Physiological and Biochemical Characteristics of Some Strains of Sulphate-reducing Bacteria

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SUMMARY

Fourteen previously unstudied strains of the genus *Desulfovibrio* and eight thermophilic strains of the genus *Desulfotomaculum* were allotted to species on the results of physiological and biochemical tests.

On DNA base composition the *Desulfovibrio* strains fell clearly into three groups, corresponding to *Dv. salexigens*, *Dv. desulfuricans* and *Dv. vulgaris*. NaCl-dependence appeared to be a constant and unique feature of *Dv. salexigens* strains, and 'sulphate-free' growth on pyruvate a constant characteristic of *Dv. desulfuricans*, although one strain of *Dv. vulgaris* also dismutated pyruvate. Fumarate dismutation showed no species correlation. 'Sulphate-free' growth on choline was often minimal and the test appeared to be of little value. A high degree of resistance to Hibitane was not a constant characteristic of *Dv. salexigens*.

The thermophilic strains showed great uniformity in DNA base composition, Hibitane resistance and 'sulphate-free' growth, and were all classified as *Desulfotomaculum nigrificans*.

INTRODUCTION

Saunders, Campbell & Postgate (1964) showed that 30 strains of non-sporulating dissimilatory sulphate-reducing bacteria could be divided into three groups defined by their deoxyribonucleic acid (DNA) base composition. With these and other data in mind, the classification of the dissimilatory sulphate-reducing bacteria was revised (Campbell & Postgate, 1965; Campbell, Kasprzycki & Postgate, 1966; Postgate & Campbell, 1966). The sporulating members were placed in a new genus, *Desulfotomaculum*, represented by three species: *Dt. nigrificans*, *Dt. ruminis* and *Dt. orientis* (Campbell & Postgate, 1965). The non-sporulating members were placed in the genus *Desulfovibrio*, represented by five species: *Dv. desulfuricans*, *Dv. vulgaris*, *Dv. salexigens*, *Dv. gigas* and *Dv. africanus* (Campbell *et al.* 1966; Postgate & Campbell, 1966).

The present paper reports the results of a study of the physiological and biochemical characteristics of 14 previously unstudied strains of the genus *Desulfovibrio* and eight thermophilic strains of the genus *Desulfotomaculum*.

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METHODS

Organisms. The following 22 strains of sulphate-reducing bacteria were obtained as ampoules of freeze-dried material from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland (NCIB):

(1) Mesophilic strains (non-sporulating; growth temperature 30°). (a) Salt-water strains: NCIB 8309 (EL AGHEILA A), 8315 (NEW JERSEY SW-8), 8316 (NEW JERSEY SW-3), 8326 (CALIFORNIA 29:137:5), 8396 (EL AGHEILA 4), 8399 (VENEZUELA), 8400 (HOSSEGOR), 8402 (EL AGHEILA 2) and 9492 (ABERDOVEY). (b) Fresh-water strains: 8456 (DENMARK, a morphologically unusual strain showing predominantly long spirillar forms), 8457 (WOOLWICH) and 8458 (BYRON).

(2) Thermophilic strains (fresh-water strains; spore-bearers; growth temperature 55°): 8356 (HOLLAND CT), 8357 (DELFT 48 T), 8359 (DELFT 3 T), 8360 (DELFT 13 T), 8361 (DELFT 15 T), 8394 (DELFT 1 T), 8706 and 8788 (unnamed strains).

Cultivation. Cultures were grown anaerobically, from freeze-dried specimens, at 30° or 55° as appropriate, in the medium of Baars (1930) containing 1.0 g. Difco yeast extract/l. and 5 mm-cysteine hydrochloride. NaCl 25.0 g./l. was added for the salt-water organisms. Details of the preparation, sterilization and pH adjustment of this medium were as given by Saleh, Macpherson & Miller (1964). After revival, stock cultures of the mesophilic strains were maintained in the medium C of Butlin, Adams & Thomas (1949) as modified by Professor J. R. Postgate (see Baker, Papiska & Campbell, 1962), and subcultured weekly. The thermophilic strains gave better growth in Baars medium, in which stock cultures were therefore maintained. Stock and experimental cultures were grown in Pyrex test tubes or flasks plugged with cottonwool and incubated in McIntosh and Fildes anaerobic jars in an atmosphere (except when stated otherwise) of 99% (v/v) H₂ + 1% (v/v) CO₂.

Stock cultures, and all experimental cultures except those in the Hibi-tane test, were tested for aerobic and anaerobic contaminants (Postgate, 1953).

Pigment detection. For cytochrome *c*₃ demonstration, fully grown 200 ml. cultures in medium C were concentrated by centrifugation and examined with the Hartridge reversion spectroscope after the addition of sodium dithionite. A strong absorption band at about 554 mμ and a weaker one at 525 mμ indicated the presence of cytochrome *c*₃. Desulfoviridin was tested for by the method of Postgate (1959a).

Maximum growth temperature. In view of the significance of thermophily in the taxonomy of sulphate-reducing bacteria, the limiting growth temperature was determined for the strains stated by the NCIB to be mesophilic. Test-tube cultures in medium C were incubated in anaerobic jars immersed in a thermostat tank initially at 37° ± 0.2°; the maximum growth temperature was observed by making serial subcultures with incubation temperature increments of 0.5°.

Resistance to Hibi-tane. The bacteriostatic concentration of Hibi-tane (Imperial Chemical Industries Ltd., Wilmslow, Cheshire) towards each strain in Baars medium was determined by the method of Saleh (1964).

Adaptation to growth in salt-water or freshwater media. Serial subcultures of the mesophilic strains were made in medium C with increments or decrements of 5.0 g./l. in NaCl concentration. Three successive subcultures were made in the final medium before adaptation was considered to be proved. In later stages of the experiment,

growth often took more than a week to appear; results were therefore not recorded as negative until three weeks after inoculation.

Pyruvate dismutation test. Strains were examined for sulphate-free growth by using the pyruvate medium of Postgate (1963*a*) containing cysteine hydrochloride. The sodium pyruvate used was recrystallized from 80% (v/v) ethanol in water. The cultures were incubated under nitrogen. Persistence of growth through five subcultures was taken as evidence of ability to grow by pyruvate dismutation.

Choline utilization. Strains were examined for anaerobic growth (under N₂) on choline, with and without 26 mM-sodium sulphate, by using the medium of Baker *et al.* (1962) containing cysteine. The same criterion of substrate utilization was used as in the pyruvate test (smell of trimethylamine is an unsuitable criterion of growth in the case of large numbers of cultures incubated together, since the gas penetrates into cultures in which no growth has occurred).

Estimation of growth. In the pyruvate, choline, and NaCl adaptation tests, growth was often scanty and therefore difficult to measure turbidimetrically or by microscopic examination. Growth in the final subculture was thus visually graded —, ± (turbidity just discernible in daylight), or +.

Buoyant density measurements. DNA was isolated and purified as described by Saunders *et al.* (1964). The buoyant density of the DNA was determined by CsCl density gradient centrifugation (Saunders *et al.* 1964) with ¹⁵N-labelled *Pseudomonas aeruginosa* DNA ($\rho = 1.742$ g. cm.⁻³) as a density reference standard. Buoyant densities and base compositions of the DNA were calculated according to the equations of Sueoka (1961).

RESULTS

All results except those of the pigment tests are summarized in Table 1.

Pigment tests. All the mesophilic strains contained cytochrome *c*₃ and desulfovibrin; the thermophilic strains contained neither pigment.

Inhibition by Hibitane. Saleh (1964) tested the ABERDOVEY strain against Hibitane and found the bacteriostatic concentration to be 10 µg./ml.; in our experiment with this strain it was 100 µg./ml. None of our strains ascribed to the species *Desulfovibrio salexigens* on DNA base composition grew in the presence of 50 µg. Hibitane/ml. Since the members of this species tested by Saleh resisted 1000 µg./ml., we checked our experimental technique by re-testing one of the strains of *Dv. salexigens* that Saleh had tested (CALIFORNIA 43:63; NCIB 8364). Our result confirmed his finding with this strain.

NaCl adaptation. Only one strain isolated from a freshwater habitat was unable to grow in 2.5% (w/v) NaCl. At the first attempt this strain (BYRON) did not survive in 2% NaCl; in a second attempt, starting again with a culture in NaCl-free medium, growth occurred in the presence of 2.0% NaCl but not 2.5%.

Pyruvate dismutation. This test was made at Urbana, Illinois, U.S.A., and at Teddington, Middlesex, England; somewhat different results were obtained. In the case of the 'doubtful' dismuter AUSTRALIA and the 'positive' dismuter EL AGHEILA A (Teddington results) the fifth subculture was examined microscopically: typical vibrios were seen. The cultures were uncontaminated.

Choline utilization. The whole choline utilization experiment was performed twice. In some cases, growth was obtained in one experiment but not the other; these are

Table 1. *Physiological and biochemical characteristics of 22 strains of sulphate-reducing bacteria*

The mesophilic strains are arranged in three groups according to their DNA base composition (% guanine + cytosine, G + C), and their classification derived from this (see Postgate & Campbell, 1966) is shown.

Name of strain	NCIB no.	Max. growth temperature	Hibitane: bacterio-static concentration ($\mu\text{g./ml.}$)	Pyruvate dismutation			Choline dismutation	Growth on choline + sulphate	% G + C in DNA	Classification from DNA composition
				Urbana expts	Tedding-ton expts	NaCl adaptation				
Mesophilic										
NEW JERSEY SW-8	8315 (S)	42.5°	25	—	—	Down to 0.25 %	—	—	44.7	<i>Desulfovibrio salexigens</i>
NEW JERSEY SW-3	8316 (S)	42.5°	25	—	—	Down to 0.25 %	—	±	45.6	
AUSTRALIA	8329 (S)	39.0°	5	—	±	Down to 0.75 %	—	+	45.6	
EL AGHEILA 2	8402 (S)	39.0°	50	—	—	Down to 0.5 %	—	±	44.6	
CALIFORNIA 29:137.5	8326 (S)	43.5°	100	±	±	Down to 0	±	±	56.3	<i>Desulfovibrio desulfuricans</i>
CALIFORNIA 29:137.11	8339 (S)	44.5°	50	+	+	Down to 0	—	±	56.3	
EL AGHEILA 4	8396 (S)	44.0°	2.5	+	+	Down to 0	±	+	56.3	
HOSSEGOR	8400 (S)	43.5°	10	+	+	Down to 0	±	—	56.3	
BYRON	8458	39.0°	5	+	+	Up to 2.0 %	±	+	56.6	<i>Desulfovibrio vulgaris</i>
ABERDOVEY	9492 (S)	42.5°	100	+	+	Down to 0	±	+	54.4	
EL AGHEILA A	8309 (S)	44.5°	2.5	—	+	Down to 0	—	+	60.2	
VENEZUELA	8399 (S)	46.0°	100	—	—	Down to 0	—	—	60.8	
DENMARK	8456	44.5°	25	—	—	Up to 2.5 %	—	—	60.2	<i>Desulfovibrio maculatum nigrificans</i> †
WOOLWICH	8457	45.0°	25	—	—	Up to 2.5 %	±	±	60.2	
Thermophilic										
HOLLAND CT	8356	*	< 0.1	+	+	*	—	—	46.6†	<i>Desulfovibrio maculatum nigrificans</i> †
DELFT 48 T	8357	*	< 0.1	±	±	*	—	—	46.6	
DELFT 3 T	8359	*	< 0.1	+	+	*	—	—	45.6	
DELFT 13 T	8360	*	< 0.1	+	+	*	—	—	45.6	
DELFT 15 T	8361	*	< 0.1	+	±	*	—	—	45.6	
DELFT 1 T	8394	*	< 0.1	+	+	*	—	—	45.6	
Unnamed	8706	*	< 0.1	+	+	*	—	—	45.6	
Unnamed	8788	*	< 0.1	+	±	*	—	—	45.6	

(S) = strain isolated from a salt-water environment. — = no growth. ± = doubtful or scanty growth. + = growth.

* Test not made; † all % G + C values for the thermophiles taken from Saunders & Campbell (1966); ‡ see text.

recorded as $\pm$ in Table 1, as also are strains which gave scanty growth. Some strains, recorded as negative, produced scanty growth and died out on or before the fifth subculture. Where positive growth occurred, it was much less than in sulphate-free pyruvate medium.

DNA-base composition. As judged by DNA base composition all 14 *Desulfovibrio* strains could be placed in the three groups of Saunders *et al.* (1964). Both marine and freshwater strains appear in group I (60% guanine + cytosine, G + C) and group II (54–56% G + C); the obligate salt-water strains all belonged to group III (45–46% G + C). The DNA base compositions of the *Desulfotomaculum nigrificans* strains were reported previously by Saunders & Campbell (1966).

DISCUSSION

The physiological and biochemical data show that the 14 strains of non-sporulating dissimilatory sulphate-reducing bacteria studied here belong to the genus *Desulfovibrio*; by using the taxonomic criteria outlined by Postgate & Campbell (1966), it was possible to place the strains into three species: *Dv. vulgaris*, *Dv. desulfuricans* and *Dv. salexigens*. The thermophilic sporulating sulphate reducers were identified as *Desulfotomaculum nigrificans* (Campbell & Postgate, 1965).

Saleh (1964) reported that the Hibitane resistance of *Desulfovibrio* strains showed a correlation with the DNA base composition. This apparent correlation did not hold for the strains examined in the present work. Moreover, it has been found that a given strain can show a tenfold variation in its Hibitane resistance at different times (or in the hands of different workers). Thus Hibitane resistance is a less useful taxonomic character for this group of organisms than was originally thought, though very high resistance still predominates in the *Dv. salexigens* group and relatively low resistance in the *Dv. vulgaris* and *Dv. desulfuricans* groups.

The readiness with which our marine and freshwater strains of *Desulfovibrio desulfuricans* and *Dv. vulgaris* could be adapted to grow either in the absence or presence of NaCl confirms the contention of Postgate & Campbell (1966) that salt relations are not of taxonomic significance in these two species. Our obligate salt-requiring strains were classified as *Dv. salexigens* (see Postgate & Campbell, 1966), though it is interesting that three of our four strains grew at lower NaCl concentrations than that stated by these authors to be required by *Dv. salexigens* (> 0.6%). It was not determined at this stage whether our strains required Na^+ or Cl^- ; a more comprehensive study of the salt relations of this species is under way.

The problems of using carbon source utilization data for speciation in the genus *Desulfovibrio* were discussed by Postgate (1959*b*), Macpherson & Miller (1963), Postgate (1965), and Postgate & Campbell (1966). The ability to grow on choline or pyruvate in the absence of sulphate appears to separate *Dv. desulfuricans* from other species of the genus *Desulfovibrio*. However, the difficulty in obtaining repeatable results with choline, coupled with the scanty growth of many strains of *Dv. desulfuricans* on this substrate, makes the use of this character questionable. Miller & Wakerley (1966) reported that the ability to grow on sulphate-free fumarate media is possessed by strains which have now been shown to belong to four of the five species of the genus *Desulfovibrio*. Fumarate dismutation is therefore not a valid taxonomic character for this group of organisms. Thus, except for dismutation of pyruvate, the utilization of carbon substrates does not appear to be useful in characterizing these organisms.

Strain EL AGHEILA A presents a taxonomic problem similar to that of Postgate's (1963*b*) oxamate-utilizing strain MONTICELLO 2. In its ability to grow on pyruvate in the absence of sulphate, EL AGHEILA A resembles *Desulfovibrio desulfuricans*; its DNA base composition, however, places it with the *Dv. vulgaris* group. We have given greater weight to the DNA base composition and have classified EL AGHEILA A as *Dv. vulgaris* (it should be noted that EL AGHEILA A does not utilize oxamate when tested by the method of Postgate, 1963*b*).

Our results with *Desulfotomaculum nigrificans* strains in sulphate-free medium confirm the previous observations of Postgate (1963*a*) and Akagi (1964) with this species.

With this study of 22 previously unclassified strains, most of the strains of the genera *Desulfovibrio* and *Desulfotomaculum* at present available from recognized culture collections have now been examined. The findings are consistent with the working classification schemes proposed for these two genera by Campbell & Postgate (1965) and Postgate & Campbell (1966).

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