CHOLINE FERMENTATION BY *DESULFOVIBRIO DESULFURICANS*[1]

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ABSTRACT

BAKER, F. D. (Western Reserve University, Cleveland, Ohio), H. R. PAPISKA, AND L. LEON CAMPBELL. Choline fermentation by *Desulfovibrio desulfuricans*. J. Bacteriol. **84**:973-978. 1962—Hayward and Stadtman pointed out that the organism they described as *Vibrio cholinicus* is closely related to *Desulfovibrio desulfuricans*. We have established that some strains of *D. desulfuricans* carry out the same fermentation of choline as does *V. cholinicus*. We have also shown that *V. cholinicus* carries out the sulfate-linked fermentation of lactate identical with that of *D. desulfuricans*. Both organisms have identical reduced cytochrome spectra, with peaks at 417 to 420, 525, and 553 mμ. *V. cholinicus* also contains the green pigment desulfoviridin, characteristic of *D. desulfuricans*, which in alkaline solution gives a red fluorescence at 365 mμ. Immunological data from cross-agglutination and absorption tests show that the two organisms have similar antigenic properties. Morphological, cultural, and biochemical studies have also demonstrated that *V. cholinicus* is indistinguishable from *D. desulfuricans*. Therefore, *V. cholinicus* should be regarded taxonomically as a strain of *D. desulfuricans*.

Hayward and Stadtman (1959) isolated an obligately anaerobic, vibrio-shaped organism, from choline enrichment cultures, which they tentatively named *Vibrio cholinicus*. This organism contained a cytochrome *c*-type pigment with absorption maxima at 418, 523, and 553 mμ, and was capable of carrying out sulfate reduction (Hayward and Stadtman, 1960). They recognized that these properties were similar to those of the sulfate-reducing vibrio, *Desulfovibrio desulfuricans*. They differentiated their isolate from this organism on the basis that it would grow on choline in the absence of sulfate, whereas *D. desulfuricans* would not grow on choline unless sulfate was present. Postgate (1952), however, has shown that some strains of *D. desulfuricans* can grow on pyruvate in the absence of sulfate. It is conceivable, therefore, that some strains of this organism will also grow on choline in the absence of sulfate. In our efforts to clarify the taxonomic position of this group of organisms (Campbell, Frank, and Hall, 1957), a comparative study of *V. cholinicus* and *D. desulfuricans* has been conducted. A preliminary report of this work has appeared (Baker, Papiska, and Campbell, 1961).

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MATERIALS AND METHODS

Organisms. Eight strains of *D. desulfuricans* and one strain of *V. cholinicus* were studied. The cultures were obtained from the following sources: *D. desulfuricans* strains 8303, El Agheila Z, Canet, and Wandle from J. R. Postgate; strains Gaz, Eau, and Sol from J. C. Senez; strain 7707 from the American Type Culture Collection; *V. cholinicus* from H. R. Hayward. All cultures were checked for purity by using the agar shake-tube method as described by Postgate (1953), and by plating on suitable media followed by incubation in an anaerobic incubator under an atmosphere of 99% H_2-1% CO_2. Stock cultures were maintained as agar stabs in the standard medium described below.

Since these organisms are obligate anaerobes, they were grown, except where noted, in the presence of a potassium carbonate-pyrogallol seal.

Media. The standard medium used was medium C of Butlin, Adams, and Thomas (1949)

as modified by Postgate (*personal communication*). The modified medium contained: KH_2PO_4, 0.5 g; NH_4Cl, 1.0 g; Na_2SO_4, 2.6 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; $CaCl_2 \cdot 2H_2O$, 0.06 g; sodium lactate, 6.0 g; yeast extract (Difco), 1.0 g; in 1 liter of distilled water. After sterilization, the medium was adjusted to pH 7.4 with sterile NaOH. Just prior to inoculation, the medium was supplemented with $Fe(NH_4)_2SO_4$ (10 μg/ml) from a filter-sterilized stock solution. For growth of the El Agheila Z and Canet strains, NaCl (20 g/liter) was added to the medium. Solid media were prepared by the addition of agar (20 g/liter). In choline fermentation studies, choline chloride (6.0 g/liter) was substituted for sodium lactate, and sulfate was omitted from the medium.

Analytical methods. Acetate and ethanol were identified and quantitated, as described by Hayward and Stadtman (1959). Choline was determined by the method of Engel, Salmon, and Ackerman (1954), trimethylamine by the procedure of Dyer (1945), lactate by the method of Barker and Summerson (1941), and hydrogen sulfide as described by Fogo and Popowsky (1949). In fermentation studies, CO_2 was estimated by gravimetric determination of $BaCO_3$.

The amount of growth in liquid media was determined by measuring the absorbancy at 480 mμ with a Bausch and Lomb spectronic 20 colorimeter.

Adenosine triphosphate (ATP) sulfurylase was prepared by the procedure of Akagi and Campbell (1962). Its activity was measured by the molybdolysis assay described by Bandurski, Wilson, and Squires (1956). Reaction mixtures contained: tris(hydroxymethyl)aminomethane (tris)-HCl buffer (pH 7.7), 25 μmoles; ATP, 5 μmoles; sodium molybdate, 20 μmoles; $MgCl_2$, 5 μmoles; yeast inorganic pyrophosphatase, 0.9 mg; enzyme, 0.05 ml; in a total volume of 0.75 ml. The inorganic pyrophosphatase employed had a specific activity of 15,000 as determined by the method of Heppel and Hilmoe (1951). After an appropriate incubation time at 30 C, the tubes were assayed for inorganic phosphate by the method of Fiske and SubbaRow (1925). Specific activity is expressed as μmoles of inorganic phosphate liberated per mg of protein per hr. Protein was estimated by the method of Lowry et al. (1951). Yeast inorganic pyrophosphatase was purified as described by Heppel and Hilmoe (1951). Adenosine-5′-phosphosulfate (APS) was synthesized by the method of Baddiley, Buchanan, and Letters (1957). Nucleotides were separated by electrophoresis on Whatman 3 MM paper in 0.03 M citrate buffer (pH 5.9) with a Spinco model R apparatus operating at 400 v for 3 hr at 4 C. Nucleotides were detected with a shortwave ultraviolet lamp. Radioactivity measurements for S^{35}-labeled APS were performed with a Vanguard model 800 paper strip counter.

Preparation of antigens and antisera. V. cholinicus and *D. desulfuricans* strain 8303 were grown in 1-liter cultures of the standard lactate medium for 48 hr, harvested aseptically by centrifugation, and washed five times with sterile 0.8% KCl. The washed cells were diluted with sterile 0.8% KCl to a reading of 360 to 400 with a no. 42 filter in a Klett-Summerson colorimeter. Rabbits were injected intravenously and into each foot pad with 1.0 ml and 0.5 ml, respectively, of the diluted antigens. The injections were repeated three times at 48-hr intervals. The animals, 1 week after the last injection, were bled via the ear vein to check the antibody titer by the agglutination reaction. When the titer reached a value of 1:1,280 to 1:2,560, the rabbits were bled from the heart, and the sera, after being heated at 56 C for 30 min to destroy complement, were tested for agglutinins. The sera were stored at -10 C.

Agglutination tests. The antigens of all strains of *D. desulfuricans* and *V. cholinicus* were prepared as described above. They were standardized to a reading of 200 with the no. 66 filter in a Klett-Summerson colorimeter. Agglutination tests were performed by adding 0.5 ml of the antigen to 0.5 ml of the diluted antiserum; controls consisted of 0.5 ml of antigen added to 0.5 ml of saline. The tubes were incubated in a water bath (37 C) for 2 hr, read, placed at 5 C overnight, and the final readings were made.

Preparation of cell-free extracts for spectrophotometry. Cells of *D. desulfuricans* (strains El Aghelia Z and Canet) and of *V. cholinicus* were harvested by centrifugation from 10-liter cultures grown in the choline medium for 48 hr at 30 C. The cells were washed three times in 0.05 M sodium dimethylglutarate buffer (pH 6.8). Cell extracts were prepared either by treatment of cells for 20 min in a 10-kc sonic oscillator or by treatment in a French pressure cell (20,000 psi). Unbroken cells and cell debris were removed by centrifugation at 25,000 $\times$ *g* for 20 min. The

cell-free extracts were examined for cytochrome pigments with a Cary recording spectrophotometer. Reduced extracts were prepared by the addition of a small amount of solid sodium hydrosulfite, just prior to examination.

RESULTS AND DISCUSSION

Carbon sources for growth. Several carbon sources were tested for their ability to support growth of *D. desulfuricans* and *V. cholinicus* in the presence and absence of sulfate. All of the organisms tested grew on lactate and pyruvate with sulfate (Table 1). Two strains of *D. desulfuricans* (El Agheila Z and Canet) and *V. cholinicus* grew on pyruvate and choline in the absence of sulfate. Although these strains reached the same absorbancy value on pyruvate in the presence and absence of sulfate, the total growth on choline in the absence of sulfate was only about one-half that obtained with sulfate. The behavior of strains El Agheila Z and Canet on pyruvate confirmed the findings of Postgate (1952). Growth on glycerol, ethanol, and malate showed strain variations, although none of the organisms could utilize these compounds in the absence of sulfate. None of the organisms could grow on formate, acetate, propionate, butyrate, succinate, citrate, tartrate, glyoxylate, glucose, fructose, sucrose, lactose, maltose, galactose, raffinose, xylose, or dulcitol, with or without sulfate, or in the presence of 15 μg per ml of sterile sodium sulfide. Postgate (1959*a*) emphasized the importance of adding sulfide to lower the Eh of the medium when studying carbon utilization by this group of bacteria.

TABLE 1. *Utilization of carbon compounds by Desulfovibrio desulfuricans and Vibrio cholinicus in the presence of sulfate*

Organism	Carbon compound					
	Lactate	Pyruvate	Choline	Glycerol	Ethanol	Malate
D. desulfuricans						
El Agheila Z.	.680*	.580†	.450†	.325	.270	.730
Canet.....	.640	.360†	.360†	.320	.235	.375
Gaz.....	.680	.350	.080	.630	.040	.060
Wandle.....	.650	.850	.040	.800	.225	.090
Eau.....	.700	.540	.420	.350	.340	.670
Sol.....	.800	.640	.400	.410	.260	.500
7707.....	.580	.500	.060	.050	.040	.100
8303.....	.780	.440	.040	.630	.230	.320
V. cholinicus...	.680	.800†	.430†	.380	.180	.060

* Absorbancy value at 480 mμ after 48 hr of incubation at 30 C.

† These strains grew without sulfate with these carbon compounds (see text).

TABLE 2. *Utilization of sulfate, sulfite, and thiosulfate by Desulfovibrio desulfuricans and Vibrio cholinicus for growth on choline and lactate*

Organism	Compound	Sulfur compound			
		Sulfate	Sulfite	Thiosulfate	None
D. desulfuricans	Choline	.460*	.660	.600	.246
E. Agheila Z	Lactate	.700	.485	.530	.100
V. cholinicus	Choline	.440	.400	.489	.235
	Lactate	.695	.710	.589	.065

* Absorbancy value at 480 mμ after 72 hr of incubation at 30 C.

TABLE 3. *Choline fermentation by growing cultures of Desulfovibrio desulfuricans and Vibrio cholinicus**

Organism	Products (mmoles/100 ml of medium)			Δ Choline
	Trimethylamine	Acetate	Ethanol	
D. desulfuricans				
El Agheila Z....	2.32	1.36	1.21	−2.52
Canet.....	2.36	1.12	1.25	−2.76
V. cholinicus.....	2.00	1.02	1.00	−2.43

* After incubation for 72 hr at 30 C, the fermentation was stopped by the addition of 10 N H_2SO_4, and products were analyzed as described in the text.

Utilization of sulfite and thiosulfate. Since *D. desulfuricans* can utilize sulfite or thiosulfate as hydrogen acceptors for growth on lactate (Postgate, 1959*a*), it was of interest to determine whether *V. cholinicus* behaved similarly. The standard lactate medium containing either 0.45% sodium sulfite or sodium thiosulfate, in place of sulfate, was inoculated with *V. cholinicus* and incubated for 72 hr at 30 C. Parallel cultures were set up with *D. desulfuricans* (El Aghelia Z and Canet) as controls. The data in Table 2 show that all three organisms utilize sulfite or thiosulfate in place of sulfate for growth on lactate.

Fermentation studies. The El Agheila Z and Canet strains of *D. desulfuricans* carry out the same fermentation of choline (in the absence of sulfate) as *V. cholinicus* does (Table 3). In agreement with Hayward and Stadtman (1959, 1960), the over-all equation describing this fermentation is: 2 choline + water → 2 trimethylamine + 1 acetate + 1 ethanol.

We next studied the ability of *V. cholinicus* to carry out the sulfate-linked fermentation of lactate characteristic of *D. desulfuricans*. Data in Table 4 demonstrate that both organisms effect this fermentation according to the following equation: 2 lactate + Na_2SO_4 → 2 acetate + 2 CO_2 + 2 H_2O + Na_2S.

Cytochrome and desulfoviridin pigments. Cell-free extracts of *D. desulfuricans* (strain El Agheila Z) and *V. cholinicus* grown in choline medium (minus sulfate) revealed the typical absorption spectrum of cytochrome c_3 (Postgate, 1956), with maxima at 419, 525, and 553 mμ, when reduced with sodium hydrosulfite. Incubation of cell extracts of these two organisms with choline resulted in a time-dependent reduction of the cytochrome, confirming the results of Hayward and Stadtman (1960) with *V. cholinicus*.

Postgate (1959*b*) showed that the green pigment desulfoviridin (absorption maxima at 411, 585, and 632 mμ) is present in *D. desulfuricans* and is absent from the other dissimilatory sulfate-reducing bacteria, *D. orientis* and *Clostridium nigrificans*. A diagnostic test for this pigment, based on its dissociation in alkaline solution to yield a photosensitive chromogenic group having a strong red fluorescence in ultraviolet light at 365 mμ, was devised by Postgate (1959*b*). Employing this test, we examined eight strains of *D. desulfuricans*, one strain of *V. cholinicus*, one strain of *D. orientis*, and eight strains of *C. nigrificans*. Only *V. cholinicus* and *D. desulfuricans* gave a positive test. These findings confirmed previous reports (Campbell et al., 1957; Postgate, 1959*b*) and further suggested that *V. cholinicus* is identical with *D. desulfuricans*.

TABLE 4. *Sulfate-linked fermentation of lactate by growing cultures of Desulfovibrio desulfuricans and Vibrio cholinicus**

Organism	Product (mmoles/100 ml of medium)			Δ Lactate
	Acetate	CO_2	H_2S	
D. desulfuricans				
El Agheila Z	4.32	4.70	2.26	−4.79
V. cholinicus	4.60	4.80	1.86	−4.81

* Fermentation apparatus set up with two traps: one trap contained 40 ml of KOH to trap CO_2; the second trap contained 1 ml of 20% cadmium acetate, absorbed on a filter-paper strip to trap H_2S. After 72 hr of incubation, the fermentation was terminated by the addition of 10 N H_2SO_4, and products were analyzed as described in the text.

TABLE 5. *Serological agglutination response of Desulfovibrio desulfuricans and Vibrio cholinicus*

Antigen	Antisera	
	D. desulfuricans 8303	*V. cholinicus*
D. desulfuricans		
El Agheila Z	1:2,560	1:2,560
Canet	1:1,280	1:1,280
Gaz	1:640	1:640
Sol	1:1,280	1:640
Eau	1:640	1:640
Wandle	1:1,280	1:1,280
7707	1:2,560	1:2,560
8303	1:2,560	1:2,560
V. cholinicus	1:1,280	1:2,560

Immunological studies. All eight strains of *D. desulfuricans* cross-agglutinated with *V. cholinicus* antiserum, and *V. cholinicus* cross-agglutinated with *D. desulfuricans* antiserum. The agglutination titer showed strain variation, but in general was in the range of 1:640 to 1:2,560 (Table 5). When either antiserum was cross-absorbed with heterologous antigen, no agglutination occurred when tested with the homologous antigens. These results demonstrated the immunological similarity of *V. cholinicus* and *D. desulfuricans*.

ATP-sulfurylase of V. cholinicus. Peck (1959), Ishimoto (1959), and Ishimoto and Fujimoto (1959) independently established that cell-free extracts of *D. desulfuricans* contain the enzyme ATP-sulfurylase, which forms APS from ATP and sulfate. They also demonstrated that APS is the form in which sulfate is reduced to sulfite. Akagi and Campbell (1962) purified the ATP-sulfurylase 30- to 40-fold from extracts of *D. desulfuricans*. The enzyme is also present in crude extracts of *V. cholinicus* (specific activity of

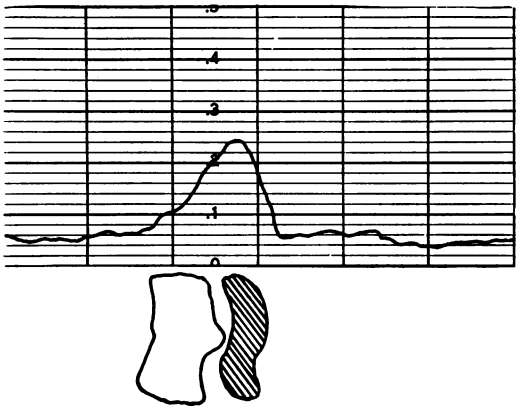

FIG. 1. *Enzymatic synthesis of sulfur^{35}-labeled adenosine-5′-phosphosulfate by purified adenosine triphosphate (ATP)-sulfurylase of Vibrio cholinicus. Reaction mixture contained: tris buffer (pH 7.7), 5 μmoles; ATP, 5 μmoles; K_2SO_4, 20 μmoles; $Na_2SO_4^{35}$ corresponding to 1.06 × 10^5 counts/min; $MgCl_2$, 5 μmoles; yeast pyrophosphatase, 0.9 mg; purified ATP-sulfurylase of V. cholinicus (0.01 mg, specific activity of 2,073). After 1 hr of incubation at 30 C under helium, the reaction was stopped by immersion of the tubes in a boiling-water bath for 90 sec. The tubes were cooled immediately and the denatured protein was removed by centrifugation. A sample of the supernatant liquid was spotted on Whatman 3 MM paper and electrophoresis carried out as described in the text. Radioactivity was detected with a Vanguard model 800 paper strip counter. Shaded area represents radioactive APS and the clear area, ATP.*

68) and can be purified by exactly the same procedure employed with *D. desulfuricans*. Incubation of the purified enzyme (specific activity of 2,073) of *V. cholinicus* with ATP and $S^{35}O_4$ gives rise to an S^{35}-labeled nucleotide which behaves identically to known synthetic APS upon paper electrophoresis. Figure 1 shows the electrophoretic pattern of the enzymatically synthesized radioactive APS. These findings are consistent with the view that *V. cholinicus* is identical to *D. desulfuricans*. Peck's (1961) report that *V. cholinicus* also contains the enzyme which reduces APS to adenosine monophosphate and sulfite (APS reductase) lends support to this conclusion.

Classification of V. cholinicus. The data presented clearly show that the organism isolated and tentatively named *V. cholinicus* by Hayward and Stadtman (1959) is identical with *D. desulfuricans*. Since the latter name has taxonomic priority, this choline-fermenting vibrio should be considered as a strain of *D. desulfuricans* and the name *V. cholinicus* should be placed in the list of *nomina rejicienda*. After the completion of these studies, a paper appeared in which Senez and Pascal (1961) independently arrived at the same conclusion.

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