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BACTERIAL FORMATION OF SULPHIDE FROM OXIDIZED FORMS OF INORGANIC SULPHUR

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SUMMARY

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Key words: bacteria, hydrogen sulphide formation, oxidized inorganic sulphur

I INTRODUCTION

Many ores have been deposited under reducing conditions with the resulting formation of sulphides. Some are simple sulphides such as pyrites (FeS_2), chalcocite (Cu_2S), sphalerite (ZnS), millerite (NiS), greenockite (CdS), sycoporite (CoS), molybdenite (MoS_2), galena (PbS) and cinnabar (HgS) while others are complex 'mixed' sulphides such as chalcopyrite (CuFeS), stannite (Cu_2FeSnS_4) cobaltite (CoAsS) and pentlandite ($[Ni,Co,Fe]_9S_8$). The weathering of sulphide ores brought about either by direct chemical action or mediated by microbial attack results in the production of water-soluble forms of sulphur such as thiosulphate and sulphate. These may then be discharged into streams and lakes. The question raised in this investigation was "In such aquatic systems are bacteria present which are capable of reducing oxidized forms of inorganic sulphur to sulphide which then reacts with base metal cations forming insoluble sulphides?". This could result formally in a resynthesis of the original ore.

In order to perceive the present investigations in perspective, a general overview of microbial sulphur metabolism is given. Clearly, only the major groups of organisms have been reviewed and even then a number of limitations and some areas of special difficulty must be taken into consideration.

1. All microbes have an absolute requirement for sulphur which is required for the biosynthesis of essential amino acids. In the present context, however, attention is focused on bacteria involved in the transformation of sulphur compounds at concentrations far higher than those required to support their anabolic activity.

2. Attention is directed almost exclusively to the metabolism of inorganic sulphur compounds and transformations of organic sulphur compounds are noted only in passing.

3. Not all groups of the bacteria discussed have been equally carefully examined and in some, only a few strains of a given taxon have been investigated. In certain cases, the organisms have been only poorly characterized so it is impossible to determine their identity.

4. Widely different methodologies have been used for studying sulphur metabolism including growth physiology, biochemical studies of enzymatic activity and analysis of metabolites. The results obtained by these various procedures, which have seldom been carried out simultaneously, are not always readily comparable.

Sulphur exists at several even oxidation levels between -2 (sulphide) and +6 (sulphate). It is convenient to discuss separately the oxidative and reductive segments of the sulphur cycle.

The oxidative segment

Four groups of organisms may be noted of which the first three are aerobes.

1. Probably the most important group of bacteria involved in the oxidation of inorganic compounds belong to the genus Thiobacillus. Although these organisms are both biochemically and physiologically heterogeneous, all are characteristically able to oxidize a variety of reduced sulphur compounds including sulphide, elemental sulphur and thiosulphate to sulphate. Oxygen and, for two species, nitrate (Thiobacillus denitrificans and Thiomicrospira denitrificans) are the electron acceptors, carbon dioxide is generally used as the source of carbon and a marked lowering of the pH accompanies growth of the cells (1).

Thiobacilli have long been recognized (2) as the cause of acidification in mine drainage water resulting from the oxidation of sulphide to sulphate with concomitant production

of acidity ($S^{2-} + 4\ H_2O \rightarrow SO_4^{2-} + 8\ H^+ + 8\ e$). In certain situations they may, however, occur in large numbers without bringing about acidification (3). Increasing interest has been devoted to exploiting this commercially in the leaching of ores (4, 33).

This is accomplished by two distinct processes: (i) by microbial oxidation of sulphide to sulphate, often simultaneously with the oxidation of Fe^{II} to Fe^{III} and (ii) by chemical reactions between Fe^{III} and the ore (e.g. UO_2). It has also been proposed that they be used for removal of unwanted pyrites from coal (5).

2. The potential role of Beggiatoa should not be underestimated even though certain aspects of metabolism have not been finally resolved (6, 32) nearly 100 years after their initial isolation (7). These are strictly aerobic organisms able to oxidize sulphide to elemental sulphur but it seems that this reaction is not utilized as a primary source of energy for growth and is a tangential metabolic activity. Although it has not been finally resolved whether these organisms are truly chemolithotrophic, it seems most likely, that they are primarily, or completely dependent on a heterotrophic mode of growth (8). One curious capability is that, under anaerobic conditions, the stored elemental sulphur may be re-reduced to sulphide.

3. The acidophilic thermophilic genus Sulfolobus (9) is an important organism in oxidation of sulphur which occurs in thermal springs but appears to be restricted to such habitats.

4. Anaerobic phototrophic bacteria belonging to the families Chromatiaceae and Chlorobiaceae are able to carry out the reduction of carbon dioxide using sulphide or elemental sulphur which are thereby oxidized to sulphate (10). In sulphide-rich anoxic lakes, these organisms may be dominant and they have attracted enormous interest over many years so that an extensive literature exists. Interestingly, it may be noted that some sulphide-tolerant cyanobacteria which normally carry out oxygenic photosynthesis have been shown

to carry out a similar reaction though in this case sulphide is oxidized only to elemental sulphur (11).

The reductive segment

The reductive segment is complex and three physiologically distinct groups of bacteria must be considered.

1. Obligately anaerobic bacteria of several genera including Desulfovibrio are able to reduce sulphate to sulphide at the expense of either lactate or several lower carboxylic acids (12). Another more recently discovered group (13) is able to use elemental sulphur in place of sulphate. In all of these organisms, sulphate or sulphur is obligately required as the electron acceptor for oxidation of the carbon source and concomitant production of sulphide. On the other hand, certain clostridia e.g. Clostridium perfringens, are able to reduce sulphite to sulphide in complex meda (14) but these organisms are not obligately dependent on this reduction for energy production and growth: this then clearly distinguishes them from the dissimilatory organisms noted above.

2. Some facultative bacteria belonging to the family Enterobacteriaceae are able to reduce thiosulphate to sulphide (15): this is found in virtually all species of Salmonella, in Edwardsiella tarda and in some species of Citrobacter (C. freundii) and Proteus (Prot. mirabilis and Prot. vulgaris). As will be discussed later, demonstration of this capability depends critically on the experimental methods for detecting sulphide production. Evidence has been presented that a few facultatively anaerobic bacteria (16, 17)may use tetrathionate, thiosulphate or sulphite as terminal acceptors for anaerobic growth on non-fermentable substrates. Anaerobic growth of strictly aerobic organisms, however, comparable to that of Pseudomonas aeruginosa or Paraccoccus denitrificans with nitrate, seems not to have been observed.

It may be noted parenthetically that dimethyl sulphoxide is able to serve as electron acceptor during anaerobic growth (18,19) and that at least superficially all of these electron acceptors function in the same way as nitrate or trimethylamine-N-oxide (20, 21).

Most members of the Enterobacteriaceae are able to reduce selenite to elemental selenium (22) and some genera e.g. Salmonella are markedly resistant to high concentrations of selenite.

3. The genus Alteromonas consists of a group of strictly aerobic Gram negative bacteria somewhat resembling the genus Pseudomonas but having a significantly lower DNA base ratio (23). Among these organisms, there exists a group (Alt. putrefaciens [24] characteristically able to produce sulphide from thiosulphate in complex media. These are phenotypically and genotypically heterogeneous but have a very wide distribution (25) and were primarily the subject of the present study. It should be stressed at the outset that very little indeed is known of the physiology of these organisms which have attracted attention primarily in connection with the spoilage of food and in the clinical environment.

II MATERIALS AND METHODS

1. Isolation of organisms

a) Anaerobic bacteria were isolated from sediment samples by dilution in saline buffer followed by spreading 0.1 ml portions onto the surface of the perfringens agar of Handford which contained (g/l): tryptone, 15: yeast extract, 5: soy peptone, 5: liver extract, 7: ferric ammonium citrate, 1: sodium sulphite, 1: Tris buffer, 1.5. The pH was adjusted to 7.3 and agar (10 g/l) added before autoclaving. Plates were incubated in an anaerobe jar for 4 d at 23°C. Black colonies were picked and purified by streaking successively on the isolation medium.

b) Isolation of aerobic and facultatively anaerobic bacteria from the water samples was carried out by two different procedures.

(i) Samples (1.0, 10 ml) were filtered through sterile 0.45 µ membrane filters which were then placed on plates of Triple-sugar-iron (TSI) agar: these were incubated at 23°C for 2 d and black colonies picked and purified by streaking on nutrient agar.

(ii) Samples (300 ml) were added to 30 ml ten times concentrated peptone water medium adjusted to pH 7.2. The completely filled bottles were then incubated anaerobically at 4°C for 4-6 weeks. Portions were spread onto deoxycholate-hydrogen sulphide-lactose (DHL) agar (26) and incubated anaerobically for 2 d at 23°C. Black colonies were picked and purified by streaking on nutrient agar.

Additional strains were also examined. These were, from the IVL Culture Collection: H_2S^+ *Escherichia coli*, *Edwardsiella tarda*, *Citrobacter freundii*, *Proteus mirabilis* and *Proteus vulgaris*. Professor L. Le Minor, Institut Pasteur Paris, kindly provided strains representing the four *Salmonella* subgenera:

I, S. typhimurium and S. enteritidis: II, S. sofia: III, S. arizonae: IV S. ochsenzoll, (S. houtenae).

2. Characterization of the strains

a) Anaerobes were not characterized biochemically and were provisionally identified only to genus on the basis of the Gram stain, tests for catalase and production of sulphide in Perfringens agar.

b) All other strains were characterized to species by methods used in this laboratory (25, 27): the facultative anaerobes using biochemical reactions carried out at 30° C and the aero organisms by their ability to hydrolyse DNA, Tween 80, gelati to decarboxylate ornithine and the ability to produce acid in an ammonium salts (ASS) medium (28) containing glucose, sucro maltose and arabinose. All of these incubations were carried out at 17°C.

3. Ability of strains to produce sulphide from oxidized inorganic sulphur sources

This was assessed by the ability to produce black insoluble ferrous sulphide in media containing the various sulphur sources and supplemented with an iron source. The concentrations of the S-sources were as follows (g/l):

Na_2SO_4	(not for anaerobes)	2.4
Na_2SO_3	(all strains)	1.0
$Na_2S_2O_3 \cdot 5H_2O$	(all strains)	2.1
$Na_2S_4O_6$	(all strains)	1.3
cysteine	(not for anaerobes)	3.0

Controls without added S-sources were always included.

a) For anaerobes, the Perfringens basal medium lacking thiosulphate was used: the S-sources were added, the pH adjusted and the medium sterilized by autoclaving. Stabs were inoculated

and incubated at 23°C: positive reactions were generally obtained within 4 d.

b) For the other organisms, different media were tried including a complex medium based on TSI agar, and the soft agar medium of Lautrop (29): these produced variable results and eventually the following procedure was developed. ASS medium without agar was supplemented with yeast extract (0.5 g/l) and ferrous sulphate (0,3 g/l): glucose and sucrose both at concentrations of 1.0 g/l were used as the carbon sources and the above S-sources added. The pH was adjusted to 7.2 and the medium sterilized by boiling and dispensed into sterile tubes, which were inoculated and incubated for up to 10 d at 23°C.

III RESULTS AND DISCUSSION

Five water samples and six sediment samples from the Garpenberg area were examined and the following pure strains of bacteria isolated:

1. from the sediment samples, 10 strains provisionally assigned to the genus Clostridium and all capable of reducing sulphite to sulphide,

2. from the water samples using membrane filtration, 6 strains of Citrobacter sp. and 1 strain of Alteromonas putrefaciens,

3. from the low-temperature enrichments, an additional 6 strains of Alteromonas putrefaciens.

The biochemical reactions of the citrobacters and alteromonads are given in Tables 1 and 2. It should be noted that the citrobacters were highly untypical for any species of this genus being strongly urease positive and extremely weakly saccharolytic. Their assignment to this genus was kindly suggested by Dr. C. Richard, Institut Pasteur, Paris. The strains of Alteromonas putrefaciens were similar to most strains isolated in this laboratory (25) and belonged to saccharolytic biotypes.

Before presenting results on sulphide formation, it seems necessary to make a digression into some methodological problems which arose. The various methods used for estimating the ability of an organism to produce sulphide from a given S-source differ not only in methodology but in sensitivity. Three widely used procedures may be distinguished.

(i) Use of a complex growth medium containing the S-source and a soluble form of iron (generally ferrous sulphate or ferric ammonium citrate) and usually solidified with agar. In this medium, sulphide formation is accompanied by the formation

of a black precipitate of ferrous sulphide which is readily seen in stab cultures. The sensitivity of this method is considered relatively low, but this procedure is reproducible and is probably the one most commonly used, for example, in Triple-sugar-iron agar, Kligler-iron agar and Perfringens agar.

(ii) A variant has been developed in which a strip of paper impregnated with a solution of lead acetate is suspended in the gas phase above a liquid culture. This is a highly sensitive method able to detect amounts of hydrogen sulphide not demonstrable by method (i). For example, some strains of aeromonas are positive using this method.

(iii) Less frequent use has been made of dense cell suspensions incubated with the S-source over short periods and using a variety of methods for detecting sulphide formation, generally method (ii) above.

The present study used the relatively insensitive method depending on visible formation of black ferrous sulphide (method (i)).

The ability of the clostridia to reduce the various S-sources in a complex medium is given in Table 3 and requires little comment except to note the apparent toxicity of tetrathionate. These results are in agreement with data in the literature for Clost. perfringens (14).

The investigations on the other strains yielded unexpectedly complex results in spite of attempts to use different test systems. The following is, threfore, a simplified summary of the results obtained.

a) Only three strains (none of which were isolated in the present study) out of a total of 34 tested were able to produce sulphide from thiosulphate in the defined liquid medium: this additionally depended on the carbon source. These strains were:

Proteus vulgaris: 1443 (glucose)
Alteromonas putrefaciens: 603 (glucose)
Citrobacter freundii: 193 (sucrose)

A careful examination of the literature reveals that the ability of enteric bacteria to produce sulphide from thiosulphate is almost invariably assessed from results using complex media containing peptones and supplemented with thiosulphate. The exception seems to be plasmid bearing strains of Escherichia coli which are H_2S^+: such strains have been reported as being able to produce sulphide from several inorganic S-sources in a defined medium (29, 30). The single strains tested in the present study was unable to do so: no explanation for this discrepancy can, at the moment be put forward.

b) Cysteine was much more widely used but its reduction depended critically on the carbon source. Using sucrose, all 4 strains of Proteus, 5 of Salmonella, 8 of Citrobacter, 1 of Escherichia coli, all strains of Alteromonas putrefaciens from Garpenberg and 2 out of 9 others were able to produce sulphide. When glucose was the carbon source, however, only the strains of Alteromonas putrefaciens gave a positive reaction and all of the representatives of the family Enterobacteriaceae were negative.

c) No strain was able to produce sulphide in the defined medium from either sulphite or tetrathionate.

Two general and important conclusions can be drawn from this investigation. First, all strains examined here were able to produce copious amounts of sulphide in a complex medium supplemented with thiosulphate. On the other hand, very few indeed

could do so in a defined medium. Second, using cysteine as S-source, a complex dependence upon the carbon substrate was clearly revealed. Such a phenomenon has not been noted previously though the difficulties with sulphide formation have been noted (31). For the clostridia, tests were carried out only in a complex medium since these organisms generally have exacting nutritional requirements and in this study, no attempt was made to develop a defined medium. Nonetheless, these strains were able to use a range of the S-sources tested.

It is concluded that substantial gaps exist in current understanding of the microbial sulphur cycle and that use of complex media for testing sulphide formation from oxidized inorganic S-sources has led to false impressions of the distribution of this capability. The answer to the question posed at the beginning of this investigation is that the relevant bacteria were present but that their capability for producing sulphide depended in a complex way on the presence of organic substrates whose role cannot at present be elucidated.

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VI TABLES 1-3

The biochemical reactions of the strains were so uniform that it was felt unnecessary to give frequencies: instead, the symbols + and - are used for positive and negative reactions respectively.

Table 1. Biochemical reactions of 6 strains of H_2S^+ Citrobacter sp.

Decarboxylation of:	arginine	-
	lysine	-
	ornithine	-
Utilization of:	citrate	-
	malonate	-
Hydrolysis of:	Urea	+
	ONPG	+
	gelatin	-
Formation of indole		-
Methyl red reaction		+
Vogues-Proskauer reaction		-
$No_3 \rightarrow NO_2^-$		+
Hydrolysis of:	esculin	-
	DNA	-
	TWEEN 80	-
Phenylalanine deaminase		-
Formation of acid from:		
	inositol	-
	sorbitol	-
	rhamnose	+
	sucrose	-
	raffinose	-
	amygdalin	-
	arabinose	+
	xylose	+
	adonitol	-
	lactose	-
	salicin	-
	cellobiose	-
	trehalose	-
	mannitol	+
	maltose	-
	sorbose	-
	glycerol	+
	mannose	-
	galactose	+

Table 2. Biochemical reactions of 7 strains of *Alteromonas putrefaciens*

Acid in TSI medium	-
Sulphide in TSI medium	+
Oxidase	+
Hydrolysis of: DNA	+
gelatin	+
TWEEN 80	+
Decarboxylation of ornithine	+
Formation of acid from: glucose	+
maltose	+
sucrose	-
arabinose	-

Table 3. Formation of sulphide by clostridia in a complex medium supplemented with various sulphur sources.

Strain nr	Sulphur source			
	Sulphate	Sulphite	Thiosulphate	Tetrathionate
1476	-	+	+	+
1477	-	+	+	NG
1478	-	+	+	NG
1479	-	+	+	-

NG: No growth