J.R.POSTGATE

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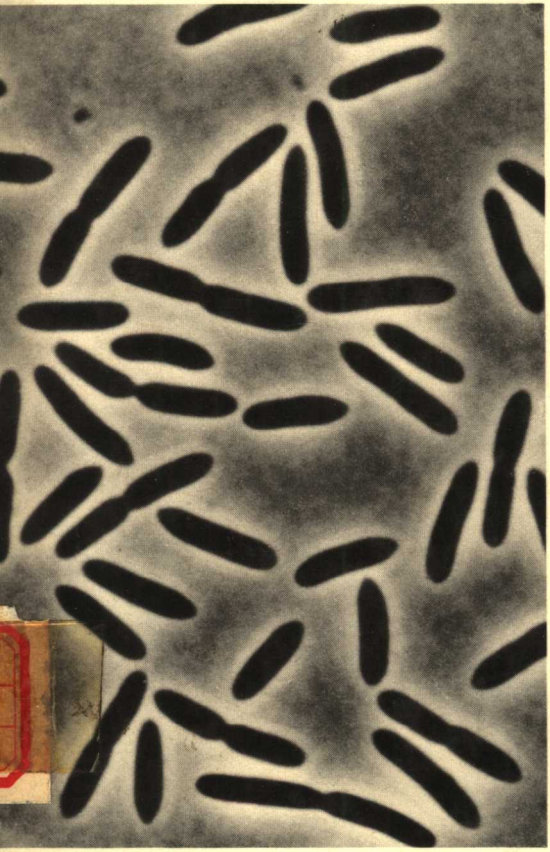

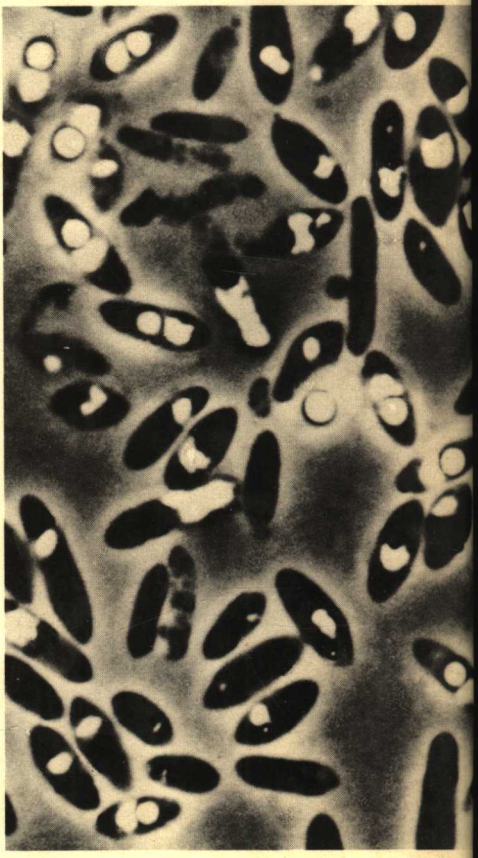

The sulphate-reducing bacteria

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Preface

The sulphate-reducing bacteria are, as I hope this monograph will demonstrate, a bizarre group of microbes of which most people, including many microbiologists, know nothing. Yet these organisms impinge on our lives in a variety of subtle, and occasionally blatant, ways. Despite their fascinating qualities they have been a somewhat neglected backwater of microbiological research: smelly, awkward to grow, intractable to isolate and count, but revealing intriguing novelties of biochemistry and physiology to those persistent enough to stick with them. The late K. R. Butlin pointed out that the Dutch, whose canals often provide so generously fetid a habitat for these bacteria, had a vested interest in knowing about them, and it is no coincidence that they were discovered by the great Dutch microbiologist M. W. Beijerinck, nor that Dutchmen such as Elion, van Delden, Baars and Kluyver laid the groundwork of today's knowledge. Butlin numbered determination among his many qualities and I owe him a lifelong debt for introducing me to these bacteria in the late 1940s, at an early stage in my scientific career, pointing my nose in the right direction and leaving me to get on with it. In due course I came to know personally nearly everyone in the world working on these bacteria: Bill Bunker, Claude ZoBell, Robert Starkey, Syd Rittenberg, Jacques Senez, Leon Campbell, Harry Peck, Jean Le Gall and a small host of others – a few I knew only by correspondence. It was a small, friendly scientific community within which rivalries and antagonisms, while not completely absent, played no important part in the accumulation and distribution of scientific information. Today, when the struggle for priority in publication has made much of scientific research a disagreeable rat-race, I recall our earlier academic calm with perhaps rose-tinted nostalgia. For there were jealousies and unseemly rushes into print, but when we met we still discussed our work freely and often exchanged manuscripts before

submitting them for publication (a practice which is certainly rare among today's scientists, at least in 'trendy' areas of research). The sulphate-reducing bacteria never really became trendy, though occasional catastrophes (a corrosion disaster, a world sulphur shortage) or a spectacular discovery (a cytochrome structure, their mixotrophy) might lead to a brief display of their talents in the popular or serious scientific press.

Inconspicuousness has its advantages, and I hope this monograph will not bring sulphate reduction too far into the forefront of competitive research. On the other hand, these are very important microbes, not only from an academic point of view but also in numerous practical ways. And it is regrettable that the student, teacher or technologist, if he seeks to find out about them, will find them dismissed in a paragraph or two of most microbiological textbooks and will have to burrow into quite obscure and ancient reviews of topics ranging from metallurgy to straight microbiology. In this monograph I have tried to compensate for that situation: to pull together in compact form the state of our knowledge of these bacteria as we approach the 1980s. I have also tried to write down some of the microbiological 'lore' necessary for handling these bacteria, such as that which gave my colleague, the late Miss M. E. Adams, green (black?) fingers, enabling her to isolate and maintain the first collection of reliably pure cultures in the world.

Microbiology is a science, but a touch of art and craft is always desirable, even essential, for progress to be made. I offer this monograph, then, as a largely but not entirely scientific handbook for those whose academic or practical compulsions have brought them face to face with these exotic forms of life.

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October 1978 John Postgate

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1

Introduction

The element sulphur is widespread on this planet and, like most of the commoner elements, it has become an essential component of the biosphere: part of the chemical structure of living things. Protoplasm contains between 0.4% and 1% of sulphur (as organic sulphur compounds), depending on the type of cell and the environment from which the cells came. For many millions of years – at least 5×10^8 years – the sulphur at the surface of the planet (available to the biosphere) has existed predominantly in an oxidized form: as sulphates in soils, rocks, rivers and seas; as sulphur oxides, a minor component of the atmosphere. To mobilize such sulphur for biological use, it must be reduced, so the biological reduction of sulphate, like biological nitrogen fixation and biological oxygen production, has become recognized as one of the critically important processes on which life on the planet depends. As far as we know, animals do not conduct this reaction: from protozoa to man, they appear to depend on plants and/or microbes for their supplies of reduced sulphur. Green plants, fungi such as yeast and many species of bacteria, unlike animals, can use sulphate as their sole source of the biological element sulphur. In so doing, they reduce the sulphate ion, bringing the sulphur atom from its fully oxidized state to its fully reduced state. This process is *assimilatory sulphate reduction*, so called because the sulphur is assimilated: it is incorporated into the organisms' protein as sulphur-containing amino acids or built into co-factors such as biotin and pantothenic acid. The biochemical pathways and means of regulation of assimilatory sulphate reduction are fairly well understood, but this subject will not form part of this monograph. Instead, I shall be concerned with a different process, of perhaps equal biological importance, conducted by a group of bacteria which, as far as we know at present, is unique in its peculiar physiology.

The name 'sulphate-reducing bacteria' is conventionally reserved

for a class of microbes which conducts *dissimilatory sulphate reduction*. In this process the sulphate ion acts as an oxidizing agent for the dissimilation of organic matter, as does oxygen in conventional respiration. A small amount of reduced sulphur is assimilated by the organism, but virtually all is released into the external environment as the sulphide ion, usually substantially hydrolysed to free H_2S. The process has also been called 'sulphate respiration', analogous to 'nitrate respiration' found among nitrate-reducing and denitrifying bacteria. In addition, it has superficial analogies to carbonate reduction, a process conducted by certain methanogenic bacteria. Sulphate respiration is not encountered on this planet outside certain specialized bacteria. To provide a quantitative guide to the difference in scale between assimilatory and dissimilatory sulphate reduction, one can make the following comparison. In conditions of sulphate limitation *Klebsiella aerogenes* yields about 200 mg dry wt organisms/mg sulphur (Postgate & Hunter, 1962); the yield with *Desulfovibrio* (a group of sulphate-reducing bacteria) depends on the carbon source but is in the region of 0.5 to 1 mg dry wt organisms/mg sulphur.

Though the sulphate-reducing bacteria have been known for over seven decades, little information about them has penetrated to conventional microbiology textbooks. Therefore this monograph, though primarily concerned with information gained during the last three decades, will necessarily allude to earlier work. These bacteria have been the subjects of periodic reviews in the specialized literature; those by Bunker (1936), Starkey & Wight (1945), Postgate (1959*a*, 1960*a*, 1965*a*) and Le Gall & Postgate (1973) form a series which may be consulted for amplification of various aspects mentioned in this monograph.

The sulphate-reducing bacteria were discovered by Beijerinck (1895); van Delden (1903) reported marine, salt-tolerant varieties and Elion (1925) described thermophilic types. Baars (1930), in a thesis which was published but which is not widely available, provided a most extensive study of these bacteria, one which is still an absorbing document though much of its information has been superseded. Such early work was reviewed by Bunker (1936) and briefly by Butlin, Adams & Thomas (1949). The sulphate-reducing

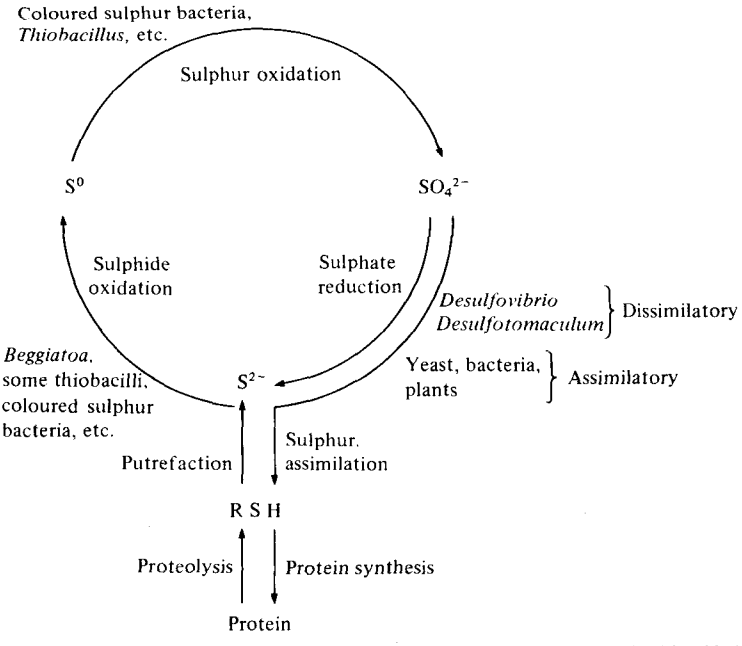

Fig. 1. The biological sulphur cycle. Sulphate (SO_4^{2-}) is reduced to sulphide (S^{2-}) by dissimilatory sulphate-reducing bacteria and provides substrates for sulphide-oxidizing bacteria who convert it, by way of elemental sulphur (S^0) back to sulphate. In assimilatory sulphate reduction, the sulphur of sulphate passes through the sulphide level of oxidation and becomes incorporated into an amino acid (RSH) before being built into plant as microbial protein. This is eaten by animals and the sulphur is eventually returned to the cycle as sulphide formed during the breakdown and putrefaction (by bacteria) of dead organisms.

bacteria are all very strict anaerobes. Some are now known to be capable of fermentative growth in the absence of sulphate, analogous to the fermentative growth of a yeast without oxygen, but none can grow with oxygen as electron acceptor, and oxygen always inhibits their growth. They grow relatively slowly compared with a common soil or water organism such as *Pseudomonas* (partly because growth of cultures is often non-exponential, see Chapter 3) but they have a remarkable capacity for survival in terrestrial and aquatic environments (see Chapter 7). They are very widely distributed, ready to become active whenever local conditions become anaerobic.

They play an important part in the biological sulphur cycle and

this must be discussed briefly before proceeding further; it is illustrated in Fig. 1. It is a formalized scheme of the chemical transformations undergone by the sulphur atom in nature through biological agencies and is analogous to the rather better known biological nitrogen cycle; it is related to, but should not be confused with, geochemical sulphur cycles (e.g. Kellog *et al.*, 1972), which describe the translocation of sulphur in various chemical forms about the planet as a result of burning fuels, putrefaction of organic matter, dissolving of soluble sulphur compounds in rain and rivers, their retention or exclusion in soils by ion exchange. The sulphate-reducing bacteria contribute to both types of cycle; in the biological version presented in Fig. 1 they by-pass assimilatory sulphate reduction and generate H_2S in sufficient amounts to support growth of the sulphide- and sulphur-oxidizing bacteria. A microbial ecosystem consisting of interdependent sulphur-oxidizing and sulphate-reducing bacteria is called a 'sulfuretum' and is discussed in more detail in Chapter 7 (p. 92).

Some historical errors

Because of the strict anaerobic habit and slow growth of sulphate-reducing bacteria, many of the earlier workers used impure cultures, albeit unintentionally; even in 1949 it seemed likely that only a few pure cultures were available in the world (Butlin *et al.*, 1949) and not all those were as pure as their proprietors believed (Postgate, 1953*a*). New methods for obtaining pure cultures are now available, which, together with more explicit criteria of purity, have somewhat eased the problem of contamination (see Chapter 3), but a few instances in which misinformation arose from use of impure cultures should be mentioned.

Desulfovibrio rubentschickii. Pure cultures of most sulphate-reducing bacteria, when utilizing ordinary carbon substrates with more than three carbon atoms per molecule (e.g. lactate or malate), dissimilate the carbon source to only the acetate level of oxidation; acetate accumulates and is not a growth substrate. Yet in crude enrichment cultures acetate slowly disappears and in nature acetate does not accumulate even where sulphate-reducing bacteria are very active. An acetate-utilizing species, *Desulfovibrio rubentschickii*, was described by Baars (1930). Exhaustive attempts to re-isolate this species failed

in several laboratories, though ordinary acetate-forming sulphate-reducing bacteria were easily detected, which suggested that *D. rubentschickii* must have been some kind of mixed commensal population (see Selwyn & Postgate, 1959). There the position rested for several years, with no further clue as to where the acetate went, until the mid-1970s, when Pfennig & Biebl (1976) discovered a 'sulphur-reducing bacterium', *Desulfuromonas acetoxidans*, which reduced the element sulphur (but not sulphate or sulphite) to sulphide at the expense of acetate.

$$4S + NaOOC.CH_3 + 3H_2O \rightarrow 4H_2S + NaHCO_3 + CO_2$$
$$(\Delta G = 5.7 \text{ kcal})^1$$

This organism provided a partial explanation of the disappearance of acetate: its oxidation with partly reduced sulphur could occur. However, a year later Widdel & Pfennig (1977) finally solved the problem by isolating a true acetate-oxidizing sulphate-reducing bacterium of the spore-forming group (*Desulfotomaculum*). They named it *Desulfotomaculum acetoxidans*; contrary to Baars's report, it does not utilize the common substrates of *Desulfovibrio* (such as lactate and pyruvate) and it is also distinctive (see Chapter 3) in other ways. Whether Baars and later workers actually had this species in their cultures will probably never be known, but the work of Pfennig and his colleagues satisfactorily showed that acetate-supported sulphate reduction can and does occur and is the property of a distinct species.

Chloropseudomonas ethylica. This 'organism' features in the scientific literature as a type of green photosynthetic sulphur bacterium. In fact it proved to be a mixed culture of a *Chlorobium* and a sulphate-reducing bacterium. Since the details of this error concern the subject of this monograph only peripherally, they will not be presented (see Gray, Fowler, Nugent & Fuller, 1972; Gray, 1977).

Interconversion of mesophilic and thermophilic types. Sulphate-reducing bacteria include both ordinary mesophilic strains and thermophilic strains able to grow at temperatures between 50 and 70 °C. Kluyver & Baars (1932) believed that these were adaptive

[1] See footnote '*', Table 3, p. 47.

variants of the same organism, a view supported by Starkey (1938) and other workers including H. J. Bunker (see Postgate, 1953*b*). However, Campbell, Frank & Hall (1956) showed conclusively that the thermophilic types were a completely different species, hitherto known as *Clostridium nigrificans* and not recognized as sulphate-reducing bacteria, a finding which ultimately led to their reclassification in the genus *Desulfotomaculum* (see Chapter 3). My unpublished experiments in 1954–56 also tended to support the views of Campbell *et al.* (1956) and I was later fortunate in being able to inspect the laboratory notes from 1936–38 of the late H. J. Bunker, when he believed he had substantiated Baars's and Starkey's findings. On close examination, it was clear that the viability of the mesophilic (30 °C) strains cultured at 55 °C had not been checked: Bunker had only given them one passage at the 'thermophilic' temperature. He used cultures of about 60 ml volume, and blackening, due to FeS precipitation (see Chapter 3), was his criterion of growth. It was a matter of simple experiment to show, in 1958, that a vigorous culture of mesophilic desulfovibrios could undergo one division, perhaps two, *while a 60–ml culture was warming up* in a conventional 55 °C incubator. Certainly it could produce enough H_2S to simulate growth during that period. Bunker's mesophiles which had 'adapted' to thermophiles were not rigorously checked for acquirement of the thermophilic character – reasonably enough in view of the published background. His thermophilic species which had 'adapted' to being a mesophilic species still grew very slowly – and this is true of most strains of *Desulfotomaculum nigrificans*. These criticisms do not apply to earlier work and it is less easy to account for the observations of Baars and Starkey; in discussing the reasons for those earlier findings, Campbell & Postgate (1965) could only conclude that the populations which apparently showed convertibility from mesophilic to thermophilic types were initially mixtures of both types.

Today, the adaptive interconversion of mesophilic and thermophilic species of sulphate-reducing bacteria must be regarded as mistaken. This view does not preclude the existence of naturally-occurring strains of unusual temperature habit and, indeed, natural isolates of both mesophilic *Desulfotomaculum* and thermophilic *Desulfovibrio* have been reported (see Chapter 3). It also does not

preclude the possibility of transformational changes to thermophilic habit of the kind reported by Lindsey & Creaser (1975) in bacilli.

Presence of syntrophic contaminants. Postgate (1953*a*) was obliged to revise a number of quantitative data covering the biochemistry of a strain because it proved to be contaminated with a non-sulphate-reducing organism. This organism was a strict anaerobe and required the presence of the sulphate reducer for growth, except in very rich media. Thus it eluded the then customary methods of checking for contaminants.

A fourth historical error did not arise from impure cultures but is nonetheless important. It concerns their status as autotrophs. Most strains can use gaseous hydrogen for the reduction of sulphate:

$$4H_2 + SO_4^{2-} \rightarrow 4H_2O + S^{2-}$$

If the energy yield of this reaction could be coupled to the assimilation of CO_2, the organisms would be capable of growing in a purely mineral environment: they would be true autotrophs. Butlin & Adams (1947) thought they had evidence for weak but real autotrophic growth of pure cultures because more bacteria grew in a mineral medium under H_2 than under N_2. Adams, Butlin, Hollands & Postgate (1951) isolated a hydrogenase-deficient variant strain which did not show improved growth under H_2. However, tests with labelled CO_2 a decade later failed to confirm autotrophy (Mechalas & Rittenberg, 1960; Postgate, 1960*b*) and Mechalas & Rittenberg concluded that the apparent autotrophy was not real. In fact, assimilation of organic impurities in the putatively mineral media was being stimulated by H_2. This process could be duplicated by substrates such as *n*-butanol, which acted as a source of H_2 but was not itself assimilated. Today these bacteria are recognized not to be true autotrophs, though they are capable of a coupled assimilation of acetate and CO_2 together, a reaction (mixotrophy) which verges upon autotrophy (see Chapter 3).

Finally, while discussing historical errors, the fact should be mentioned that all viable counts of sulphate-reducing bacteria reported before 1955 were probably incorrect, as well as many reported after that date; this matter is discussed further in Chapter 3.

2

Classification

The two well-established genera of sulphate-reducing bacteria, *Desulfovibrio* and *Desulfotomaculum*,[1] seem to be quite unrelated to each other, and any affinities they may have to other groups of bacteria have become obscured in the course of evolution. A third genus, *Desulfomonas*, is very like *Desulfovibrio* (see below). The genus *Desulfovibrio* is the best known, largely because its members are usually somewhat easier to isolate and purify; they are usually mesophilic and can be halophilic; they do not form spores. Earlier synonyms for this genus were *Spirillum*, *Microspira*, *Vibrio* and *Sporovibrio*;[2] the type species is *Desulfovibrio desulfuricans*. The second genus, now known as *Desulfotomaculum*, may be mesophilic or thermophilic but naturally-occurring halophilic strains[3] are not known. The thermophilic species, *Desulfotomaculum nigrificans*, was earlier known as *Clostridium nigrificans*. All members of the genus form spores. Both genera are Gram-negative.

The taxonomy of the sulphate-reducing bacteria is in an unsatisfactory state, having become confused in the 1920s to 1940s by the prevalence of impure cultures and the use of inappropriate culture media (see Chapter 3). These points matter, because impure cultures are capable of metabolizing a wider range of carbon sources than are pure ones (e.g. Kimata, Kadota & Hata, 1955*b*) and the presence or absence of a reducing agent influences considerably the apparent range of carbon sources attacked (Grossman & Postgate, 1953).

[1] Being of Latin derivation, *Desulfovibrio* and *Desulfotomaculum* are etymologically correct spellings; *Desulphovibrio* and *Desulphotomaculum* are incorrect, though they appear in some British journals. Sulphur ought to be spelled 'sulfur', since the Romans had no 'ph', but it is a century too late for this error to be corrected.

[2] Pochon & de Barjac (1954) assigned the name *Sporovibrio ferro-oxidans* to a spore-forming vibrio that oxidized Fe^{2+} anaerobically at the expense of nitrate reduction. The report was brief and the strain was lost; whether it had any biological relationship to the spore-forming sulphate-reducing bacteria is not known.

[3] Since I wrote this chapter, Nazina & Rozanova (1978) reported a natural halophilic *Desulfotomaculum* from an oil stratum.

Baars (1930) regularly added sterile H_2S-water to his cultures; the reducing effect of this may account for the fact that his cultures used a wide range of carbon sources for growth, whereas most subsequent workers have recorded very limited fermentative abilities. A small amount of yeast extract is sometimes added to cultures to enhance growth; when tested in media with yeast extract, a strain will sometimes show a wider substrate range.

Pure cultures have been available for some decades now, and so have prescriptions for suitable media, but still the taxonomic picture is unsatisfactory. At the root of the problem is the relatively small number of diagnostic properties that one can assign. The primary taxonomic character is dissimilatory sulphate reduction; in a monumental survey of 92 isolates using 116 biochemical characters, Skyring, Jones & Goodchild (1977) found only 26 subsidiary characters to be of taxonomic value, and several of those were of only limited use. Nomenclature must be based on taxonomy, but if the taxonomy is faulty what can one do? Table 1 is a working classification based on that of Campbell & Postgate (1965, 1969) and Postgate & Campbell (1966), up-dated with newly named species even where these are of uncertain status. It is not inconsistent with the statistical analysis of Skyring *et al.* (1977) but future taxonomic study will no doubt impose subdivision and deletions on the scheme given in Table 1. Not all the types listed in Table 1 are accepted in the eighth edition of *Bergey's manual of determinative bacteriology* (Buchanan & Gibbons, 1974); new editions should be consulted to up-date the information in Table 1.

Several features of Table 1 require amplification and an appropriate commentary follows.

Morphology

Morphologically most desulfovibrios are curved (Figs. 2*a*, 3*a*) and most desulfotomacula are straight (Figs. 2*b*, 3*b*), but departures from the rule exist. For example, *Desulfotomaculum orientis* was incorrectly classified as a desulfovibrio when first isolated because of its curved appearance (Adams & Postgate, 1959), while the Berre strains and the rather unusual strain Norway 4 of *Desulfovibrio desulfuricans* are usually straight. Both genera are prone to pleomorphism in old

Table 1. *A key to the classification of sulphate-reducing bacteria*

Character	*Desulfotomaculum* (spore-formers)				
	nigrificans	*orientis*	*ruminis*	*antarcticum*[1]	*acetoxidans*[6]
Form	Rod	Curved rod	Rod	Rod	Rod
Flagella	Peritrichous	Peritrichous	Peritrichous	Peritrichous	Polar, thick
Spores	+	+	+	+	+
Principal cytochrome	*b*	*b*	*b*	*b*	*b*
Desulfoviridin	−	−	−	−	...[4]
% guanine + cytosine	45	42	46	...[4]	37
Growth with:					
lactate plus sulphate	+	+	+	+	−
pyruvate minus sulphate	+	−	+	...[4]	−
formate plus sulphate	−	−	+	−	...[4]
acetate plus sulphate	−	−	−	−	+
glucose plus sulphate	−	−	−	+	−
Gelatinase	−	−	−	+	...[4]
NaCl requirement	−	−	−	−	−
Thermophily	+	−	−	−	−
Hibitane resistance[3] (mg/litre)	0.25	0.25	1	...[4]	...[4]