STUDIES ON THERMOPHILIC SULFATE REDUCING BACTERIA

I. IDENTIFICATION OF *Sporovibrio desulfuricans* AS *Clostridium nigrificans*¹

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Received for publication October 15, 1956

Elion (1924) was the first to isolate a thermophilic sulfate reducing bacterium in pure culture. He named the organism *Vibrio thermodesulfuricans*, and reported that it was capable of sulfate reduction between 30 and 65 C, with an optimum of 55 C. Baars (1930) and Kluyver and Baars (1932) reisolated *V. thermodesulfuricans*, and carried out a comparative study of this organism and *Vibrio desulfuricans*. They reported that it was possible to adapt cultures of *V. desulfuricans*, initially capable of growth only at 30 C, to grow up to 55 C by gradually increasing the incubation temperature. The reverse phenomenon was also demonstrated with *V. thermodesulfuricans*. They concluded that *V. thermodesulfuricans* should be regarded as a temperature adapted strain of *V. desulfuricans*. The sulfate reducing bacteria are now placed in the genus *Desulfovibrio*, created by Kluyver and Van Niel (1936).

Starkey (1938) isolated cultures from sewage, mud, and soil at 30 and 55 C. Contrary to the findings of earlier investigators, all of the cultures isolated at 55 C were large, slightly curved, spore-forming rods, while those isolated at 30 C were typical asporogenous short vibrios. The cultures isolated at 30 C were motile by a rapid progressive movement and had one (or two) polar flagellum(a). Those isolated at 55 C had a rotating motility with only a slight progressive movement, and possessed from 1 to 10 or more flagella scattered around the cell. Cultures isolated at 30 C failed to grow when transferred directly to 55 C, but the cultures isolated at 55 C

grew when transferred to 30 C. When the 55 C cultures were transferred to 30 C, they underwent striking morphological changes. The cultures appeared first as long filaments, changing to chains of vibrios, then to shorter vibrios and finally to the small vibrios characteristic of the cultures originally isolated at 30 C. Starkey proposed the new genus *Sporovibrio* for the anaerobic curved cells of vibrio shape which produced endospores.

Werkman and Weaver (1927) and Werkman (1929) isolated *Clostridium nigrificans* and showed that it was responsible for the sulfide spoilage of canned corn. *C. nigrificans* is a thermophilic organism with an optimum growth temperature of 55 C. Some strains will grow at temperatures as low as 35 C (Sealey, 1951). Although Werkman and Weaver stated that *C. nigrificans* was a gram positive rod, most investigators have reported that it is gram negative (Sealey, 1951). Sealey reported that hydrogen sulfide was produced by this organism in a medium in which sulfate was the only source of sulfur.

Due to the similarity of *C. nigrificans* (Werkman and Weaver, 1927; Werkman, 1929; Sealey, 1951) to the organism described by Starkey (1938) as *Sporovibrio desulfuricans*, a comparative study of these organisms and *Desulfovibrio desulfuricans* has been carried out. A preliminary report of this work has appeared (Campbell *et al.*, 1956).

EXPERIMENTAL METHODS

Organisms. In order to avoid confusion the cultures referred to in this paper as *S. desulfuricans* are the large, slightly curved, sporulating rods whose optimum growth temperature is 55 C. Those referred to as *D. desulfuricans* are small, curved, nonsporulating rods whose optimum growth temperature is 30 C.

¹ Scientific Paper No. 1530, Washington Agricultural Experiment Stations, Pullman, Washington. Presented at the 56th General Meeting of the Society of American Bacteriologists, Houston, Texas, April 29–May 3, 1956.

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Five strains of *C. nigrificans*, four strains of *S. desulfuricans*, and two strains of *D. desulfuricans* were studied. The cultures were obtained from the following sources: (a) *C. nigrificans* strain 3750, Mr. C. B. Denny, National Canners Association, Washington, D. C.; strain 7946, A.T.C.C.; strains 55, 106, and 134, Dr. O. B. Williams; (b) *S. desulfuricans* strain 14T, Dr. R. L. Starkey; strain 7757T, A.T.C.C.; strains 8351 and 8395, Dr. J. R. Postgate; (c) *D. desulfuricans* strain 7757, A.T.C.C.; strain 344, Dr. C. E. ZoBell. All cultures were checked for purity.

Stock cultures of *C. nigrificans* and *S. desulfuricans* were maintained in a medium containing peptone (Difco), 5.0 g; yeast extract (Difco), 4.0 g; Na_2SO_4 , 1.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 g; glucose, 10.0 g; agar, 20.0 g; in 1 L distilled water, pH 7.2 to 7.4 (PYG) (Postgate, 1951). Stock cultures of *D. desulfuricans* were carried in the same medium except that sodium lactate (3.5 g) was substituted for glucose (PYL).

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

Media. The medium used in most of the work was that of Starkey (1938) and contained peptone (Difco), 5.0 g; glucose, 10.0 g; Na_2SO_4 , 1.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 g; tap water, 1 L, pH 7.2. This medium will be referred to as medium SG. Occasionally glucose was replaced by sodium lactate (3.5 g). This medium is designated as medium SL. All media used in the characterization studies were supplemented with Na_2SO_4 , 1.5 g and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 g per L.

Characterization methods. Fermentation reactions on carbohydrates and alcohols were determined in the basal medium of Reed and Orr (1941). After incubation for 5 days at the appropriate temperature, acid production was measured with a Beckman pH meter.

Tests for proteolytic activity were carried out as follows: (1) liquefaction of coagulated egg albumin, proteolysis of alkaline egg medium, blackening of brain medium, and reddening of beef heart tissue as described in the *Manual of Methods for Pure Culture Study of Bacteria* (1944); (2) action on litmus milk in litmus milk medium (Difco); (3) gelatin liquefaction in the medium of Reed and Orr (1941); (4) indole formation in the medium of Reed and Orr (1941). Indole was deter-

mined, after incubation for 48 hr, with Kovac's reagent.

Nitrate reduction was determined in the medium of Reed and Orr (1941) after incubation for 24 and 48 hr. Sulfanilic acid and α -naphthylamine were used to determine nitrite.

Hemolytic activity was determined using 5 per cent defibrinated rabbit blood in a blood-agar base medium (Difco). Streak plates were examined for hemolysis after incubation for 5 days in a Brewer anaerobic jar in an atmosphere of hydrogen.

Estimation of growth and hydrogen sulfide. The amount of growth in liquid media was determined by optical density measurement at 525 μ with a Bausch and Lomb "spectronic 20" colorimeter.

The starch-iodine method described by Starkey (1938) was used for the quantitative determination of hydrogen sulfide.

Preparation of antigens. Two strains of *C. nigrificans*, three strains of *S. desulfuricans*, and one strain of *D. desulfuricans* were selected for immunological studies. The organisms were grown in medium SG. After incubation for 48 hr the cells were harvested by centrifugation and washed 5 times with 0.01 M potassium phosphate buffer, pH 7.4; the packed cells were stored at -10°C . Somatic and flagellar antigens were prepared according to the methods described by Kolmer *et al.* (1951) and stored at 5°C .

In testing the specificity of H and O antisera against H and O antigens, marked agglutination of the O antigens occurred when they were mixed with saline. It was, therefore, necessary to prepare another type of antigen to test the O antisera. Phenolized antigens were prepared by suspending 1.0 ml of the packed cells in 20 ml of 5 per cent phenol in saline. The suspension was incubated at 37°C for 24 hr and diluted 15 times with 0.5 per cent phenol in saline. This preparation showed no agglutination in saline.

Immunization procedures. Two rabbits were used for each H and O antigen. No naturally occurring antibodies for the antigens were present. Intravenous injections of 0.5 ml and then 1.0 ml were given every other day until a total of 9.5 ml had been given. One week after the last injection the animals were bled, and the sera were tested for agglutinins by the slide agglutination test. All of the rabbits received another series of injections until they had received 6.0 ml more

antigen. The rabbits were then bled from the heart, and the sera were tested for agglutinins by test tube procedures. The sera were stored at -10°C .

Agglutination tests. 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 37°C water bath for 2 hr, read, then placed at 5°C overnight at which time the final readings were made.

Preparation of cell free extracts for spectrophotometry. Cultures of *C. nigrificans* and *S. desulfuricans* were grown in medium SG for 48 hr at 55°C . *D. desulfuricans* was grown in medium SL for 48 hr at 30°C . The cells were harvested by centrifugation, washed 3 times with 0.01 M phosphate buffer, pH 7.4, and placed at -10°C until used. Cell extracts were prepared by grinding washed cells with twice their weight of alumina at 0°C for 20 min. The paste was extracted with 2.0 ml of 0.10 M phosphate buffer, pH 7.4, per g of bacteria, and centrifuged in a "servall" centrifuge at 10,000 rpm for 20 min to remove unbroken cells and cell debris. Appropriate dilutions of the yellow supernatant were used to determine cytochrome pigments, using a Beckman DU spectrophotometer. Reduced extracts were prepared by the addition of a small amount of solid sodium hydrosulfite just prior to examination.

RESULTS AND DISCUSSION

The formation of hydrogen sulfide from sulfate, sulfite, and thiosulfate. To determine if *C. nigrificans* could reduce sulfate, sulfite, and thiosulfate to hydrogen sulfide under the same conditions as *S. desulfuricans*, the following experiment was carried out. Four strains of each organism were inoculated into the following media: (1) medium SG; (2) medium SG except that the sulfate was replaced by 1.0 g of Na_2SO_3 , (3) medium SG except that the sulfate was replaced by 1.0 g of $\text{Na}_2\text{S}_2\text{O}_3$, (4) medium SG except that the sulfate was omitted. Medium (4) and uninoculated media served as controls. The amount of hydrogen sulfide produced was determined after incubation for 48 hr at 55°C . The data obtained are presented in table 1, and are typical of replicate determinations. These data clearly show that *C. nigrificans* and *S. desulfuricans* reduce

sulfate, sulfite, and thiosulfate under the same conditions.

The effect of sulfate and sulfite concentration on growth of *C. nigrificans* and *S. desulfuricans*. It was found in the above experiments that little or no growth occurred in the absence of sulfate, sulfite, or thiosulfate. We then checked the effect of sulfate and sulfite concentration on the growth of these organisms. The medium employed contained 0.5 per cent peptone, 1.0 per cent glucose, and the desired amount of the compound being studied in tap water, pH 7.2. All cultures gave a typical growth response curve with sulfate and sulfite. Half maximum growth was obtained with 20 μmoles of sulfate per ml and with 33 μmoles of sulfite per ml.

Hydrogen donors for sulfide production from sulfate. The ability of *C. nigrificans* and *S. desulfuricans* to utilize glucose, ethanol, lactate, malate, or pyruvate as hydrogen donors for sulfate reduction was tested. Four strains of each organism were inoculated into medium SG, except that glucose was replaced by the appropriate donor (10.0 g per L). After incubation for 48 hr at 55°C the amount of hydrogen sulfide produced was determined. All cultures utilized glucose, ethanol, lactate, and pyruvate as donors for sulfate reduction. Malate was utilized only slightly or not at all. Pyruvate and lactate were the best donors for most of the cultures. Pyruvate was better

TABLE 1

Hydrogen sulfide production from sulfate, sulfite, and thiosulfate by Clostridium nigrificans and Sporovibrio desulfuricans

Organism	Compound		
	$=\text{SO}_4$	$=\text{SO}_3$	$=\text{S}_2\text{O}_3$
<i>C. nigrificans</i> strain			
55.....	205*	138	138
106.....	244	136	114
3750.....	196	141	107
7946.....	191	124	157
<i>S. desulfuricans</i> strain			
14 T.....	208	138	128
7757 T.....	205	150	167
8351.....	121	117	189
8395.....	253	128	189

* Mg H_2S per L. Values are corrected for H_2S production in absence of sulfate, sulfite, or thiosulfate. Conditions as described in text.

TABLE 2

Serologic agglutination responses of Clostridium nigrificans, Sporovibrio desulfuricans, and Desulfovibrio desulfuricans

O Antiserum	O Antigens Showing Agglutination	H Antiserum	H Antigens Showing Agglutination
<i>C. nigrificans</i>		<i>C. nigrificans</i>	
7946	7946, 14 T, 8351	7946	7946, 3750, 14 T, 8395
3750	None	3750	3750, 7946, 14 T, 8395
<i>S. desulfuricans</i>		<i>S. desulfuricans</i>	
14 T	14 T, 7946, 8351, 8395	14 T	14 T, 7946, 3750, 8351, 8395
8351	8351, 7946, 14 T, 8395	8351	8351, 7946, 14 T, 8395
8395	8395, 7946, 14 T, 8351	8395	8395, 7946, 3750, 14 T, 8351
<i>D. desulfuricans</i>		<i>D. desulfuricans</i>	
7757	7757	7757	7757

than lactate except for *C. nigrificans* strain 106 and *S. desulfuricans* strain 8395.

Characterization studies. Morphological, cultural, and biochemical studies revealed that all strains of *C. nigrificans* and *S. desulfuricans* possessed the properties described by Werkman and Weaver (1927) and Sealey (1951) for *C. nigrificans*. Furthermore, they were distinctly different from *D. desulfuricans*. The latter organism is a much smaller curved rod and is actively motile, possessing a single polar flagellum. The optimum growth temperature of this organism is 30 C, although growth can be obtained at 50 C by gradually increasing the incubation temperature as described by Baars (1930). *D. desulfuricans* does not form spores at 30 C, nor can spores be demonstrated in cultures adapted to 50 C. It was also found that *D. desulfuricans* is much more active in reducing sulfate at 35 C than adapted cultures of *C. nigrificans* or *S. desulfuricans*, and is equally as active at 45 C.

Immunological studies. Homologous and cross reactions were run against all H and O antisera with the H and O antigens of each strain studied. The data obtained are summarized in table 2. It may be seen that, with few exceptions, *C. nigrificans* and *S. desulfuricans* showed cross reactions in both somatic and flagellar antisera but not with the antisera of *D. desulfuricans*. Similarly, *D. desulfuricans* reacted only with homologous H and O antisera and showed no cross reactions with *C. nigrificans* or *S. desulfuricans*. These data show that *C. nigrificans* and *S. desulfuricans* are closely related, and that they are distinctly different from *D. desulfuricans*. It will be noted, however, that the O antigen of *C. nigrificans*

strain 3750 did not give a homologous reaction in 3750-O antiserum nor with any of the other strains tested. Apparently the O antigen preparation of this organism was not antigenic and did not stimulate antibody formation in the animals used in the immunization experiments. This point is being investigated.

The absence of cytochromes in C. nigrificans and S. desulfuricans. Postgate (1954a, b; 1955; 1956), Ishimoto *et al.* (1954) and Ishimoto and Koyama (1955) have demonstrated the presence of cytochromes in *D. desulfuricans* and have shown that these pigments are necessary for sulfate, sulfite, and thiosulfate reduction. We, therefore, examined cell free extracts of *C. nigrificans* and *S. desulfuricans* for the presence of cytochromes. For comparative purposes *D. desulfuricans* was also examined. With *D. desulfuricans* peaks were observed at 415–420, 525, 550–555, 580, and 630–632 m μ , confirming the findings of previous investigators. Figure 1 shows the spectrum obtained with *C. nigrificans* strain 3750, and is typical of that obtained with all cultures of *C. nigrificans* and *S. desulfuricans* investigated. It may be seen that cytochromes are completely absent in these organisms, although the presence of protohaem is indicated by the peak observed at 560–562 m μ . These findings have been confirmed by Postgate (*personal communication*) with six strains of *S. desulfuricans*.

Effect of temperature on morphology. Starkey (1938) reported that temperature greatly influenced the morphology of *Sporovibrio* cultures. We reexamined this point by preparing electron micrographs of strains of *C. nigrificans* and *S. desulfuricans* grown at 35 and 55 C and *D. desulfuri-*

cans grown at 35 and 50 C. Figure 2 shows *D. desulfuricans* grown at 35 C with the typical curved rod and single polar flagellum. At 50 C the organism has the same morphology. Figures 3 and 4 show *S. desulfuricans* and *C. nigrificans* respectively, grown at 55 C. Note that the organisms are much larger than *D. desulfuricans*, and that they have peritrichous flagella. When grown at 35 C the size, shape and flagellation pattern of *S. desulfuricans* and *C. nigrificans* are identical to those observed at 55 C. Thus, with the organisms employed in this study, we were unable to confirm the findings of Starkey (1938) that temperature

Figure 1. Absorption spectrum of cell free extract of *Clostridium nigrificans*.

Figure 2. *Desulfovibrio desulfuricans* strain 7757 grown at 35 C; $\times 18,540$.

Figure 3. *Sporovibrio desulfuricans* strain 8351 grown at 55 C; $\times 8,610$.

Figure 4. *Clostridium nigrificans* strain 3750 grown at 55 C; $\times 8,610$.

causes a change in the morphology of the sulfate reducing bacteria.

Classification. Data presented clearly show that the spore forming, thermophilic, sulfate reducers are a distinct group of organisms and are not temperature induced variants of *D. desulfuricans*. Furthermore the cultures isolated and designated *Sporovibrio* by Starkey (1938) are identical with those described by Werkman and Weaver (1927) as *C. nigrificans*. Since the latter name has taxonomic priority the thermophilic, spore forming, sulfate reducers should be properly named *Clostridium nigrificans*.

ACKNOWLEDGMENT

The authors wish to thank those who supplied the cultures used in this investigation. Sincere

appreciation is expressed to Dr. Owen B. Weeks of the University of Idaho for his helpful suggestions in the preparation of specimens for electron microscopy and to Mr. E. E. Spiker who made the electron micrographs.

SUMMARY

It was shown that *Clostridium nigrificans* can reduce sulfate, sulfite, and thiosulfate to hydrogen sulfide. The presence of cytochrome pigments in *Desulfovibrio desulfuricans* was confirmed and the absence of these pigments in *C. nigrificans* and *Sporovibrio desulfuricans* was established.

Morphological, cultural, immunological, and biochemical studies revealed that *C. nigrificans* and *S. desulfuricans* are identical, and that they are distinctly different from *D. desulfuricans*. Since the name *C. nigrificans* has taxonomic priority, the spore-forming, thermophilic, sulfate reducers should be properly named *C. nigrificans* and the name *Sporovibrio desulfuricans* should be placed in the list of *nomina rejicienda*.

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