the presence of cysteine (Table 1). Pyruvate was also produced from cysteine. The method for measuring pyruvate involved an initial pretreatment with acidic mercuric sulfate, which, according to Kredich et al. (9), causes the degradation of a product of cysteine desulfhydrase activity, 2-methyl-2,4-thiazolidinedicarboxylic acid (conjugate of pyruvate and cysteine). In this study, when samples were assayed for pyruvate without pretreatment with acidic mercuric sulfate, only 65% of the pyruvate was detectable, which indicated that only a portion of the pyruvate was present in the free form. Even with the acidic mercuric sulfate pretreat-

TABLE 1. Cysteine-degrading activity in cell extracts of rumen isolates of *D. desulfuricans* grown in the presence and absence of added cysteine^a

Isolate	Cysteine hydrochloride in the medium	Cysteine degradation sp act ^b	
		Sulfide	Pyruvate
ATCC 27774	-	0.62	0.47
	+	3.42	1.48
BH	-	1.88	0.45
	+	2.64	0.50

^a The bacteria were subcultured four times at 24-h intervals in the presence and absence of 0.05% (wt/vol) cysteine hydrochloride before the experiment. The bacterial dry weights in culture media at harvest for strain BH were 0.20 mg ml⁻¹ and 0.40 mg ml⁻¹, respectively, for cells grown in the absence and presence of cysteine; those for strain ATCC 27774 were 0.18 mg ml⁻¹ and 0.19 mg ml⁻¹, respectively.

^b Specific activity is defined as units per milligram of protein.

ment, less pyruvate was detected than would be expected for a cysteine desulfhydrase type of reaction in which the products expected are sulfide, pyruvate, and ammonia in equimolar amounts.

Sulfide was produced from cysteine and sulfate by actively growing cultures (Fig. 1A) and from cysteine by stationary-phase cultures (Fig. 1B). The low initial production of sulfide from cysteine by the actively growing culture (Fig. 1A) may have been due to a high utilization of cysteine and sulfide. During the later stages when growth slowed, sulfide production exceeded the growth requirement. The decrease in free sulfide observed in the stationary-phase culture at 200 min after addition of cysteine (Fig. 1B) was probably due to the utilization of sulfide by sulfur-limited cells, faster than it was produced, after an initial adaptation period.

When isolates ATCC 27774 and BH were diluted in agar medium in roll tubes with added FeCl₂, cysteine plus FeCl₂, or Na₂SO₄ plus FeCl₂, black colonies were visible in tubes of all three treatments after 3 days of incubation. In tubes with FeCl₂ alone added, only the colonies were black, whereas in the presence of cysteine black colonies with surrounding black precipitates were visible earlier. The precipitation of FeS in agar media in the absence of added cysteine and Na₂SO₄ probably resulted from the degradation

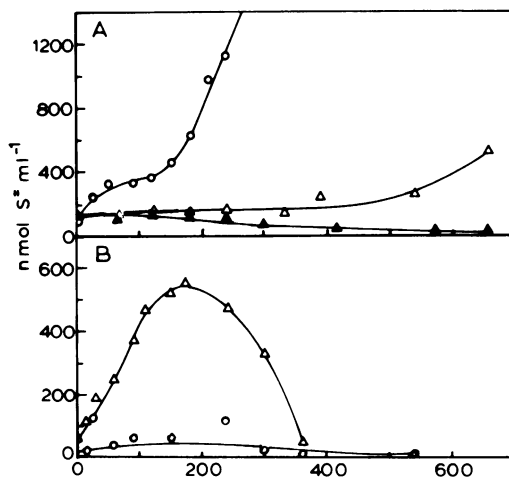


FIG. 1. Sulfide production from added sulfate and cysteine by *D. desulfuricans* BH cells previously grown in a sulfur-deficient medium. (A) Growing cells (0.08 mg [dry weight] ml⁻¹) at the time of additions; (B) stationary-phase cells (0.29 mg [dry weight] ml⁻¹) at the time of additions. (△) Cysteine hydrochloride, 6.8 μmol ml⁻¹, (○) sodium sulfate, 10 μmol ml⁻¹; (▲) no additions.

of cysteine in protein present in the yeast extract.

Cysteine catabolism by a marine *Desulfovibrio* was reported in 1954 by Senez and Leroux-Gilleron (18). To my knowledge, no further information on this enzymatic activity by *Desulfovibrio* spp. has become available in the intervening time. Knowledge of the presence of cysteine-degrading activity is important because inclusion of cysteine as a reducing agent in either the culture medium or a reaction mixture could lead to errors in the measurement of sulfide production from sulfate. In the past, it has been claimed that cysteine could be used as a reducing agent for determining colony counts with pure cultures (see reference 15); however, this method must be interpreted with caution, since FeS precipitates would arise from either or both sulfate and cysteine metabolism. The inclusion of cysteine in media for the enumeration of natural samples (e.g., reference 8) for *Desulfovibrio* requires testing the isolated colonies for sulfide production in the absence of cysteine (14); otherwise, the results could be in error. The use of thioglycollic acid as the reducing agent has been favored since it avoids this inconvenience (16). Due to the inhibitory nature of thioglycollic acid (5, 14), perhaps dithiothreitol would be a better reducing agent.

The desulfuration of cysteine to hydrogen sulfide by bacteria is usually attributed to the action of the enzyme cysteine desulfhydrase, although cystathionase (17), tryptophanase (13), and more complicated pathways (12) may also contribute to this process. The data presented here are insufficient to rule out any one of these possibilities as the mechanism of cysteine degradation in the *Desulfovibrio* isolates; consequently, additional work is necessary to characterize the precise nature of the enzyme(s) involved.

Desulfovibrio species have been found within the rumen at 10^7 to 10^8 cells per ml (6, 7). In these high numbers, they account for the dissimilatory sulfate-reducing activity observed in the rumen (1, 6) and may also contribute significantly to cysteine degradation within that environment (1).

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