A Survey of Microorganisms for the Ability to Methylate Mercury and Further Investigations into Factors Affecting Mercury Methylation

A Thesis
Submitted to
The Faculty of Graduate Studies
University of Manitoba
Department of Microbiology

In Partial Fulfillment
of the Requirements for the Degree of
Master of Science

By George W. Meichel

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George W. Meichel

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

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Dedication

I dedicate this thesis to my beautiful wife Margaret. Without her love, support, and understanding, none of this would have been possible.

Abstract

Methyl mercury is a global pollutant of great environmental concern. The methylation of inorganic mercury to form methyl mercury is a process known to be performed by a number of bacteria. This project was intended to survey a number of bacteria for; cobalamin content, ability to methylate mercury, and to investigate molybdate as a microbial inhibitor, and in particular its mode of action on SRB. For species testing positive for the ability to methylate mercury, further investigations were conducted to examine physical factors affecting methylation activity. These factors were, low total mercury content, pH, substrate composition, temperature, gas phase, and nickel concentration. Of the organisms surveyed for mercury methylation, some of which have been shown by other workers to methylate mercury, only two SRB species were capable of methylation. *Desulfobulbus propionicus* and *Desulfovibrio* sp. B-203, demonstrated mercury methylation; however, both these SRB were known previously to methylate mercury. Investigation of the effect molybdate on the growth of bacteria indicated that molybdate has no effect on the bacteria tested, with the exception of two methanogen species, *Methanococcus thermolithotrophicus* and *Methanospirillum hungatei*, and all the SRB tested. The SRB were inhibited under both fermentative and respiratory growth conditions. Increased sensitivity to molybdate under fermentative conditions and decreased sensitivity while respiring on NO_3, for those SRB capable of fermentative growth or respiration using NO_3 as a terminal electron acceptor, was seen in some cases. Results of the cobalamin content survey demonstrate differences in cobalamin content for SRB grown fermentatively or while respiring. Investigation utilizing CVAFS indicate that *Desulfobulbus propionicus* is capable of mercury methylation at total mercury concentrations comparable to those found in pristine natural environments. For the variables of pH, substrate composition, and gas phase, mercury methylation was seen to be directly proportional to biomass, indicating methylation as a function of growth rate. Increased nickel concentration produced a slight increase in the amount of mercury methylated. The effect of temperature demonstrated methylation as low as 4° C with increasing rates of methylation as temperature increased.

Chapter 1

Introduction

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Section 1.1

Mercury: a global pollutant

Mercury is a global pollutant of great environmental concern. It is neuro-toxic in both organic and inorganic forms; the organic form is many times more toxic than the inorganic form (D'Itri and D'Itri 1977). The most common form of organic mercury is methylmercury, which is formed in the environment from inorganic mercury by bacteria (Fitzgerald *et al.* 1990). Inorganic mercury in soils and sediment is derived from weathering of minerals, from natural mercury cycling through the atmosphere as Hg^0 and from industrial processes and fossil fuel burning (Matheson 1979). Organic mercury compounds are also produced industrially, mainly as phenyl mercury acetate, a fungicide (Robinson and Tuovinen 1984). Organic forms of mercury undergo bioaccumulation in the aquatic food chain (National Academy of Sciences 1978). This accumulation is a serious health and economic concern for people and wildlife that consume fish from waters containing mercury in the organic form.

The earliest work investigating the dangers of organic mercury compounds began in the early 1950's, with incidents involving point source contamination of sites with organic and inorganic mercury compounds. This information on early mercury work has been reviewed by D'Itri and D'Itri (1977). Following these incidents of high level contamination, high levels of methyl mercury were seen in people. Minimata Bay remains as one of the most horrific examples of methyl mercury poisoning on record. The bay received effluent from a plastics factory for a number of years. This effluent contained mercuric chloride used in the production of vinyl chloride as well as a number of organo-mercurial compounds including methyl mercury. Methyl mercury became concentrated in the fish and shellfish of the bay and consumption of a normal diet of these fish, resulted in severe health problems in the residents of Minimata Bay, including birth defects in newborns, severe neurological impairment, and even death.

In 1965, Westermark discovered high concentrations of mercury in fish in Swedish lakes that received inorganic mercury as well as phenyl mercury as the result of industrial pollution (Westermark 1965). In 1966, Westoo determined that the mercury in these fish was almost entirely methyl mercury. With this, investigations into the causes of mercury transformation, especially the transformation of Hg^{2+} to methyl mercury began.

Currently it is understood that most of the mercury entering ecosystems is inorganic mercury and the most common problem of mercury in fish is methyl mercury, so understanding the transformation of inorganic mercury to methyl mercury is key.

Section 1.2

Early evidence of mercury biotransformation

In the late sixties, the work of Jensen and Jernelöv (1969), in a series of aquaria sediment experiments, demonstrated that the conversion of inorganic mercury to methyl mercury was a biological phenomenon, favoured under anaerobic conditions and possibly mediated by bacteria. Wood *et al.* (1968), demonstrated methylation of mercury with extracts of methanogenic bacteria. Methanogens were thought to be likely candidates for microbially mediated methylation of mercury because they are anaerobes and have high concentrations of methylcobalamin (see Fig. 1.1), the only known C_1 carrier capable of transferring a methyl group in the carbanion form necessary for the formation of methyl mercury (Wood 1974).

This demonstration of methylation in methanogen extracts, plus the anaerobic location of methylation implicated methanogens as possible agents in the transformation of inorganic mercury to methyl mercury. It would seem a reasonable assumption based on these two lines of evidence that inorganic mercury was converted to the methyl form by the actions of methanogenic bacteria; however, Wood *et al.* (1968) failed to demonstrate methylation of mercury by intact cells of methanogens. Second, his work involved the addition of large concentrations of exogenous methylcobalamin to methanogen

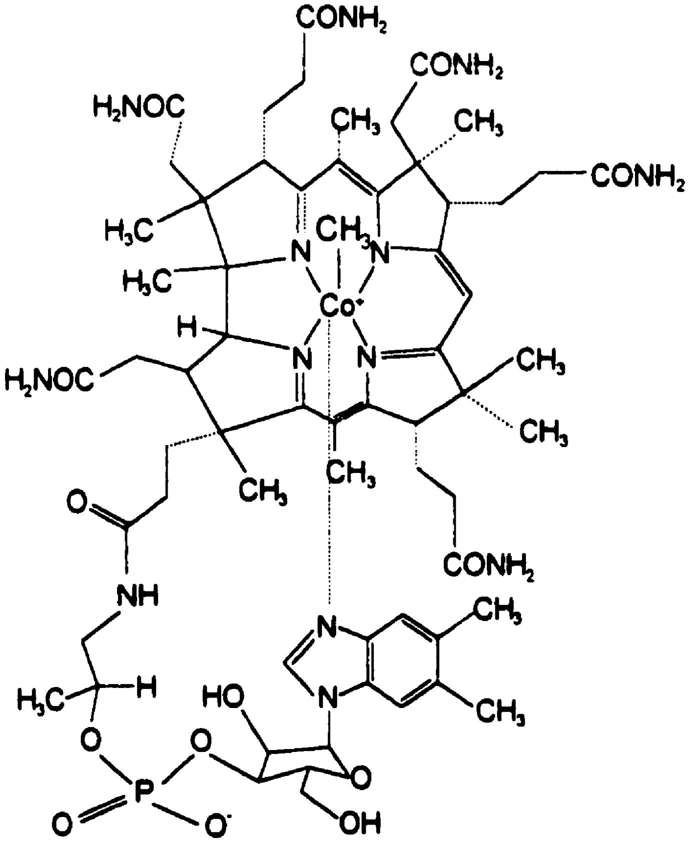

Figure 1.1 **Structure of the corrinoid methylcobalamin (Krautler et al. 1988)**

cell extracts. Third, Wood *et al.* (1968) used an acid precipitation step prior to the extraction of the organomercury compounds from the cell extract methylcobalamin reaction mixture. In later work, Berman *et al.* (1990) and others demonstrated the spontaneous methylation of mercury by methylcobalamin in an acidic buffer (Choi and Bartha 1993, DeSimone *et al.* 1973). Wood *et al.* (1968) also demonstrated the abiotic methylation of mercury under mild reducing conditions in an NH_4Cl solution by reacting methylcobalamin and propylcobalamin with mercury and producing mono and dimethyl and mono and dipropyl mercury, respectively. To date no pure cultures of intact cells of methanogens have been shown to methylate mercury *in vivo*. Although the flaws in Wood's experimental demonstration of enzymatic mercury methylation have been mentioned in published work, Choi *et al.* (April 1994) researchers are still mentioning the role methanogens play in the biologically mediated methylation of mercury and have cited Wood as verification (Drake *et al.* 1996). In addition they often cite the paper that mentions Wood's experimental flaw (the use of exogenous methylcobalamin) (Choi, *et al.* April 1994) and the paper that provides evidence excluding methanogens from environmental mercury methylation, at the same time without ever mentioning the contradiction (Drake *et al.* 1996).

Section 1.3

Mercury methylation in the environment

As was mentioned previously, the incidence of human poisoning led to the investigation of methyl mercury. Following that, many incidence of mercury contamination of fish stocks led to the investigation of mercury methylation in aquatic systems. It was found that, in addition to lakes receiving point source contamination of mercury compounds, lakes receiving little or no anthropogenic mercury, but experiencing acid rain, also showed elevated levels of methyl mercury in fish (Wren and MacCrimmon 1983, Akielazek and Haines 1981). It is important to know that there are orders of magnitude difference in contamination levels between the Minimata Bay incident and in lakes receiving no point source pollution. Elevated levels of methyl mercury in fish are also seen in hydro electric reservoirs, whose construction results in the flooding of large areas of ground (Bodaly *et al.* 1984, Bodaly and Hecky 1979). High methyl mercury concentrations were also seen in fish at the upper trophic levels (Gilmour and Henry 1991, Bodaly and Hecky 1979).

These observations led to the investigation of factors affecting rates of mercury methylation. The vast majority of research done on mercury methylation has not focused on the microbial physiology of mercury methylation, but rather on factors affecting mercury methylation as it occurs in natural environments, especially in lake sediments (Porvari and Verta 1995, Stordal and Gill 1995, Regnell 1994, Gilmour *et al.* 1992, Gilmour and Henry 1991, Kerry *et al.* 1991, Baldi *et al.* 1989, Steffan *et al.* 1988, Korthals and Winfrey 1987, Xun *et al.* 1987, Ramlal *et al.* 1986, Furutani and Rudd 1980). Water chemistry affecting mercury speciation and availability, nutrient levels such as phosphorous, dissolved organic carbon, and sulphate, water pH, water E_h, and total mercury concentration, are all factors that have been investigated in relation to mercury methylation (Kelly *et al.* 1995, Regnel 1994, Gilmour and Henry 1991, Steffan *et al.* 1988, Compeau and Bartha 1985, Compeau and Bartha 1984). From the results of these studies it is hard to make quantitative comparisons between differing aquatic systems because the methods for measuring mercury methylation, such as ^{203}Hg addition do not give quantitative information, *ie.* they do not give actual rates but rather potential or relative rates of mercury methylation. At best qualitative comparisons can be achieved, although they provide useful information they do not provide the whole story on mercury methylation.

Section 1.4

Microorganisms that methylate mercury

In the early seventies a great deal of work focused on identifying organisms that could methylate mercury. These investigations showed that a number of bacterial and fungal cultures could methylate mercury *in vivo*. Bacteria such as *Escherichia coli*, *Pseudomonas fluorescens*, *Bacillus megaterium*, *Aerobacter aerogenes*, *Mycobacterium phlei*, *Enterobacter aerogenes* and *Clostridium cochlearium*, were shown to methylate mercury (Pan-Hou *et al.* 1980, Hamdy and Noyes 1975, Rowland *et al.* 1975, Vonk and Sijpesteijn 1973). Unidentified species of the genera Streptococci, Staphylococci, Lactobacilli, Bacteroides, and Bifidobacteria were shown to methylate mercury (Hamdy and Noyes 1975, Rowland *et al.* 1975). Fungi such as *Neurospora crassa*, *Aspergillus niger*, and *Scopulariopsis brevicaulis* also showed mercury methylating ability (Hamdy and Noyes 1975, Rowland *et al.* 1975, Vonk and Sijpesteijn 1973, Landner 1971). Although the various efforts involving the above named organisms demonstrated their ability to methylate mercury under the laboratory conditions used, none of the experiments determined whether these organisms are of any significance when it comes to the methylation of mercury in the environment. These experiments were not conducted under environmental conditions and population mixes, and second they were not conducted using environmentally relevant mercury concentrations. Thus the contribution of different bacterial species to the production of methyl mercury in the environment were unknown.

In 1985 Compeau and Bartha (1985) demonstrated that sulphate reducing bacteria are significant methylators of mercury in anoxic salt marsh sediment. In earlier work Compeau and Bartha found methylation to be confined to the anoxic and reduced portions of salt marsh sediments (Compeau and Bartha 1984). They determined that both sulphate reducers and methanogens were active in these portions of the sediments and proceeded to investigate the respective contributions of each of these bacterial groups to methyl mercury production. They accomplished this through the use of specific inhibitors of sulphate reducing bacteria (SRB) and methanogens. When they added 2-bromoethane sulfonate (BES), a known specific inhibitor of methanogens (Sparling and Daniels 1987) they found an increase in mercury methylation rates. When they added sodium molybdate, a known inhibitor of SRB (Oremland and Capone 1988), they found an inhibition of mercury methylation. From the results of the inhibitor experiments Compeau and Bartha (1985) concluded that SRB were responsible for mercury methylation in salt marsh sediments.

From these salt marsh sediments, Compeau and Bartha isolated in pure culture a SRB that could methylate mercury and identified it as a strain of *Desulfovibrio desulfuricans*. They designated it strain LS (low salt) in reference to its estuarine origin. This isolate is able to methylate mercury under anaerobic, reduced conditions (Compeau and Bartha 1985). Interestingly it methylates best at low sulphate concentrations. Since this time other workers have been able to isolate and identify other strains of SRB, including *Desulfobulbus propionicus*, that methylate mercury (Henry 1992). At present C. Gilmour has in excess of 60 different isolates of mercury methylating SRB (Gilmour and Henry 1991, per. comm. Gilmour).

Section 1.5

Phylogenetics of the Sulphate Reducing Bacteria

The group of bacteria collectively known as the Sulphate Reducing Bacteria (SRB), are a diverse collection of Gram positive and Gram negative strictly anaerobic eubacteria (Postgate 1984, Pfennig *et al.* 1981). They share the obvious defining trait of dissimilatory sulphate reduction. Beyond this and their strict anaerobic nature the SRB as a group demonstrate a number of physiological abilities not all of which are shared from species to species nor strain to strain (Postgate 1984, Pfennig *et al.* 1981). Traits that differ include such things as range of carbon sources for growth, the extent to which these carbon sources are oxidized (complete or incomplete), the range of terminal electron acceptors other than SO_4^{2-} (eg. nitrate, thiosulphate, sulphite, tetrathionate, and elemental sulphur), the ability to grow fermentatively, and the ability to methylate mercury (Compeau and Bartha 1985, Postgate 1984, Pfennig *et al.* 1981).

Early classification of the SRB by necessity was done using morphology, nutritional similarities, chemical data such as serology, genomic G+C content, and pigment type (Postgate 1984, Pfennig *et al.* 1981). This classification work resulted in the establishment of 11 genera of SRB (Devereux *et al.* 1989).

More recently 16S rRNA studies have been employed to investigate the phylogenetic relationships between the various genera of SRB (Devereux *et al.* 1989). The 16S rRNA studies are generally consistent with the established taxonomic groupings, however; they do show the extent of phylogenetic diversity among the SRB *ie.* even when 16S rRNA sequences support a taxonomic grouping there is still a great deal of diversity within the 16S rRNA sequence data (Devereux *et al.* 1989). An example of this is *Desulfotomaculum ruminis* and *D. orientis* sharing only 83% sequence homology (Devereux *et al.* 1989).

Using the previously mentioned classification traits and the 16S rRNA sequence relationships, a number of *Desulfotomaculum* species have been grouped with the Gram positive endospore forming eubacteria. Based on the ability to form spores and rRNA similarity a clear affiliation exists with the Gram positive endospore forming eubacteria, although the 16S rRNA sequences indicate the genus is quite diverse. The gram-negative non-spore forming SRB seem to cluster within the delta subdivision of the purple bacteria (proteobacteria)(Devereux *et al.* 1989).

Within genera, 16S rRNA studies demonstrate a high degree of phylogenetic distance amongst members, suggesting in some instances separate lineages for the members of some of the SRB genera (Devereux *et al.* 1989). Separate lines of lineage are suggested for members of the genus *Desulfotomaculum* based on 16S rRNA sequence comparisons, and the wide range of G+C content values found within the genus (Devereux *et al.* 1989).

Among the genus *Desulfovibrio*, significant phylogenetic diversity exists (Devereux *et al.* 1989). The genus contains both complete and incomplete oxidizers as well as species differences in pigment content (Postgate 1984, Pfennig *et al.* 1981). Thus, in the strains of the species *Desulfovibrio desulfuricans* considerable phenotypic as well as physiological diversity exists (Devereux *et al.* 1990).

Mercury methylation has been reported in a variety of SRB species, notably *Desulfobulbus propionicus*, and *Desulfovibrio desulfuricans*. It is important to remember that not all SRB methylate mercury and that not all members of a genera, or strains of a species, that contain members that methylate mercury, share this ability (Gilmour and Henry 1991). In considering the extent of phylogenetic diversity exhibited within the SRB it is not surprising that we find situations where traits are not shared between, what would at first appear to be, closely related organisms and contrarily we find traits shared among organisms that are only distantly related. This is particularly pertinent to mercury methylation as it provides a possible explanation of why methylation is not seen in all strains of a species which has demonstrated methylation in one or more strains and yet methylation is seen in strains of distantly related species.

Section 1.6

Proposed mechanisms of mercury methylation

Many cellular compounds are capable of transferring methyl groups in biochemical reactions. They are tetrahydrofolate, methionine CoA, 2-mercaptoethanesulfonic acid, F430 (nickel cofactor), and corrinoid coenzymes (Wood 1974). Of these only the methylated corrinoids have been implicated in the methylation of mercury, because they are the only C_1 carrier capable of transferring methyl groups as carbanions. The carbanion form is required for the methylation of mercury.

$$CH_3^- + Hg^{2+} \rightarrow CH_3Hg^+$$

Corrinoids are coenzymes comprised of a tetrapyrrole derived corrin ring (see Fig. 1.1)(Stupperich *et al.* 1990). The centre of the corrin ring contains a cobalt ion which is the active site of the molecule. Corrinoids are involved in many enzymatically mediated methyl transfers, such as methionine synthesis, nucleic acid modification, production of methane by methanogens, and fixation of CO_2 in the acetyl-CoA pathway (Kräutler *et al.* 1988, Wood *et al.* 1986, Eden and Fuchs 1982).

Section 1.7

Desulfovibrio desulfuricans LS

Work with *D. desulfuricans* LS has shown the presence of cobalamin, a corrinoid coenzyme. The methylated form of this corrinoid (methylcobalamin) isolated from *D. desulfuricans* LS, has been shown to methylate mercury *in vitro* in a pH 4.5 acetate buffer with Hg^{2+} (Choi and Bartha 1993). ^{14}C labelling studies using pyruvate, formate, serine, and HCO_3^-, indicate the operation of the acetyl-CoA synthase pathway (Fig. 1.2)(Choi *et al.* 1994, Berman *et al.* 1990).

When assayed for, enzymes of the acetyl-CoA synthase pathway were present. From this evidence, Choi *et al.* (1994) have proposed the production of methyl mercury via a modification of the acetyl-CoA pathway where the methyl group originally destined for acetyl-CoA formation instead becomes the methyl group of methyl mercury (see Fig. 1.2)(Choi *et al.* 1994). There are weak points in the presentation of this scheme as a general model for mercury methylation in organisms other than *D. desulfuricans* LS. i)

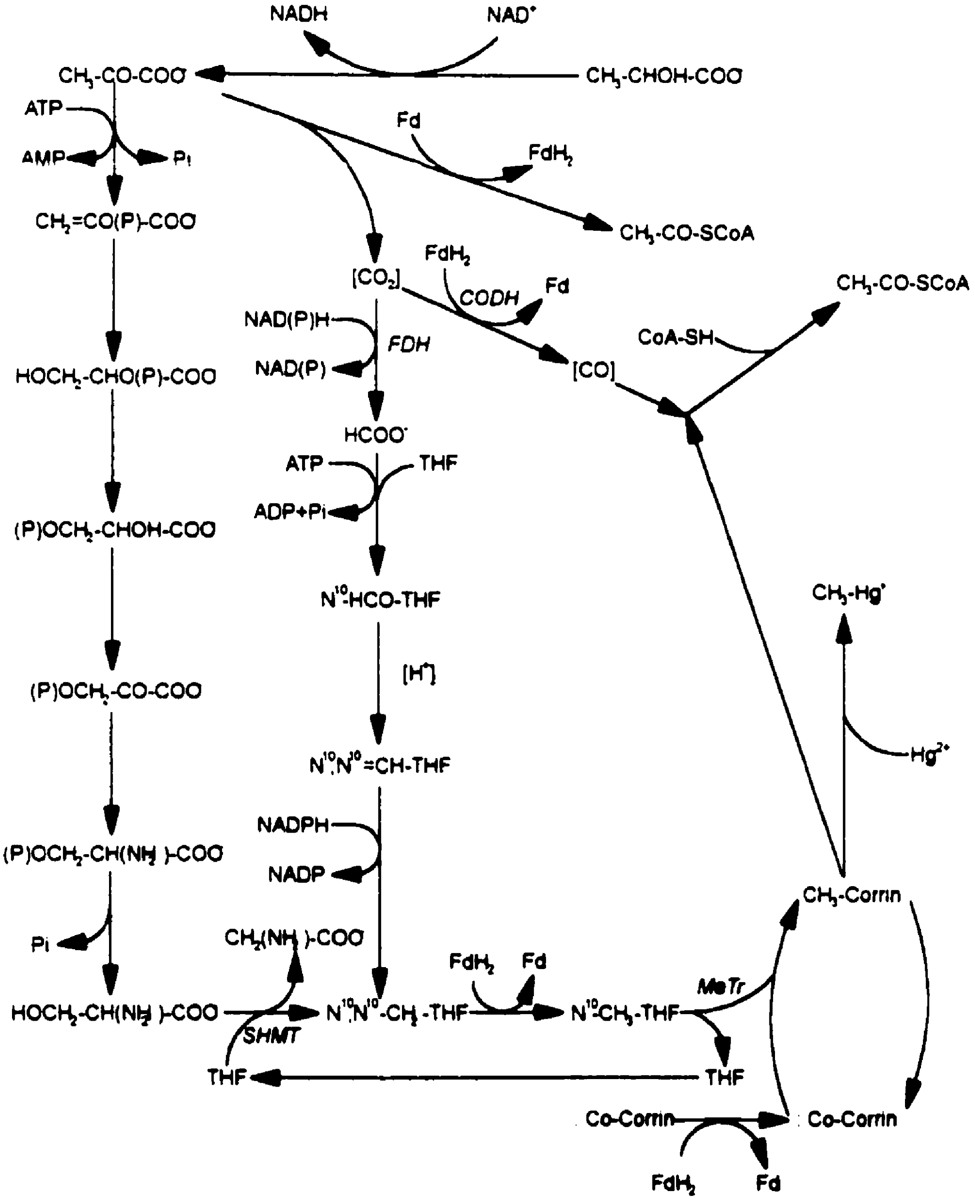

Figure 1.2 The proposed metabolic pathway for mercury methylation in *Desulfovibrio desulfuricans* LS. Fd, ferredoxin; FDH, formate dehydrogenase; CODH, carbon monoxide dehydrogenase; MeTr, methyltransferase; SHMT, serine hydroxymethyltransferase (Choi et al. 1994)

The acetyl-CoA pathway in many sulphate reducers including *D. desulfuricans* LS is a very minor pathway, as levels of enzymes specific to the pathway are low, yet this species methylates mercury (Choi *et al.* 1994). ii) While methanogens and acetogens are both known to have several thousand fold greater concentrations of cobalamin, and carry out acetate synthesis via the acetyl-CoA pathway as a major biosynthetic reaction, no members of either group have been shown to methylate mercury (Daniels *et al.* 1984, Choi *et al.* 1994).

Choi *et al.* (1994) have isolated from *Desulfovibrio desulfuricans* LS, a 40-kDa corrinoid protein that is capable of methylating Hg^{2+} at rates up to 600 fold greater than that of free methylcobalamin. They propose that an enzyme specific to mercury methylation exists, which they term mercury methyltransferase. They propose that this transferase enzyme works in conjunction with the 40-kDa corrinoid protein to methylate mercury, or alternately that the 40-kDa corrinoid protein contains within itself the so called mercury methyltransferase enzyme. The absence of this mercury methyltransferase enzyme, whether it be a separate enzyme or a part of the 40-kDa corrinoid protein, would explain why organisms with much greater concentrations of methylcobalamin and enzymes specific to the acetyl-CoA pathway are incapable of mercury methylation. However, Choi *et al.* (1994) provide no evidence to suggest that this methyltransferase enzyme (whether it is a separate enzyme or a part of the 40-kDa corrinoid protein) is not just the normal methyltransferase enzyme required for the transfer of the methyl group from cobalamin to carbon monoxide in the formation of CH_3-CO-SCoA.

Section 1.8

Reasons for mercury methylation

One of the least understood questions surrounding mercury methylation is why organisms methylate mercury at all. One proposed explanation is that mercury methylation functions as a detoxifying process (Pan-Hou and Imura 1982). Mercury methylation as a detoxifying mechanism seems counterintuitive, because methyl mercury is more toxic than Hg^{2+}; however, methyl mercury is less soluble than Hg^{2+}, and thus diffuses out of the cell more readily, thereby lowering the overall mercury concentration in the immediate environment of the cell (Boudou *et al.* 1991). Evidence suggesting methyl mercury diffuses more rapidly across cell membranes than does Hg^{2+}, and thus leaves the cell more rapidly, comes from phospholipid bilayer studies using chlorinated forms of methyl mercury and Hg^{2+}, (CH_3HgCl and $HgCl_2$) respectively (Boudou *et al.* 1991). Only one study supporting methylation as detoxification mechanism exists, that of Pan-Hou and Imura (1982). Pan-Hou and Imura (1982) found that a vitamin B_{12} auxotroph of *Clostridium* *cochlearium*, incapable of methylation and cured of the demethylation plasmid showed greater sensitivity to Hg^{2+} than did the parent strain, which was capable of methylation but also cured of the demethylation plasmid.

The demethylation plasmid is the plasmid containing the *mer* operon. The *mer* operon is composed of several genes, the most important being *mer*A and *mer*B, which code for a Hg reductase and an organomercury lyase, respectively (Brinkman and Olson 1988). The remaining genes are involved in transport and in regulating the expression of *mer*A and *mer*B (Weber 1993). Opponents of the idea that mercury methylation is a detoxifying mechanism, cite the increased toxicity of methyl mercury and the existence of a more efficient detoxifying mechanism, *ie.* the *mer* operon, which as mentioned above has both a demethylating capacity and a mercury volatilization capacity (Weber 1993, Brinkman and Olson 1988). When induced, and only nanomolar concentrations of mercury are required to induce *mer* transcription, the *mer* operon produces enzymes for the cleavage of methyl mercury resulting in the production of CH_4 and Hg^{2+} (Selifonova *et al.* 1993, Robinson and Tuovinen 1984). The Hg^{2+} is then reduced to Hg^0 which has an extremely low vapour pressure and quickly escapes to the atmosphere.

Section 1.9

Abiotic mercury methylation

Since the chemical methylation of mercury via methylcobalamin, under acidic conditions has been demonstrated, and no single definitive model for the microbial methylation exists, some of the possible alternative explanations for the methylation of mercury will be discussed.

Carbocation donors such as (dimethylsulfonio)propionate and methyl iodide may methylate Hg_2^{2+} via a radical mediated process resulting in a one-electron oxidation of Hg_2^{2+} (Weber 1993).

$$Hg_2^{2+} + CH_3I \rightarrow CH_3Hg^+ + HgI^+$$

Methylcobalamin, mono, di, and tri methyl tins, and humic matter have also been suggested as possible abiotic methylators of mercury (Weber 1993). As mentioned earlier methylcobalamin methylates mercury abiotically under acidic conditions; however, little is known about the abundance of methylcobalamin in the environment, outside of living organisms. Evidence that does exist does not seem to indicate a correlation between cobalamin concentration and methyl mercury concentration in the environment (Regnel and Tunlid 1991). Methyl transfers from the various methyl tins to mercury have been demonstrated in the lab, but no experimental evidence for environmental mercury methylation via methyl tins exists (Weber 1993). The common occurrence of humic matter in aquatic systems and the demonstration of *in vitro* methylation of mercury by humic matter, indicate that humic matter is the most likely of abiotic candidates for methylation of mercury (Weber 1993); however, Berman and Bartha (1986) demonstrated that humic matter was responsible for less than 1/10 of the methyl mercury formed from anoxic estuarine sediments.

Section 1.10

Difficulties in conducting research on mercury methylation

Like any type of research, there are inherent difficulties in studying mercury methylation. Some of these difficulties appear when trying to relate what is known about the microbiology of mercury methylation with what is seen in the environment. Some of the difficulties exist due to limitations in analytical technique. Others exist due to difficulties in controlling variables of microbial activity. A few examples of these difficulties follow.

All the work done to date on the microbiology of mercury methylation in pure culture used concentrations of mercury several hundred to several thousand fold higher than what is seen in natural systems not receiving point source contamination, or even in very polluted areas exhibiting high levels of methyl mercury in the fish at the higher trophic levels. Thus it must be asked, are the laboratory experiments relevant to what is occurring in the environment? In laboratory experiments, using sediments, increasing mercury concentrations from 10 to 100 ppm resulted in an increase in methylation (Jensen and Jernelöv 1969). While in experiments with *Desulfovibrio desulfuricans* LS, increases in inorganic mercury from 0.1 to 10.0 µg per ml, under fermentative conditions and 10.0 to 50.0 µg per ml for sulphate reducing conditions, resulted in decreases in methyl mercury production (Choi and Bartha 1993). In the environment, total mercury proves to be a poor indicator of methyl mercury concentrations from system to system, suggesting that mercury methylation activity varies in different aquatic environments (Kelly *et al.* 1995). Yet the addition of mercury to sediments results in an increase in methylation in the system (Furatani and Rudd 1980).

Contradictory results achieved using molybdate as an inhibitor of SRB *ie.* near complete inhibition of mercury methylation or a near complete lack of inhibition, would tend to indicate that other organisms are capable of mercury methylation in the environment (Matilainen 1995, Compeau and Bartha 1985). This contradicts the widely held belief that sulphate reducing bacteria are the only significant methylators of mercury in the environment. Yet with the exception of an iron reducer no other organisms have been isolated that demonstrate a significant role in mercury methylation in the environment (per. comm. Gilmour).

In conjunction with this, contradictory results exist for work involving the stimulation of mercury methylation by SRB, with addition of sulphate (Winfrey and Rudd 1990). A potential explanation exists for this, although it is difficult to overcome experimental limitations in trying to pinpoint the answer. SRB when growing on sulphate produce H_2S, which has a high binding affinity for mercury and is thought to reduce its bioavailability. Thus sulphate stimulation of an aquatic system may in fact reduce the amount of mercury available for biomethylation such that no change in mercury methylation rates occur, yet the methylating potential has increased due to increases in the SRB population.

SRB that methylate mercury and can grow fermentatively, convert mercury to methyl mercury in the greatest proportion to added mercury when growing in the absence of sulphate (Choi and Bartha 1993). Attempts to measure mercury methylation in pure culture at environmentally realistic concentrations have been limited until recently, due to the lack of suitably sensitive detection equipment (Alli *et al.* 1994). Mercury concentrations in aquatic systems, showing significant levels of methyl mercury, can be at or near the detection limit of even the most sensitive equipment. The difficulty is further compounded when trying to develop growth media free of mercury contaminants such that one can achieve realistic environmental concentrations.

Section 1.11

Objectives

The objectives of this thesis project were derived from the following observations:

i) The evidence implicating SRB as the principle methylators of mercury in estuarine sediments is based on experiments using molybdate as a specific inhibitor of SRB.

ii) Pure culture work with SRB shows that they methylate best when SO_4^{2-} is low or absent and under these conditions molybdate would not be expected to inhibit SRB because it is thought to work through competition with SO_4^{2-}.

iii) Many genera of microorganisms other than SRB have been shown in the past to methylate mercury in pure culture at high total mercury concentrations.

iv) Past work investigating mercury methylation in pure culture and the majority of work on methylation in water and sediment have all been done using high total mercury concentration.

Therefore I have proposed to;

i) investigate the specificity of molybdate inhibition, and its mode of action;

ii) investigate factors affecting mercury methylation in pure culture;

iii) conduct a new survey of a variety of microorganisms including a selection of lab strains, and environmentally significant microorganisms, at lower mercury concentrations, using Hg^{203} techniques more sensitive than past work but still not at environmental concentrations;

iv) investigate the ability of organisms to methylate at total mercury concentrations approximating mercury concentrations seen in the environment.

Chapter 2

Specificity of the effect of molybdate on the growth of sulphate reducing and other bacteria

Section 2.1

Introduction

Molybdate has long been held as a specific inhibitor of sulphate reducing bacteria (SRB)(Postgate 1952). The initial research on molybdate inhibition of SRB was economically driven for industrial needs (Ormeland and Capone 1988), such as the prevention of corrosion of steel structures, fouling of hydrocarbon fuels, and pollution of deep water wells and domestic hot water systems (Postgate 1984). With the ability to inhibit the growth of SRB responsible for these problems, molybdate has been an effective industrial tool and research has continued in this direction (Hamilton 1985).

Since its initial consideration for industrial use, molybdate has also been frequently used in environmental microbiology as a means of isolating the effects of SRB in mixed natural populations (Ormeland and Capone 1988). The first use of molybdate as a specific inhibitor of SRB for environmental studies was by Ormeland (1976). Others have used molybdate for similar purposes (see Ormeland and Capone 1988, for a detailed review).

There are thought to be two different modes of action for the inhibition of SRB by oxyanions. Molybdate, as a member of the Group VI oxyanions, acts as an analogue of sulphate, and competes for uptake by sulphate reducers creating a competitive inhibition situation (Furuska 1961, Postgate 1952). A further biochemical inhibition occurs once the molybdate is in the cell (Oremland and Capone 1988, Taylor and Oremland 1979). The first step in the sulphate reduction pathway requires the activation of the sulphate through association with the enzyme ATP-sulfurylase (Peck 1959). The energy cost of the activation of the sulphate is one ATP, producing adenosine-5'-phosphosulphate (APS) (Peck 1959). This ATP is recovered in subsequent reductions (Voordouw 1995 for review, Peck 1960). In the presence of molybdate one ATP is spent to produce an adenosine-5' phosphomolybdate complex (APMo) via ATP-sulfurylase, which is unstable and quickly dissociates, without the recovery of ATP, and without participating in the further reductions required to produce additional ATP (Wilson and Bandurski 1958). Molybdate can then go on to form another APMo complex repeating this futile cycle. By causing the hydrolysis of ATP via ATP-sulfurylase, molybdate causes a reduction of the cellular ATP content (Taylor and Oremland 1979, Peck 1962).

Even though molybdate has been used extensively as a specific inhibitor of SRB in ecological studies, a number of questions about its use remain. For example, what are the effects of molybdate on the activities of other bacteria? Jones *et al.* (1982) reported the inhibition of methanogenesis in freshwater systems at molybdate concentrations of 20 mM. Concentrations of molybdate ranging from 2 mM to 100 mM have been used to successfully inhibit sulphate reduction in a variety of systems (Ormeland and Capone 1988). Wolin and Miller (1980) report the inhibition of hydrogenase synthesis in *Ruminococcus albus* by molybdate at a concentration of 0.01 mM, and significant inhibition of growth at a molybdate concentration of 0.1 mM. This suggests further investigation into the specificity on molybdate inhibition would be prudent.

More importantly, what are the effects on SRB while growing in the absence of sulphate? SRB, depending on the strain, can grow on a wide variety of substrates either fermentatively or with alternate terminal electron acceptors, in low sulphate environments (Postgate 1984), but it is not known whether molybdate inhibits SRB under these conditions. In the fermentative mode of growth it is obvious that a means other than the sulphate reducing pathway is being utilized. Also respiratory growth on terminal electron acceptors other than sulphate may indicate the operation of metabolic pathways other than that which is operating while respiring on sulphate. There is minimal experimental data on the effects of molybdate on SRB while growing on fermentative substrates or alternate electron acceptors. Thus we do not know whether molybdate additions inhibit SRB in general, or only affect those cells currently using sulphate as a terminal electron acceptor. This is an important question because the test of effectiveness of molybdate in environmental studies is inhibition of sulphate reduction (measured by the conversion of $^{35}SO_4^{2-}$ to $^{35}S^{2-}$) with no test for activity of the SRB carrying out other types of energy metabolism.

The present work is undertaken in order 1) that a broader understanding of the effects of molybdate on organisms other than SRB may be gained, and 2) to investigate the effects of molybdate on SRB when grown in the absence of sulphate. To this end inhibition studies on a wide range of bacteria were undertaken, as well as inhibition studies of SRB grown on a range of substrates. The effects of molybdate on ATP levels of *D. desulfuricans* and B-203, a *Desulfovibrio* species capable of methylating mercury, grown on a variety of substrates and then exposed to molybdate were investigated. A key question is whether the sulphate reduction pathway, which interacts with molybdate, is operational in the absence of sulphate. Thus experiments were also done to determine if selected SRB continually express the sulphate reducing pathway or if it must be induced by the presence of sulphate. If it is inducible, is molybdate also an inducer?

Section 2.2

Materials and Methods

i) Organisms examined

The following organisms were tested for inhibition by molybdate; *Acetobacterium woodii* DSM 1030, *Desulfovibrio baarsii* DSM 2075, *D. desulfuricans* DSM 1924, *D. desulfuricans* Essex 6 DSM 642, *D. desulfuricans* ATCC 27774, *D. vulgaris* DSM 644, *Desulfobulbus propionicus* DSM 2032, *Desulfotomaculum guttoideum* DSM 4024, *D. ruminis* DSM 2154, *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* MP 180, *Lactobacillus plantarum* ATCC 8014, *Methanococcus thermolithotrophicus* DSM 2095, *Methanosarcina barkeri* strain Fusaro DSM 804, *Methanosphaera stadtmanae* DSM 3091, *Methanospirillum hungaeti* strain GP1 DSM 1101, *Myxococcus xanthus* DK 1050, *Pseudomonas fluorescens* 493, *Rhodospirillum rubrum* ATCC 11170, *Staphylococcus aureus* 96, and *Streptococcus faecalis* 97.

The non-sulphate reducing eubacteria tested comprise a variety of Gram negative and Gram positive species. Among the Gram negative species is included *Myxococcus xanthus*, an organism suggested to be phylogenetically related to the SRB (Devereux *et al.* 1989). The remaining Gram negative organisms as well as the Gram positive are all species, or members of genera that in the past have been shown to methylate mercury (Hamdy and Noyes 1975, Rowland *et al.* 1975, Vonk and Sijpesteijn 1973), with the exception of *Rhodospirilum rubrum*, which was chosen for its ability to grow phototrophically under anaerobic conditions and its ability to oxidize H_2S to sulphate.

Methanogens were chosen for investigation due to their early implication in mercury methylation (Wood *et al.* 1968). Of four species of methanogens examined, representatives of the three recognised orders of methanogens are present (Vogels *et al.* 1988). *Methanococcus thermolithotrophicus* is a member of the group Methanococcaeles, *Methanospirillum hungaeti* and *Methanosarcina barkeri*, are members of the group Methanomicrobeailes, and *Methanosphaera stadtmanae*, is a member of the group Methanobacteriales. Methanogens and SRB are both strict anaerobes and can be found occupying the same anaerobic environments. Inhibition of methanogenesis has been reported previously with molybdate concentrations of 20 mM (Jones *et al.* 1982).

ii) Determination of growth

To determine the effects of molybdate on the growth of the listed microorganisms, with the exception of *M. xanthus*, all were grown anaerobically in triplicate in 9 ml of the required media, and inoculated with a 10% (1 ml) inocula from a mid-log phase culture. Growth was measured via changes in optical density at 660 nm in a LKB spectrophotometer (Novaspec II). Balch-Wolfe tubes, 15 ml nominal capacity, Bellco stoppered with butyl rubber stoppers and aluminum crimp tops were used as the culture vessels. Prior to degassing and autoclaving, molybdate was added from stock solutions of 1 M, 0.1 M, 0.01 M, and 0.001 M to achieve final concentrations of 30 mM, 20 mM, 10 mM, 1 mM, 0.1 mM, and 0.01 mM. 0.1 ml of the appropriate stock was added to 8.9 ml of media to achieve concentrations of 0.01 mM to 10 mM and 0.2 and 0.3 ml of the 1 M stock was added to 8.7 ml of media to achieve concentrations of 20 mM and 30 mM respectively. 0.1 ml distilled water was used to bring the volume up to 9.0 ml where required. All cultures were incubated in the dark, with the exception of *Rhodospirillum rubrum*, which was incubated under incandescent light. *M. xanthus* was grown aerobically in 50 ml of media in side arm flasks. Molybdate was added to the media prior to autoclaving. From a 1 M stock, 1.5, 1.0, and 0.5 ml were added to 48.5 ml of media, with distilled water used to bring the final volume up to 50 ml where necessary, to achieve concentrations of 30 mM, 20 mM, and 10 mM, respectively. From 0.1 M, 0.01 M, and 0.001 M stocks, 0.5 ml was added to 48.5 ml of media. Volume was made up to 50 ml with distilled water to achieve molybdate concentrations of 1.0 mM, 0.1 mM, and 0.01 mM. Growth was measured in a Klett Summerson photocolourimeter. Media formulations and growth conditions are listed in appendices A, B, and C.

iii) Induction of sulphate reduction pathway via molybdate

To determine if molybdate has the ability to induce the sulphate reduction pathway in fermentatively growing SRB, it was first determined if cells of *Desulfovibrio desulfuricans* DSM 1924, *Desulfovibrio desulfuricans* ATCC 27774, *D.*

vulgaris DSM 644, *Desulfobulbus propionicus* DSM 2032 and *Desulfotomaculum ruminus* DSM 2154, continuously express the enzymes required to reduce sulphate, even when grown fermentatively, or if these enzymes need to be induced by the presence of sulphate. Cells were grown in batch culture in 500 ml of fermentative media (appropriate for the organism) in 1 litre Pyrex reagent bottles (Corning) (see appendices A, B, and C, for media formulations, solutions and growth conditions). Cells were harvested at late log phase in a Coy anaerobic chamber (Coy Laboratory Products Inc. 22 Metty Drive, Ann Arbor Michigan 48103) with an atmosphere of 90:10 N_2/H_2, then placed in O-ring sealed centrifuge bottles and centrifuged at 4,225 x g for 15 minutes. Again in an anaerobic chamber the supernatant was discarded and the pellet resuspended in 100 ml of harvesting buffer (see appendix B for formulation) then dispensed in 50 ml aliqouts to two, 100 ml serum bottles, stoppered with butyl rubber stoppers and aluminum crimp tops. To each of the bottles, 5 ml of a fumarate sulphate solution was then added. For *Desulfobulbus propionicus* a lactate sulphate solution was used in place of the fumarate sulphate solution as *D. propionicus* is known to grow fermentatively on lactate. The serum bottles were then incubated in a water bath at the appropriate temperatures (see appendix C) for the duration of the experiment. Samples were then drawn at time zero and then at 2 minute intervals and analyzed for sulphide production via the method of Truper and Schlegel (1964) with sample and reagent volumes being reduced by 1/10 for convenience.

iv) Determining ATP content

To determine the effect molybdate has on cellular ATP content of cells growing on a variety of fermentative substrates, and terminal electron acceptors, *Desulfovibrio desulfuricans* DSM 1924 and *D. desulfuricans* B-203 were used. *D. desulfuricans* DSM 1924 grows fermentatively on a wide variety of substrates and can utilize a variety of terminal electron acceptors. *D. desulfuricans* B-203 grows fermentatively as well as respires sulphate, and is known to methylate mercury. They were grown in five, 500 ml batch cultures in 1 L Pyrex reagent bottles (Corning) (see appendices A, B, and C for media formulations, amendments and growth conditions). Cultures were grown to near the end of log phase and harvested in a Coy anaerobic chamber with an atmosphere of 90:10 N_2/H_2, then placed in O-ring sealed centrifuge bottles and centrifuged at 4,225 X g for 15 minutes. Again in an anaerobic chamber the supernatant was discarded and the pellets are resuspended in 100 ml harvesting buffer and dispensed in 20 ml amounts to four, 100 ml nominal capacity serum bottles stoppered with butyl rubber stoppers and aluminum crimp tops. Molybdate was added to two of the bottles to a final concentration of 10 mM. All four bottles received 2.5 ml of the nutrient solution appropriate for the nutrient medium on which they were grown (see appendices A and B for formulations). The bottles were then incubated for one hour at 30° C. Cultures were then passed once at 12,000 psi through a French pressure cell (SLM Instruments) and analyzed for ATP content using the luciferase luciferin assay (Kimmich *et al.* 1975).

Section 2.3

Results and Discussion

i) Determination of molybdate inhibition specificity

No inhibition was seen for any of the non-SRB eubacterial species tested (see Figures 2.1 through 2.9 at end of chapter) at concentrations up to 30 mM molybdate. Results for the methanogens tested are shown in Table 2.1, as derived from Figures 2.10 through 2.13 (see end of chapter).

In this study, two of the tested methanogens, *Methanococcus thermolithotrophicus* and *Methanospirillum hungatei*, showed significant inhibition of growth, as determined by culture turbidity, at molybdate concentrations much lower than 20 mM. This may give cause to reconsider the use of molybdate as a means of isolating the activities of the methanogens from those of SRB when working with mixed natural populations. This bears special significance in the area of mercury methylation, as the use of molybdate was the

Table 2.1: Methanogens tested for inhibition of growth by molybdate

Species	Inhibition[1] by molybdate
Methanosarcina barkeri	no inhibition up to 30 mM[2]
Methanosphaera stadtmanae	no inhibition up to 30 mM[2]
Methanococcus thermolithotrophicus	inhibition at ≥ 0.1 mM[2]
Methanospirillum hungatei	inhibition at ≥ 1.0 mM[2]

[1] Inhibition as determined by culture turbidity as compared to control containing only trace amounts of molybdate as media component (see appendix for media formulation).

[2] mM = millimolar

Experimental determinations are average of triplicates.
Media for *Methanococcus thermolithotrophicus* and *Methanospirillum hungatei* contains no sulphate. The medium for *Methanosphaera stadtmanae* contains $(NH_4)SO_4$ (see appendix for media formulation).

means of attributing the role of environmentally significant mercury methylators to the SRB (Compeau and Bartha 1985).

ii) The effect of molybdate on the growth of SRB with and without sulphate

The inhibition of SRB while respiring on a variety of terminal electron acceptors and while growing on a variety of fermentative substrates are shown in tables 2.2 and 2.3, respectively. Data for these tables are drawn from figures 2.14 through 2.38 (see end of chapter). In results reported here, SRB grown on nitrate, thiosulphate, sulphite, fumarate, lactate, and pyruvate, were inhibited by molybdate at various concentrations. As can be seen from table 2.2, SRB growing with terminal electron acceptors other than sulphate were inhibited by molybdate, with growth on thiosulphate the most sensitive to molybdate. Table 2.3 shows the inhibition of several SRB while growing fermentatively. For the SRB represented in tables 2.2 and 2.3, notice that inhibition by molybdate occurred at lower concentrations for fermentative growth than it did for growth on sulphate.

Such data are in apparent conflict with previous work on the inhibition of SRB by molybdate. It was proposed that molybdate inhibited these organisms only when growing on sulphate (Newport and Nedwell 1988). Inhibition of SRB by molybdate was thought not to occur while the SRB were

Table 2.2: Inhibitory molybdate concentrations on sulphate reducing bacteria while growing on various terminal electron acceptors

Organism	SO_4^{2-}	NO_3^-	S_2O_3
Desulfovibrio desulfuricans (DSM 1924)	10 mM[1]	10 mM[1,5]	0.1 mM[1]
Desulfovibrio desulfuricans Essex 6 (DSM 642)	1.0 mM[1]	Poor Growth	0.1 mM[1]
Desulfovibrio desulfuricans (ATCC 27774)	1.0 mM[1]	1.0 mM[1,4]	0.1 mM[1]
Desulfovibrio vulgaris (DSM 644)	0.1 mM[1,4]	1.0 mM[1,4]	N/D[3]
Desulfovibrio baarsii (DSM 2075)	1.0 mM[1]	N/A[2]	N/D[3]
Desulfotomaculum ruminis (DSM 2154)	1.0 mM[1]	N/A[2]	N/D[3]
Desulfotomaculum guttoideum (DSM 4024)	10 mM[1]	N/D[3]	N/D[3]
Desulfobulbus propionicus (DSM 2032)	1.0 mM[1]	N/A[2]	N/D[3]

[1] mM = millimolar

[2] N/A = not applicable organism does not grow on this substrate

[3] N/D = not determined

[4] Partial inhibition, complete inhibition seen at 10 fold higher molybdate concentration

[5] Partial inhibition

Experimental determinations are average of triplicates

Table 2.3: Inhibitory molybdate concentrations on sulphate reducing bacteria while growing fermentatively

Organism	Fumarate	Pyruvate	Lactate
Desulfovibrio desulfuricans (DSM 1924)	1.0 mM[1]	0.1 mM	N/A[2]
Desulfovibrio desulfuricans Essex 6 (DSM 642)	1.0 mM[1]	0.1 mM	N/A[2]
Desulfovibrio desulfuricans (ATCC 27774)	1.0 mM[1]	1.0 mM	N/A[2]
Desulfovibrio vulgaris (DSM 644)	1.0 mM[1]	N/D[3]	N/A[2]
Desulfotomaculum ruminis (DSM 2154)	1.0 mM[1]	N/D[3]	N/A[2]
Desulfotomaculum guttoideum (DSM 4024)	0.1 mM[1]	N/D[3]	N/A[2]
Desulfobulbus propionicus (DSM 2032)	N/A[2]	N/D[3]	0.1 mM

[1] mM = mM molybdate

[2] N/A = not applicable organism does not grow on this substrate

[3] N/D = not determined

Experimental determinations are average of triplicates growing fermentatively. Newport and Nedwell (1988) reported lack of inhibition of SRB while growing fermentatively, however; they employed a method other than culture turbidity to determine if inhibition had occurred. They investigated the effects of molybdate on fermentatively growing cultures of *Desulfovibrio vulgaris*, strains NCIB 11779 and NCIB 94442, *Desulfotomaculum ruminus*, strain NCIB 8452, and *Desulfotomaculum desulfuricans*, strains NCIB 8372, NCIB 9335 and NCIB 8307. The method they employed involved the introduction of molybdate to cultures with an initial cell density, determined with a haemocytometer, of 10^5 cells/ml. After a five day incubation, cultures with a cell density less than $5 X 10^7$ cells/ml were considered to be inhibited. Using this method changes in cell number, *ie*. growth, can occur and still be considered inhibited if a specified cell density is not met. Using optical density as a measure of growth to determine if inhibition occurs shows changes in cell number over time and allows the comparison of both growth rate and final cell density at the end of the incubation for the various concentrations of molybdate used. So instances of partial inhibition can be distinguished from complete inhibition. Partial inhibition being reduced growth rate and or reduced cell density at the end of incubation, while complete inhibition would be seen as no change in cell density over the incubation period.

iii) Induction of the sulphate reduction pathway

Since molybdate has been shown to inhibit SRB while growing fermentatively it was of importance to determine if molybdate could induce the expression of the sulphate reduction pathway in SRB that do not constitutively express the pathway.

Of the five SRB shown in table 2.4, two, *Desulfovibrio desulfuricans* DSM 1924 and *D. vulgaris* DSM 644, were shown not to express continually the capacity to reduce sulphate. *Desulfovibrio desulfuricans* DSM 1924 and *D. vulgaris* DSM 644 required 40 and 20 minutes respectively, after exposure to sulphate to induce the enzymes required for sulphate reduction. Thus when growing fermentatively in the absence of sulphate they lack the ability to reduce sulphate and must have exposure to sulphate in order to gain the ability to reduce sulphate.

As can be seen in Table 2.4 molybdate does not induce sulphate reduction in these SRB. This is significant as inhibition of SRB by molybdate was thought to occur via interference with the normal functioning of the sulphate reduction pathway. While growing fermentatively neither *Desulfovibrio desulfuricans* DSM 1924 or *D. vulgaris* DSM 644 express the sulphate reduction pathway nor does molybdate induce this pathway as determined by the measurement of S_2. Molybdate must then cause inhibition by some other means, as both these organisms are inhibited by molybdate when growing

Table 2.4: The result of sulphate spike on sulphate reducing bacteria after growth on fermentative medium

Organism	Time Required Prior to Initial Sulphate Reduction (Minutes)	Induction by Molybdate 10 mM
Desulfovibrio desulfuricans (DSM 1924)	40	NO
Desulfovibrio desulfuricans (ATCC 27774)	0	--
Desulfovibrio vulgaris (DSM 644)	20	NO
*Desulfobulbus propionicus** (DSM 2032)	0	--
Desulfotomaculum ruminus (DSM 2154)	0	--

*Methylates mercury

fermentatively (Table 2.3).

iv) The effect of molybdate on cellular ATP content

Table 2.5 shows the effect of molybdate on the ATP content of two SRB species. One of which methylates mercury (*Desulfovibrio* sp B-203). Both were tested while growing fermentatively on fumarate and while respiring on sulphate. *Desulfovibrio desulfuricans* (DSM 1924) was also tested while respiring on nitrate, thiosulphate, and sulphite. As can be seen from the table molybdate reduces the amount of ATP under both fermentative and respiratory conditions, with fermentative conditions being particularly sensitive. It is important to know that for *Desulfovibrio desulfuricans* DSM 1924 the sulphate reduction pathway is not expressed while growing fermentatively and yet cellular ATP content is drastically reduced when growing fermentatively on either fumarate or pyruvate. This indicates that molybdate must obviously act as an inhibitor in some other fashion then that suggested by Newport and Nedwell (1988) and Taylor and Oremland (1979).

Table 2.5: Effects of molybdate on ATP levels of *Desulfovibrio desulfuricans* DSM 1924 and *Desulfovibrio* sp. B-203 grown on various substrates

Substrate	**ATP (ng/mg prot) Minus Molybdate**	**ATP (ng/mg prot) Plus Molybdate**[1]	**Percent of Control**
***Desulfovibrio desulfuricans* DSM 1924**			
Sulphate	26905 ± 0.9%	5905 ± 10.3%	22 ± 10.3%
Nitrate	30300 ± 4.7%	25450 ± 0.6%	84 ± 4.7%
Thiosulphate	30447 ± 3.2%	1447 ± 12.0%	5 ± 12.4%
Sulphite	14187 ± 6.0%	1485 ± 0.01%	10.5 ± 6.0%
Fumarate	16678 ± 4.2%	0 ± 6.3%	0 ± 7.6%
Pyruvate	22935 ± 2.3%	0 ± 6.9%	0 ± 7.3%
***Desulfovibrio* sp. B-203**			
Sulphate	10638 ± 7.5%	0 ± 5.0%	0 ± 9.0%
Fumarate	36642 ± 3.4%	0 ± 0.3%	0 ± 3.4%

[1] After one hour incubation with molybdate

Section 2.4

Conclusions

Molybdate had no effect on a wide range of eubacterial species at concentrations that were inhibitory to a number of SRB. Molybdate inhibited all the SRB tested regardless of whether they were growing fermentatively or respiring. Molybdate did not induce the expression of the sulphate reduction pathway in SRB that do not continually express the pathway, yet molybdate caused the reduction of cellular ATP content of *Desulfovibrio desulfuricans* DSM 1924, a species that does not continually express the sulphate reduction pathway. It can be concluded that molybdate does effectively inhibit SRB, even when grown in the absence of sulphate and when the sulphate reduction pathway is not in operation. A mechanism for inhibition by molybdate other than what is thought to occur for growth on sulphate is obviously responsible for the inhibition seen in the absence of sulphate.

Molybdate also inhibited *Methanococcus thermolithotrophicus* and *Methanospirrillum hungatei*. Although inhibition of methanogenesis due to molybdate has been previously reported (Jones *et al*. 1982) this is the first reporting of particular species of methanogens being inhibited by molybdate. Molybdate does inhibit some methanogens at concentrations well within the range of molybdate concentrations often used to inhibit and therefore isolate the activities of SRB. This may call into question the use of molybdate to separate the environmental activities of SRB and methanogens. This is particularly pertinent in areas of methyl mercury research in which molybdate was used to assign the role of environmentally significant mercury methylation to the SRB. Particularly when earlier work has demonstrated mercury methylation in laboratory experiments with a number of non-SRB eubacterial species, and fungi, as well as methanogen cell extracts (Hamdy and Noyes 1975, Vonk and Sijperstein 1973, Landner 1971).

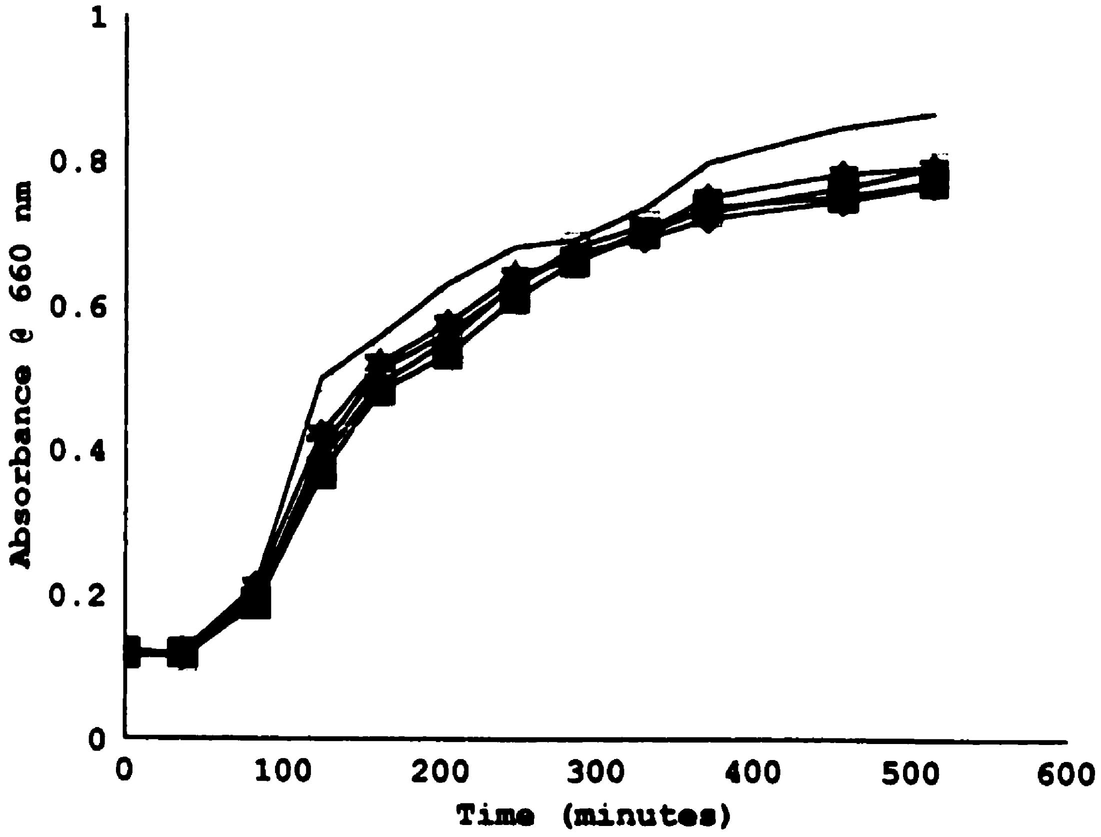

Figure 2.1. The effect of molybdate on the growth of *Bacillus subtillis* ATCC 6051 while growing anaerobically on yeast peptone broth. ☐ control, 1.0 mM molybdate, ■ 10 mM molybdate, ● 20 mM molybdate, + 30 mM molybdate. Values are the average of triplicates.

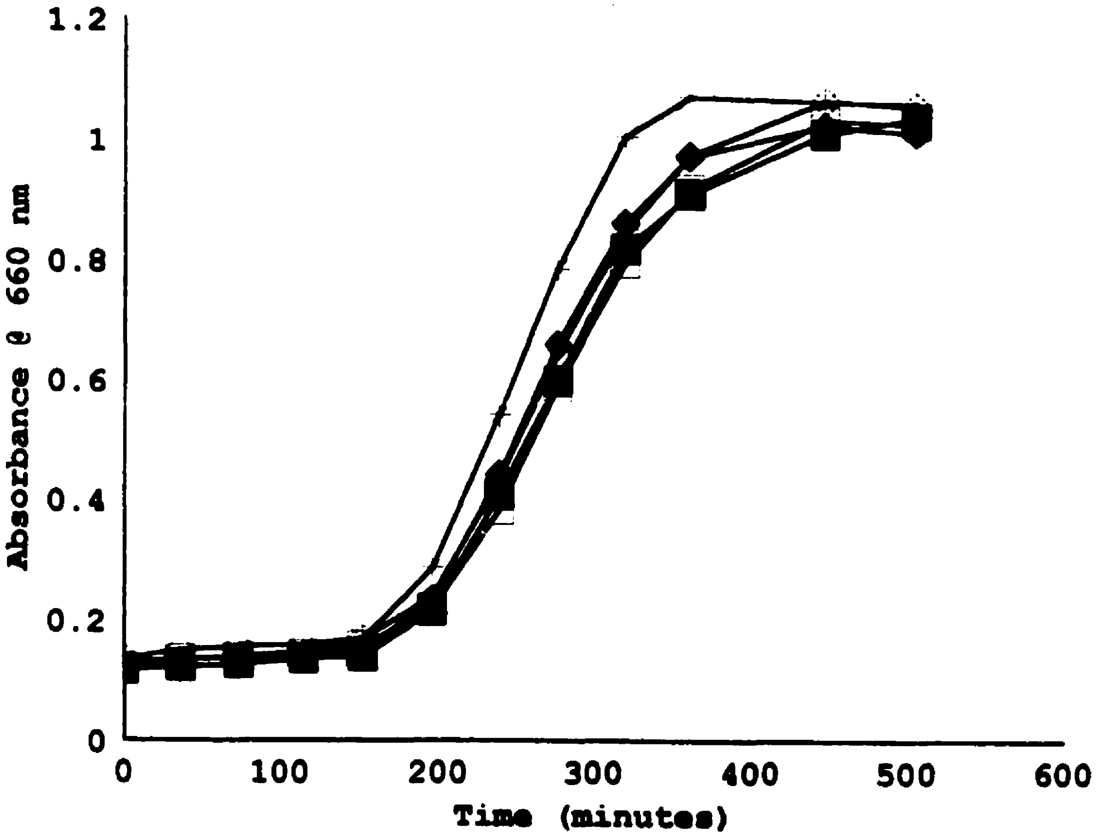

Figure 2.2. The effect of molybdate on the growth of *Enterobacter aerogenes* ATCC 13048 while growing anaerobically on yeast peptone broth. ☐ control, · 1.0 mM molybdate, ■ 10 mM molybdate, ◆ 20 mM molybdate, + 30 mM molybdate. Values are the average of triplicates.

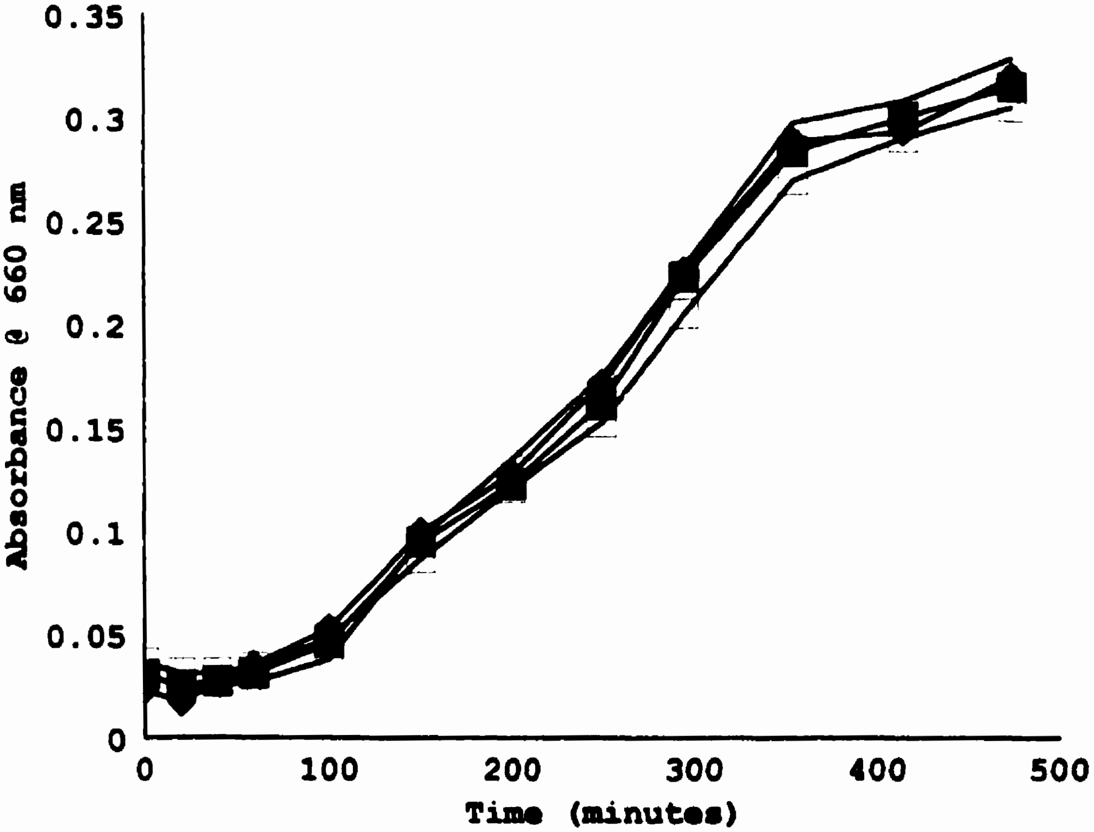

Figure 2.3. The effect of molybdate on the growth of *Escherichia coli* MP 180 while growing anaerobically on yeast peptone broth. □ control, 10 mM molybdate, ■ 20mM molybdate, ◆ 30 mM molybdate. Values are the average of triplicates.

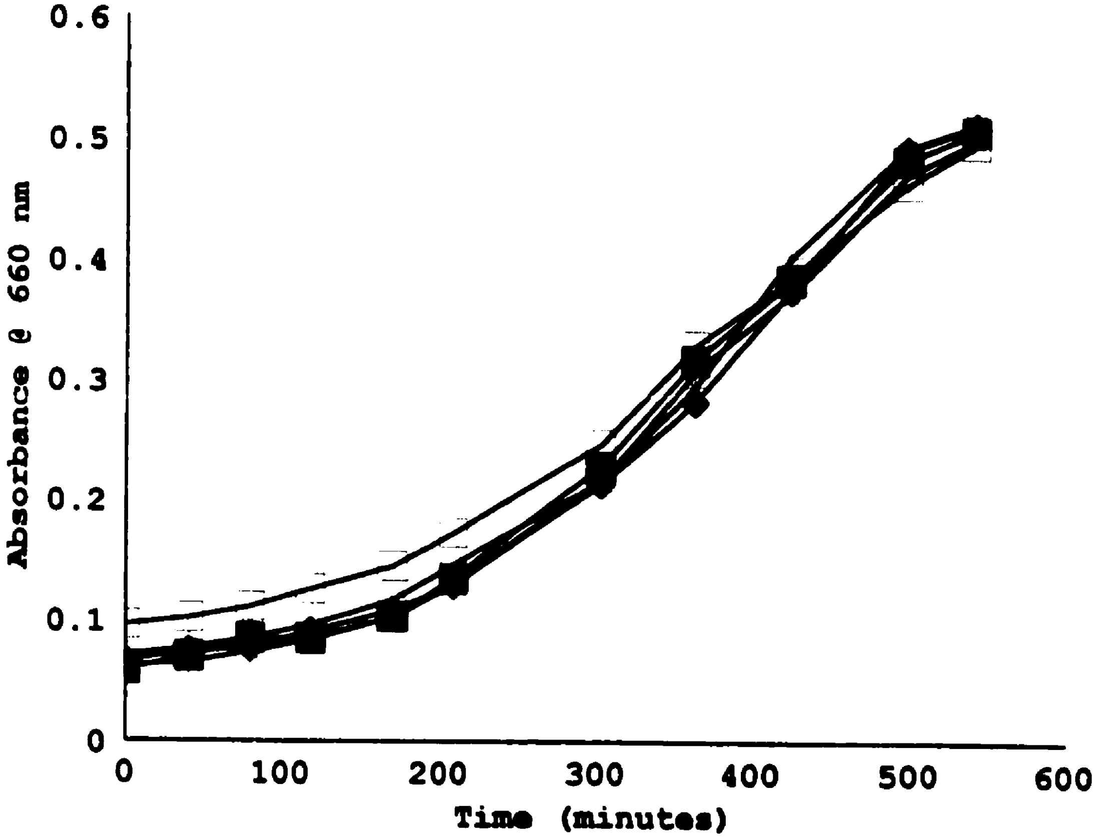

Figure 2.4. The effect of molybdate on the growth of *Lactobacillus plantarum* ATCC 8014 while growing anaerobically on yeast peptone broth. □ control, 1.0 mM molybdate, ■ 10 mM molybdate, ◆ 20 mM molybdate, + 30 mM molybdate. Values are the average of triplicates.

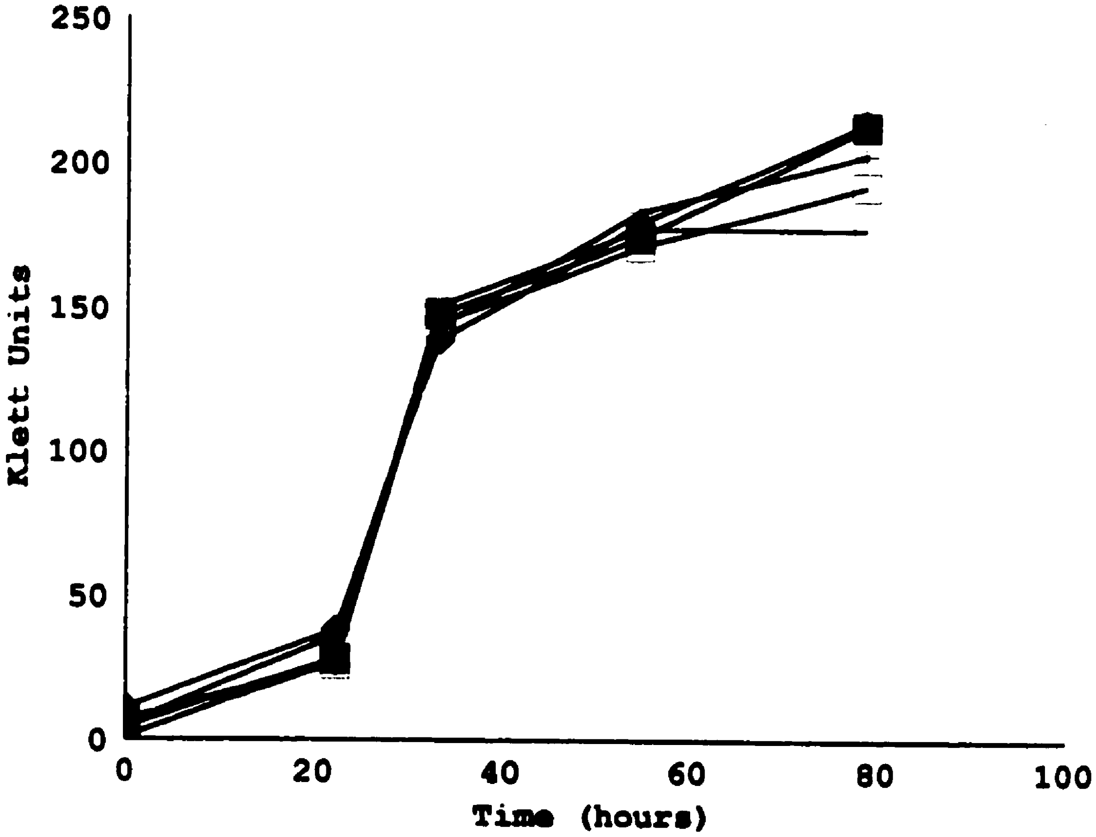

Figure 2.5. The effect of molybdate on the growth of *Myxococcus xanthus* DK 1050 while growing aerobically on casitone and mineral salts broth. □ control, ◇ 1.0 mM molybdate, ■ 10 mM molybdate, ◆ 20 mM molybdate, + 30 mM molybdate. Values are the average of triplicates.

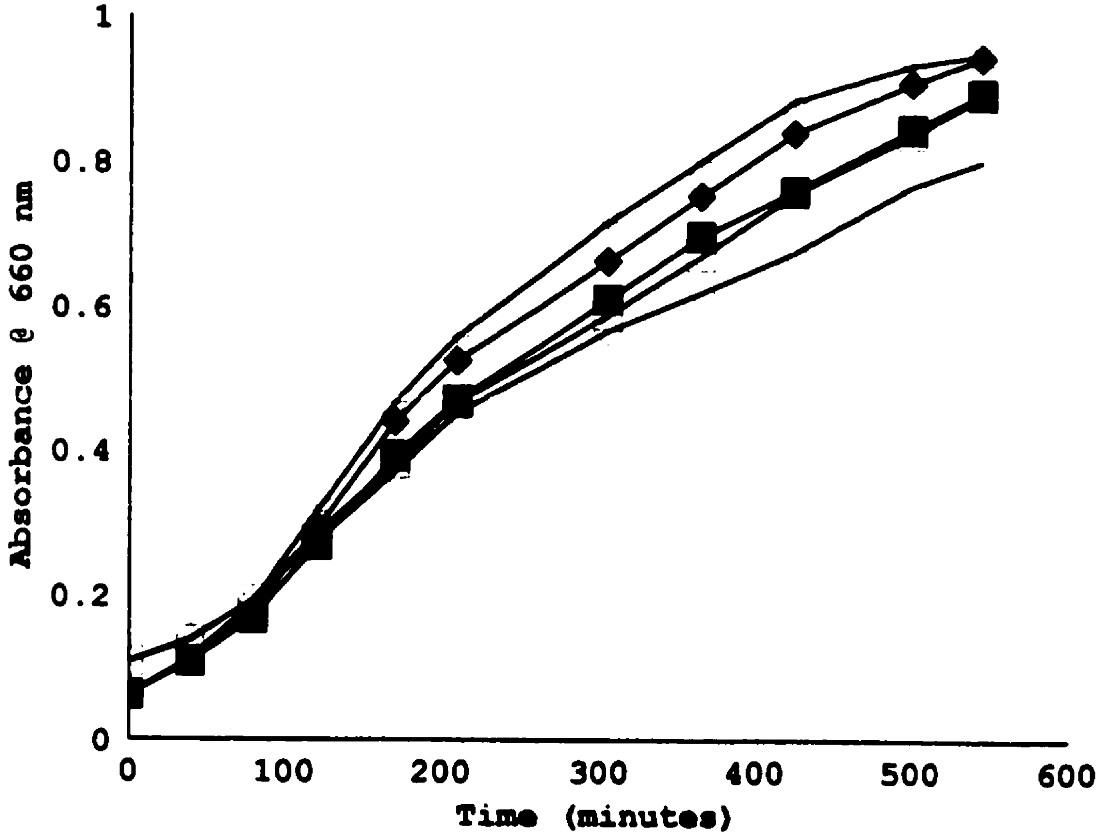

Figure 2.6. The effect of molybdate on the growth of *Pseudomonas fluorescens* 493 while growing anaerobically on yeast peptone broth. □ control, 1.0 mM molybdate, ■ 10 mM molybdate, ◆ 20 mM molybdate, + 30 mM molybdate. Values are the average of triplicates.

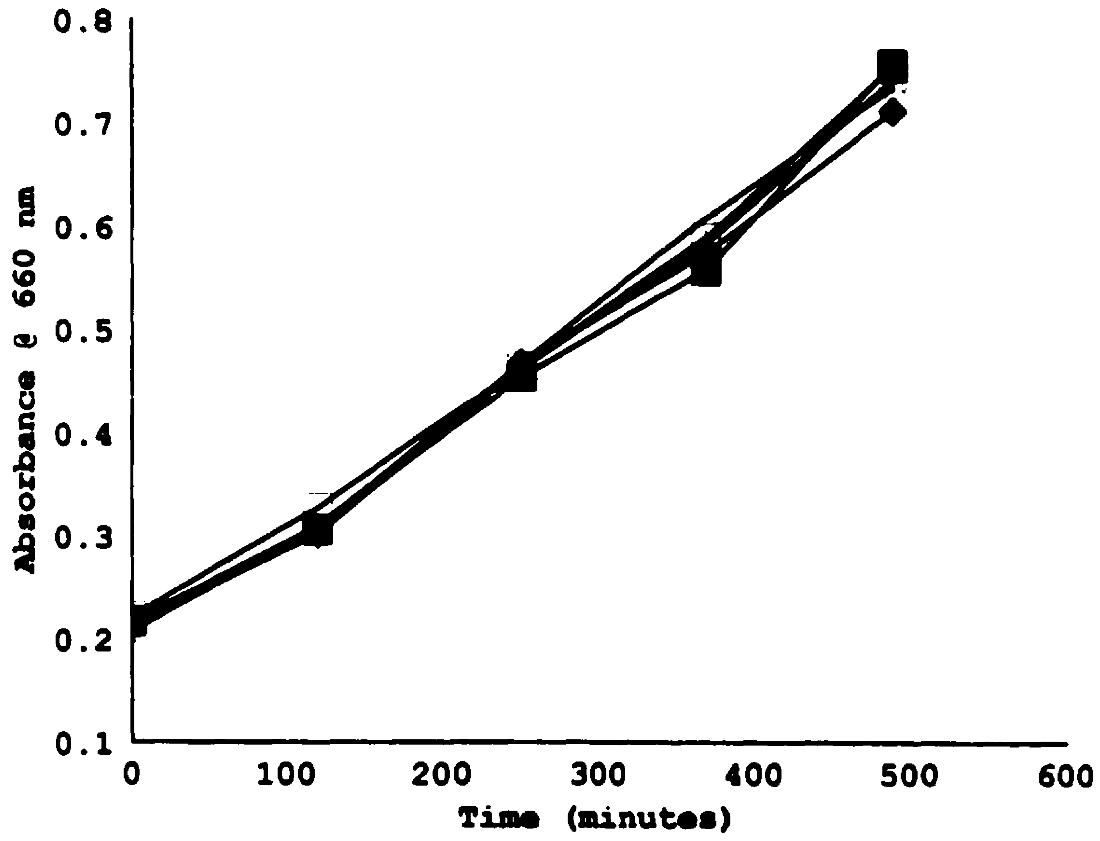

Figure 2.7. The effect of molybdate on the growth of *Rhodospirillum rubrum* ATCC 11170 while growing phototrophically under anaerobic conditions. □ control, 1.0 mM molybdate, ■ 10 mM molybdate, ◆ 20 mM molybdate, + 30 mM molybdate. Values are the average of triplicates.

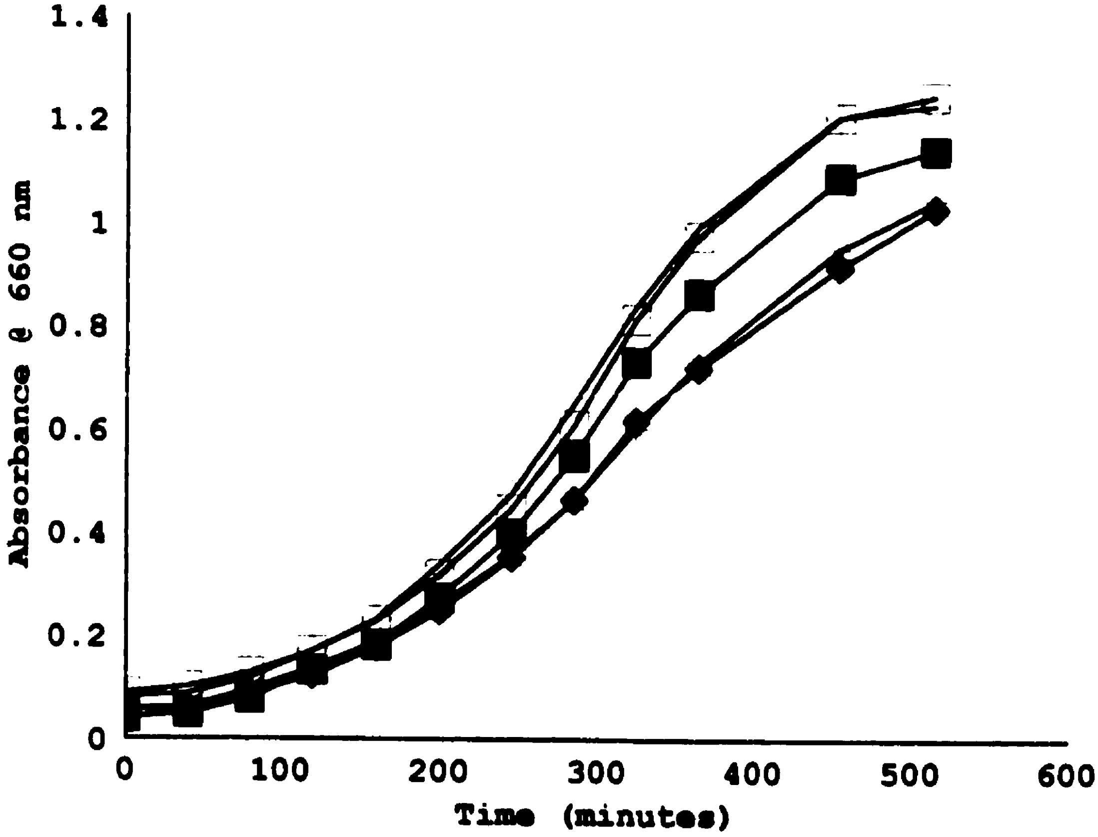

Figure 2.8. The effect of molybdate on the growth of *Staphylococcus aureus* 96 while growing anaerobically on yeast peptone broth. □ control, · 1.0 mM molybdate, ■ 10 mM molybdate, ◆ 20 mM molybdate, + 30 mM molybdate. Values are the average of triplicates.

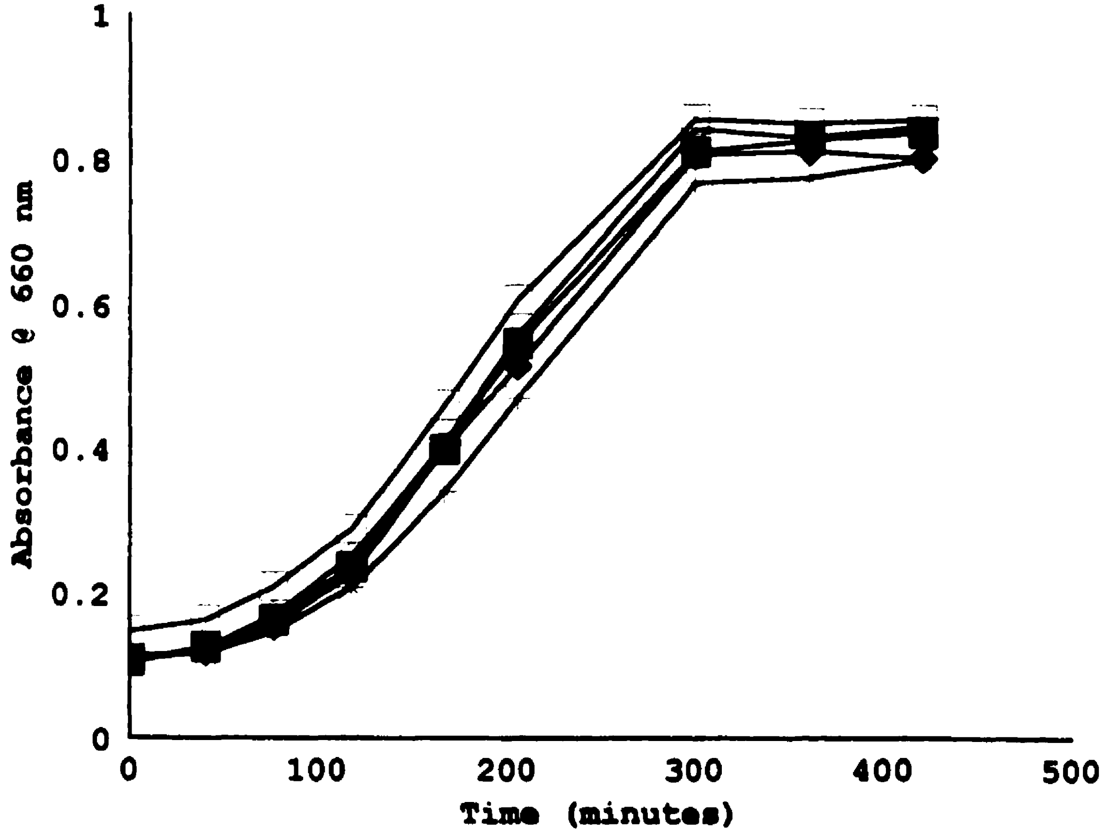

Figure 2.9. The effect of molybdate on the growth of *Streptococcus faecalis* 97 while growing anaerobically on yeast peptone broth. □ control, · 1.0 mM molybdate, ■ 10 mM molybdate, ◆ 20 mM molybdate, + 30 mM molybdate. Values are the average of triplicates.

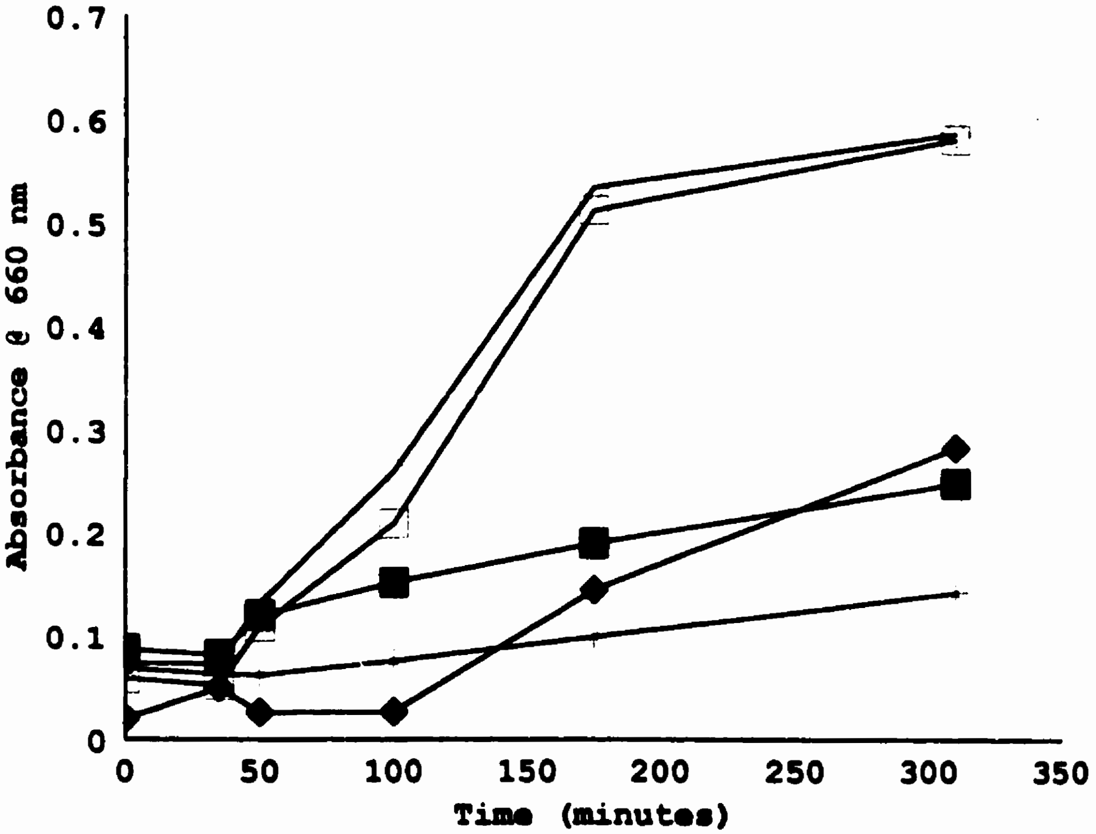

Figure 2.10. The effect of molybdate on the growth of *Methanococcus thermolithotrophicus* DSM 2095 while growing anaerobically. □ control, 1.0 mM molybdate, ■ 10 mM molybdate, ◆ 20 mM molybdate, + 30 mM molybdate. Values are the average of triplicates.

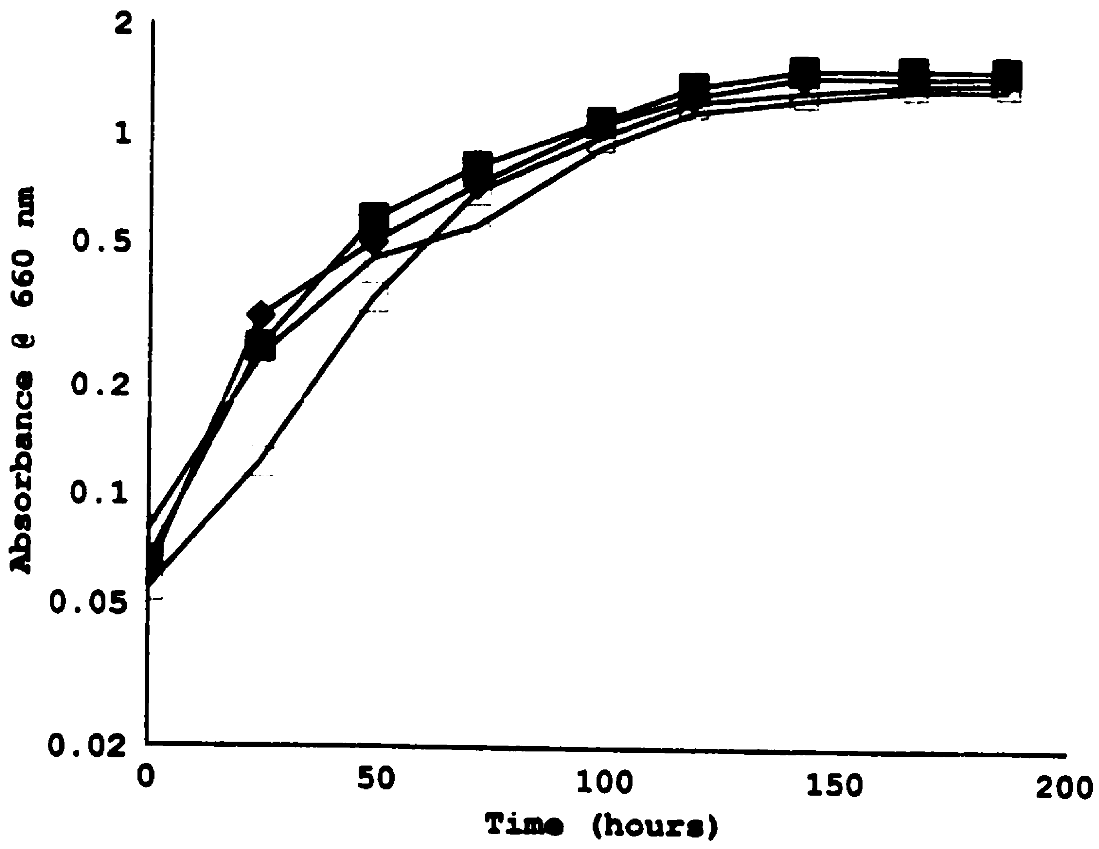

Figure 2.11. The effect of molybdate on the growth of *Methanosarcina barkerii* DSM 804 while growing anaerobically. ☐ control, 10 mM molybdate, ■ 20 mM molybdate, ◆ 30 mM molybdate. Values are the average of triplicates.

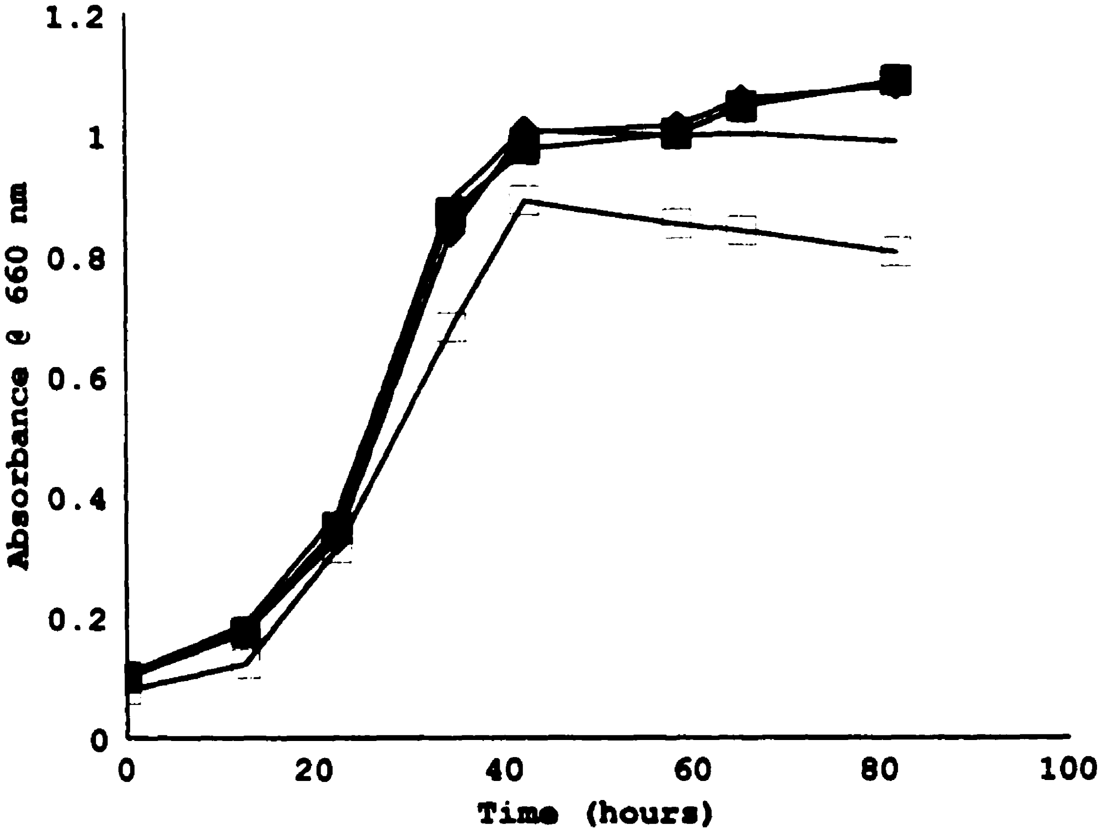

Figure 2.12. The effect of molybdate on the growth of *Methanosphaera stadtmanae* DSM 3091 while growing anaerobically. □ control, ○ 10 mM molybdate, ■ 20 mM molybdate, ◆ 30 mM molybdate. Values are the average of triplicates.

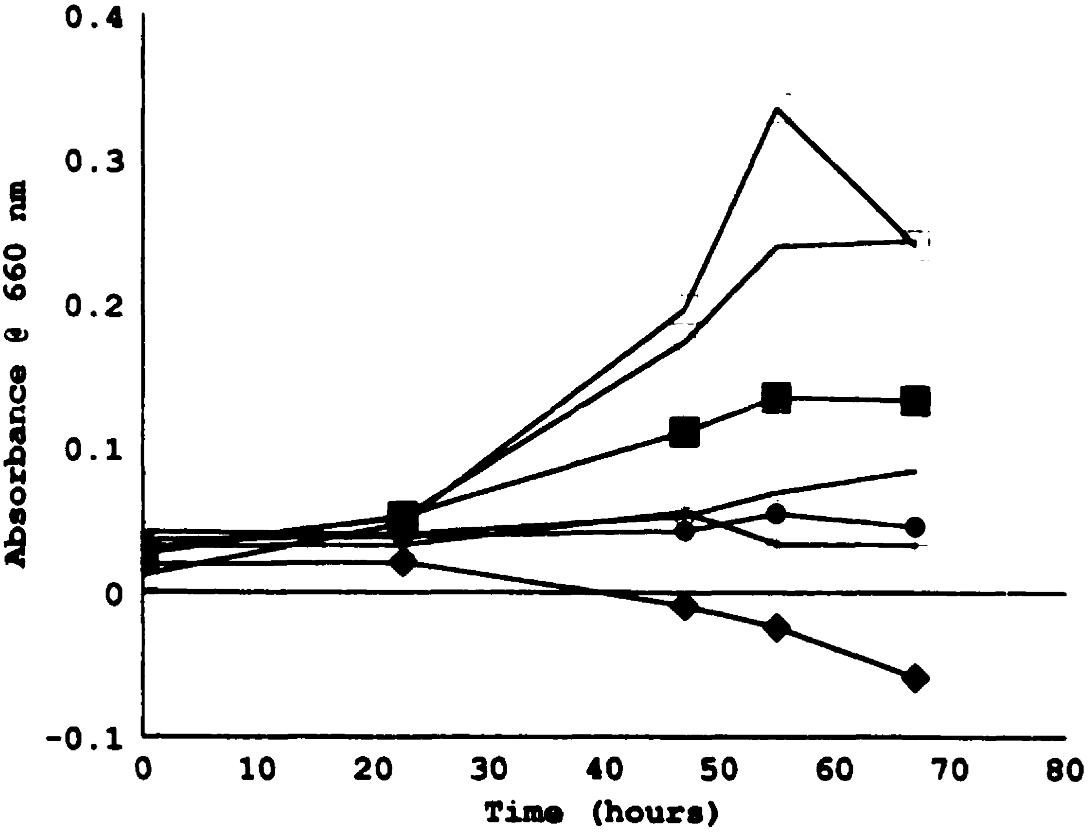

Figure 2.13. The effect of molybdate on the growth of *Methanospirilum hungatei* DSM 1101 while growing anaerobically. □ control, 0.01 mM molybate, ■ 0.1 mM molybdate, ◆ 1.0 mM molybdate, 10 mM molybdate, ● 20 mM molybdate, + 30 mM molybdate. Values are the average of triplicates.

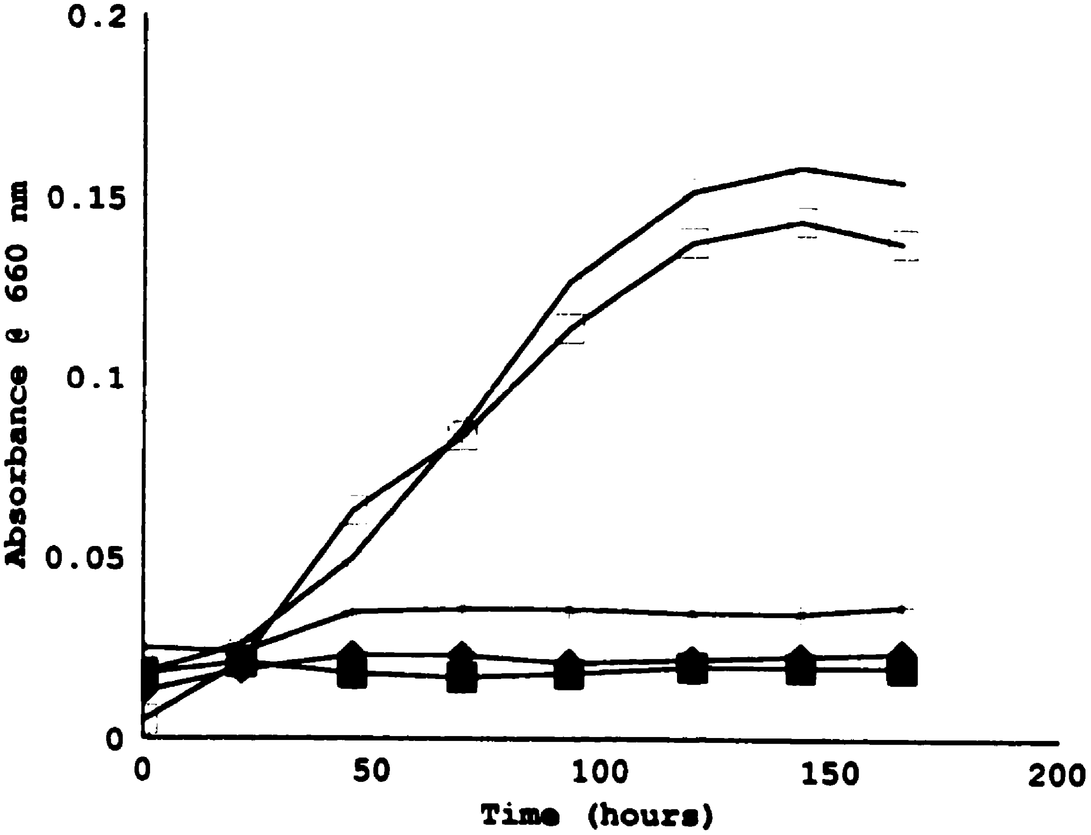

Figure 2.14. The effect of molybdate on the growth of *Desulfobulbus propionicus* DSM 2032 while growing fermentatively on lactate. ☐ control, ◆ 0.01 mM molybdate, ■ 0.1 mM molybdate, ● 1.0 mM molybdate, + 10 mM molybdate. Values are the average of triplicates.

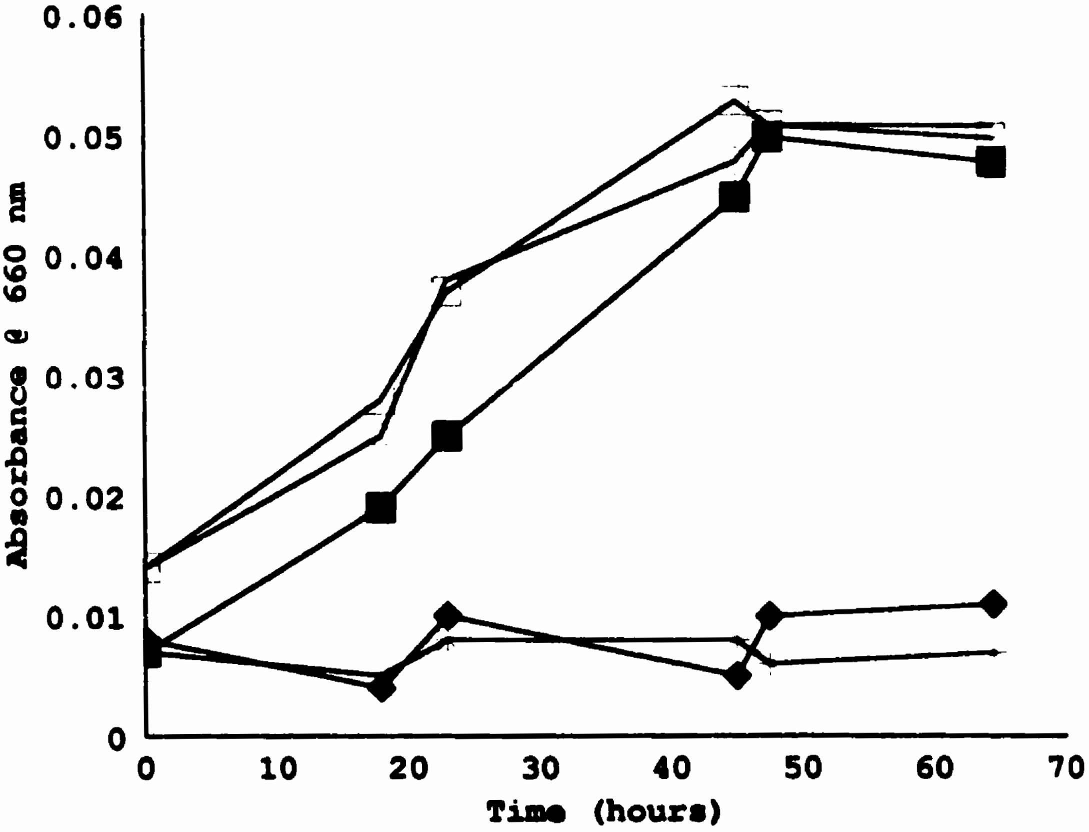

Figure 2.15. The effect of molybdate on the growth of *Desulfobulbus propionicus* DSM 2032 while growing on lactate and sulphate. □ control,
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◆ 1.0 mM molybdate, + 10 mM molybdate.
Values are the average of triplicates.

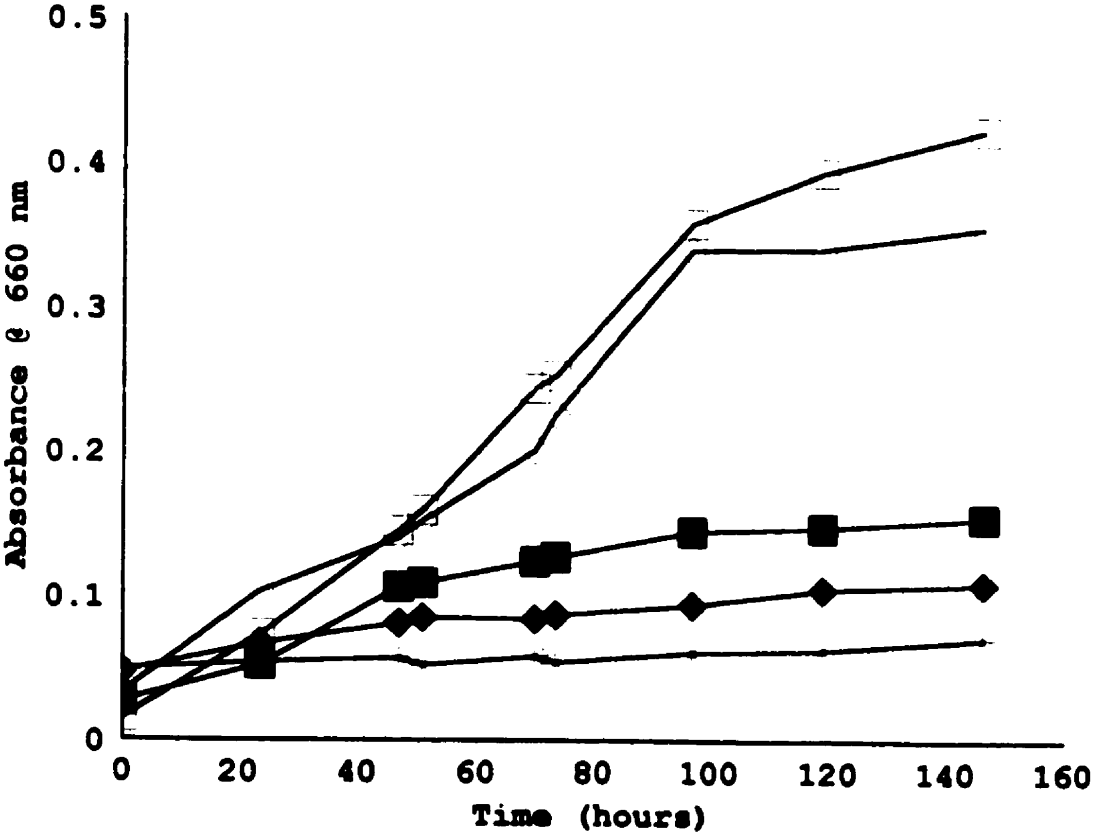

Figure 2.16. The effect of molybdate on the growth of *Desulfotomaculum guttoideum* DSM 4024 while growing fermentatively on fumarate. □ control, ◇ 0.01 mM molybdate, ■ 0.1 mM molybdate, ◆ 1.0 mM molybdate, + 10 mM molybdate. Values are the average of triplicates.

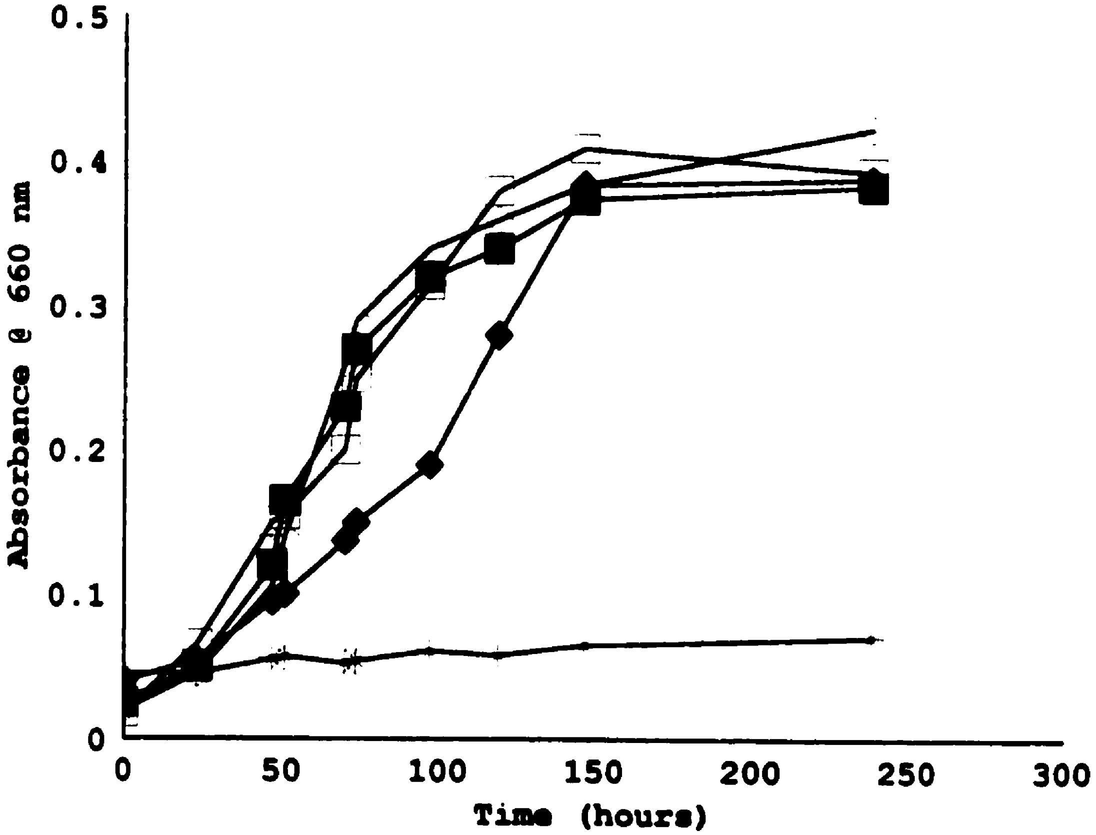

Figure 2.17. The effect of molybdate on the growth of *Desulfotomaculum* guttoideum DSM 4024 while growing on lactae and sulphate. ☐ control, ◇ 0.01 mM molybdate, ■ 0.1 mM molybdate, ● 1.0 mM molybdate, * 10 mM molybdate. Values are the average of triplicates.

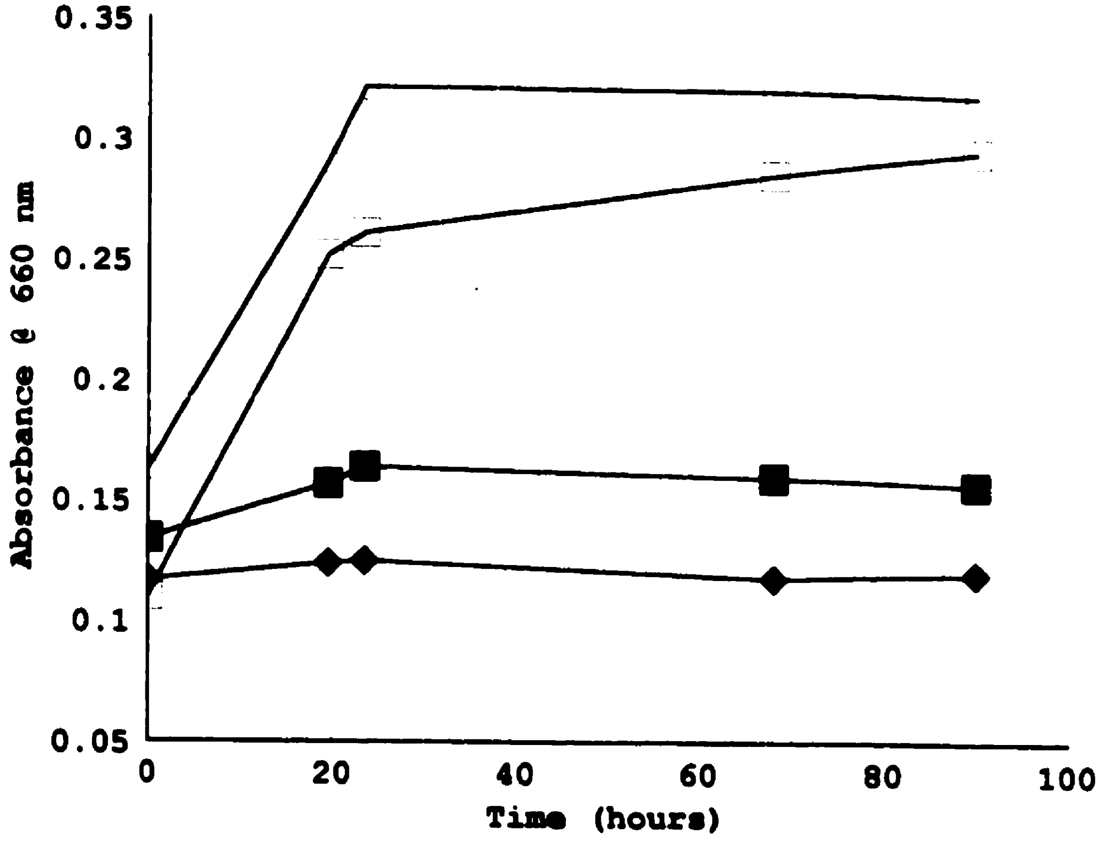

Figure 2.18. The effect of molybdate on the growth of *Desulfotomaculum* ruminus DSM 2154 while growing fermentatively on fumarate. □ control, ◇ 0.1 mM molybdate, ■ 1.0 mM molybdate, ◆ 10 mM molybdate. Values are the average of triplicates.

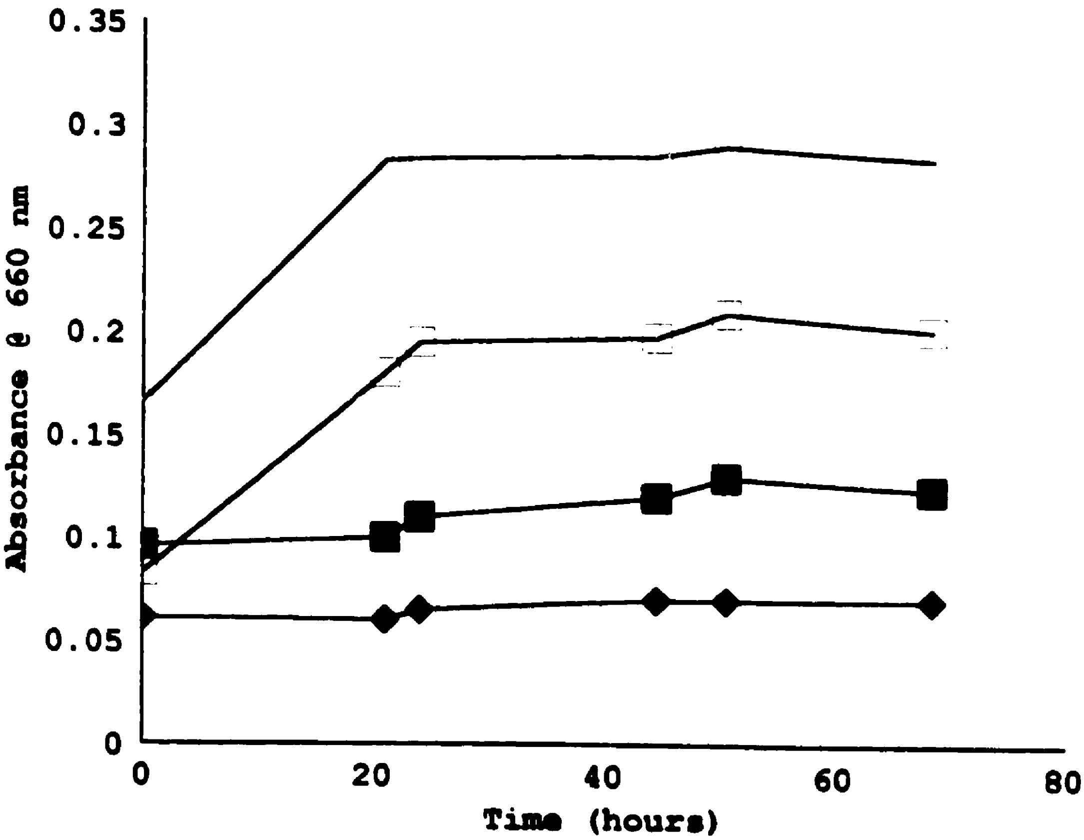

Figure 2.19. The effect of molybdate on the growth of *Desulfotomaculum* ruminus DSM 2154 while growing on lactae and sulphate. ☐ control, 0.1 mM molybdate, ■ 1.0 mM molybdate, ◆ 10 mM molybdate. Values are the average of triplicates.

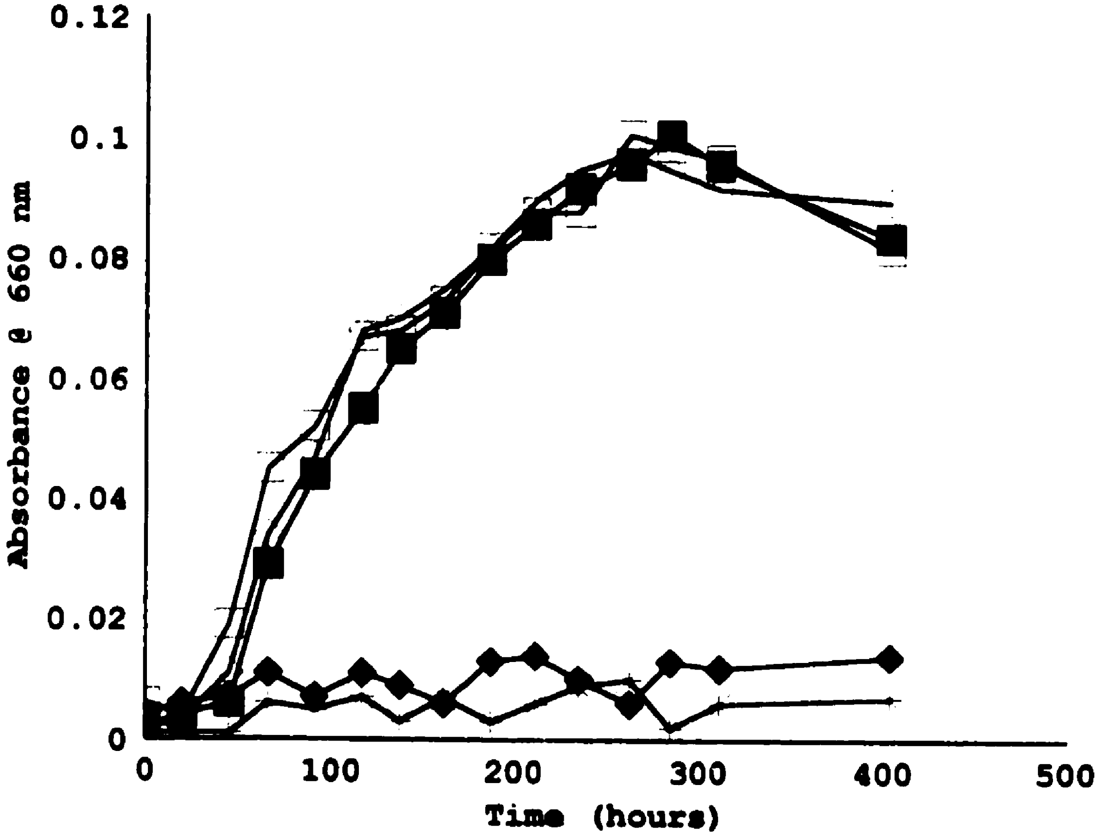

Figure 2.20. The effect of molybdate on the growth of *Desulfovibrio baarsii* DSM 2075 while growing on lactae and sulphate. ☐ control, ◇ 0.01 mM molybdate, ■ 0.1 mM molybdate, ◆ 1.0 mM molybdate, + 10 mM molybdate. Values are the average of triplicates.

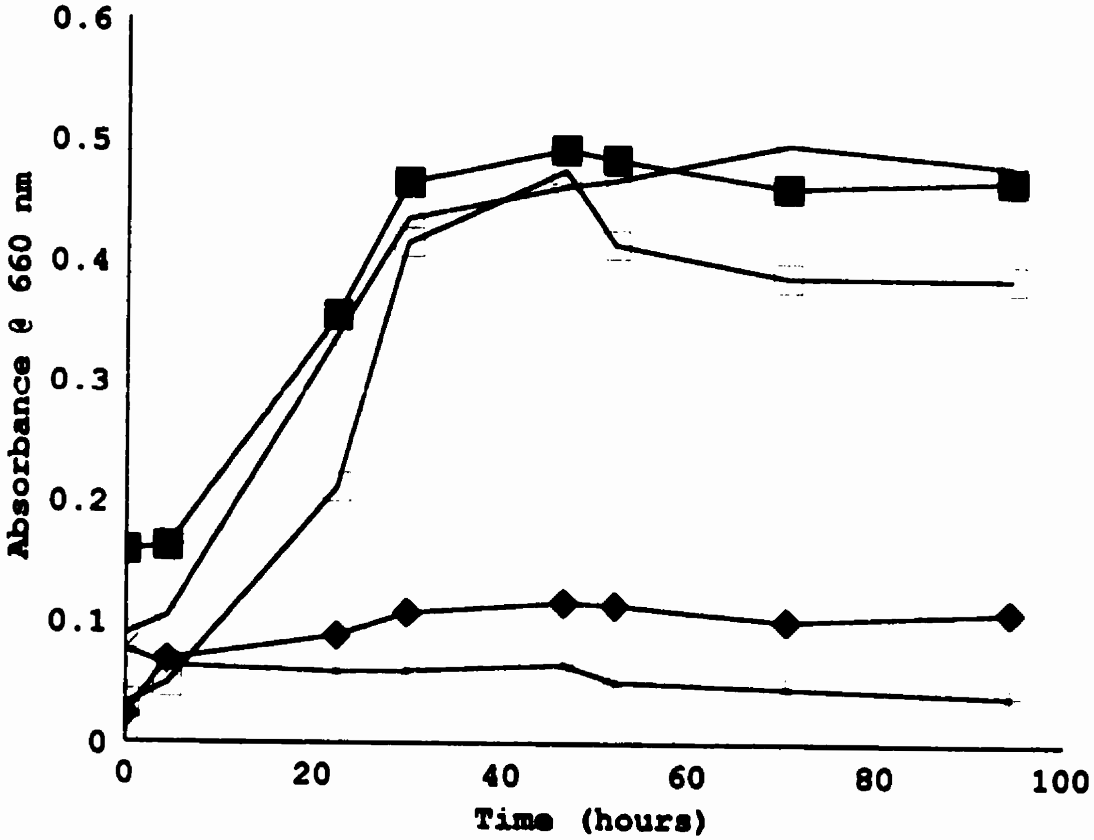

Figure 2.21. The effect of molybdate on the growth of *Desulfovibrio desulfuricans* DSM 1924 while growing fermentatively on fumarate. □ control, · 0.01 mM molybdate, ■ 0.1 mM molybdate, ◆ 1.0 mM molybdate, + 10 mM molybdate. Values are the average of triplicates.

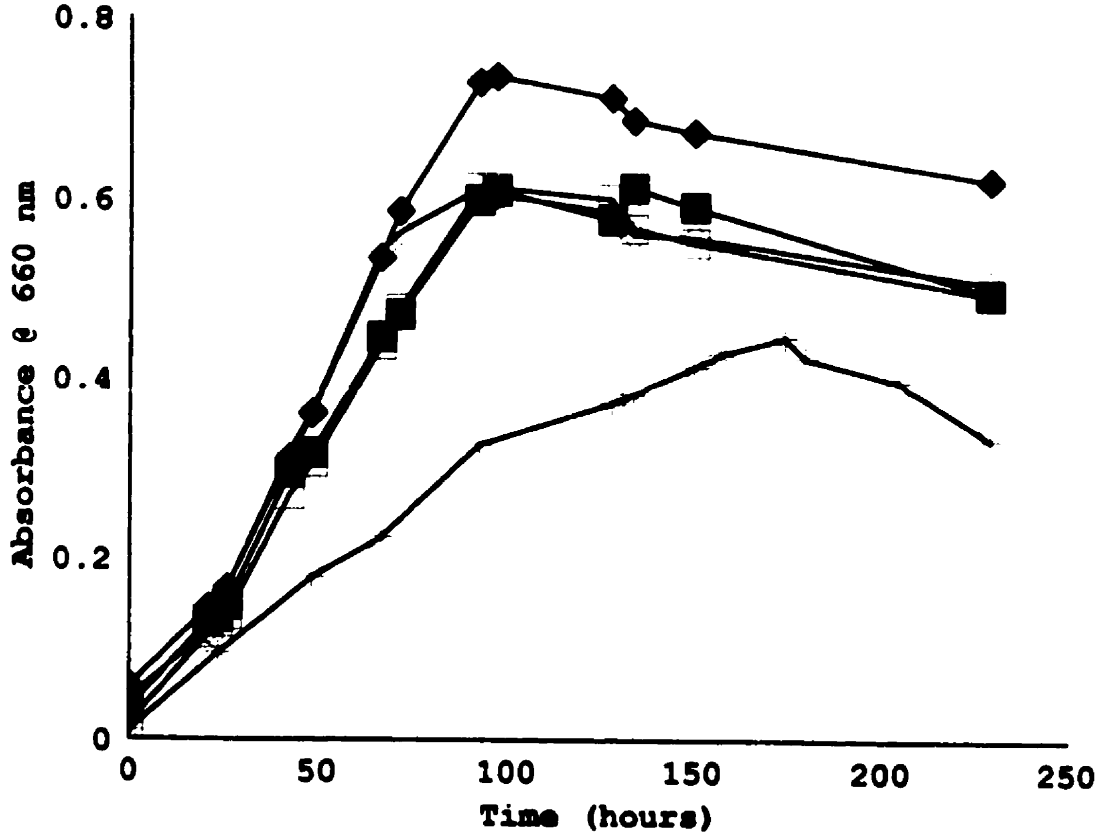

Figure 2.22. The effect of molybdate on the growth of *Desulfovibrio desulfuricans* DSM 1924 while growing on lactate and nitrate. □ control, ◇ 0.01 mM molybdate, ■ 0.1 mM molybdate, ◆ 1.0 mM molybdate, + 10 mM molybdate. Values are the average of triplicates.

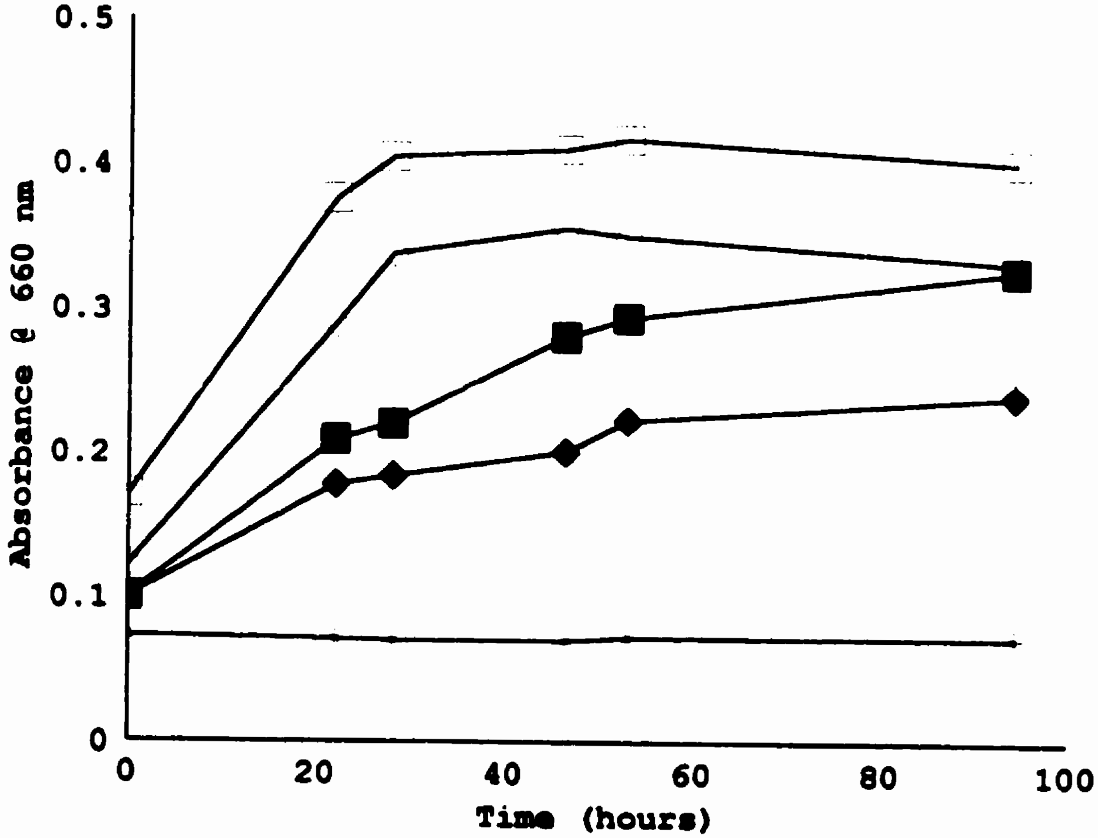

Figure 2.23. The effect of molybdate on the growth of *Desulfovibrio desulfuricans* DSM 1924 while growing fermentatively on pyruvate. □ control, · 0.01 mM molybdate, ■ 0.1 mM molybdate, ◆ 1.0 mM molybdate, + 10 mM molybdate. Values are the average of triplicates.

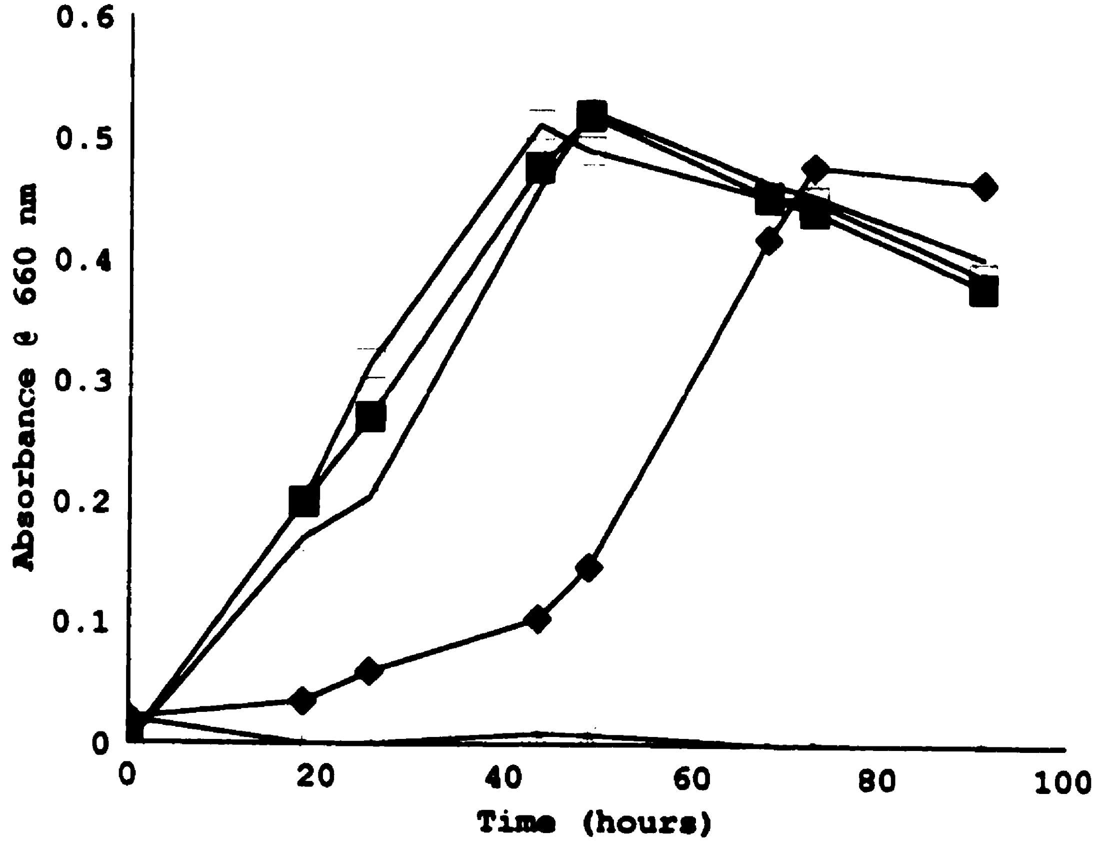

Figure 2.24. The effect of molybdate on the growth of *Desulfovibrio desulfuricans* DSM 1924 while growing on lactate and sulphate. ☐ control, . 0.01 mM molybdate, ■ 0.1 mM molybdate, ◆ 1.0 mM molybdate, + 10 mM molybdate. Values are the average of triplicates.

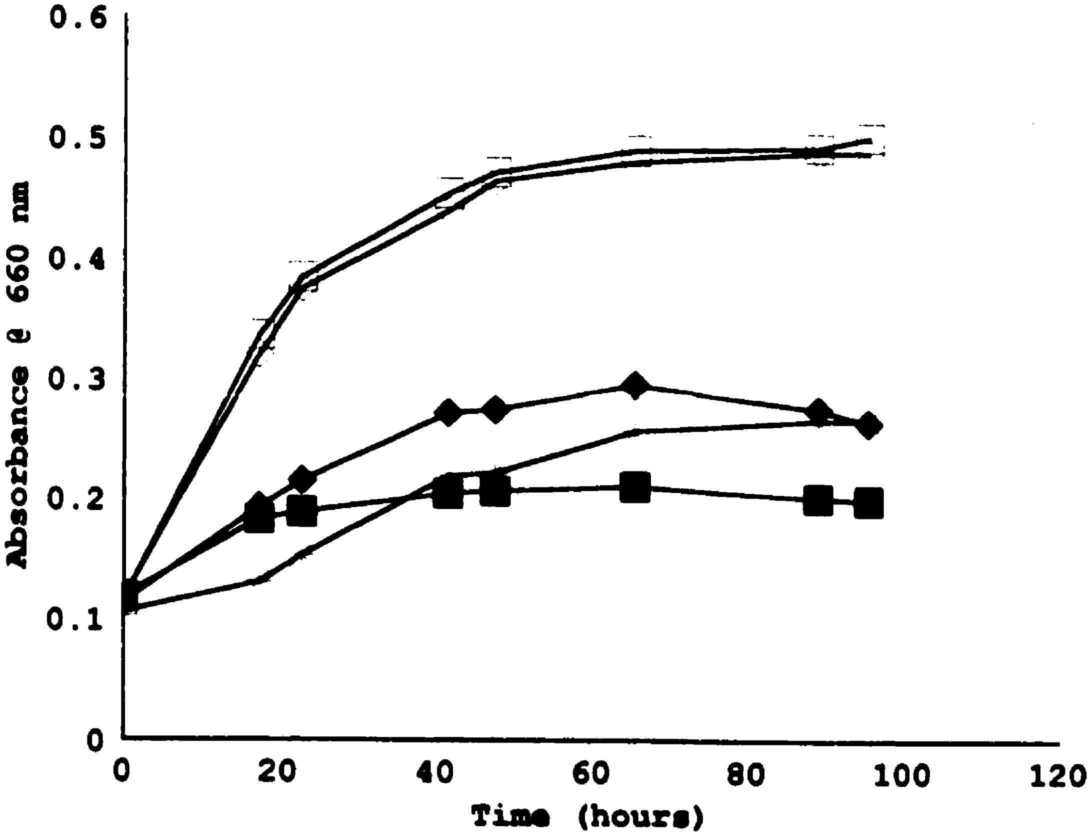

Figure 2.25. The effect of molybdate on the growth of *Desulfovibrio desulfuricans* DSM 1924 while growing on lactate and thiosulphate. □ control, 0.01 mM molybdate, ■ 0.1 mM molybdate, ◆ 1.0 mM molybdate, + 10 mM molybdate. Values are the average of triplicates.

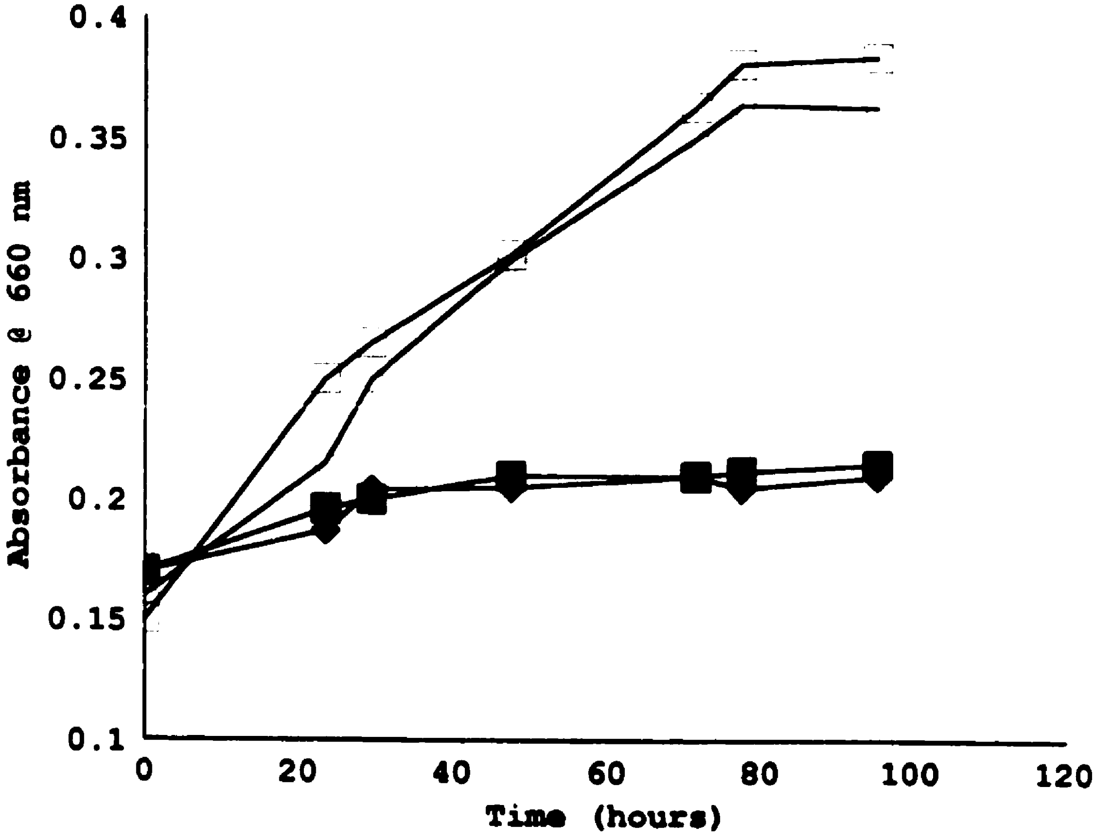

Figure 2.26. The effect of molybdate on the growth of *Desulfovibrio desulfuricans* ATCC 27774 while growing fermentatively on fumarate. ☐ control, 0.1 mM molybdate, ■ 1.0 mM molybdate, ● 10 mM molybdate. Values are the average of triplicates.

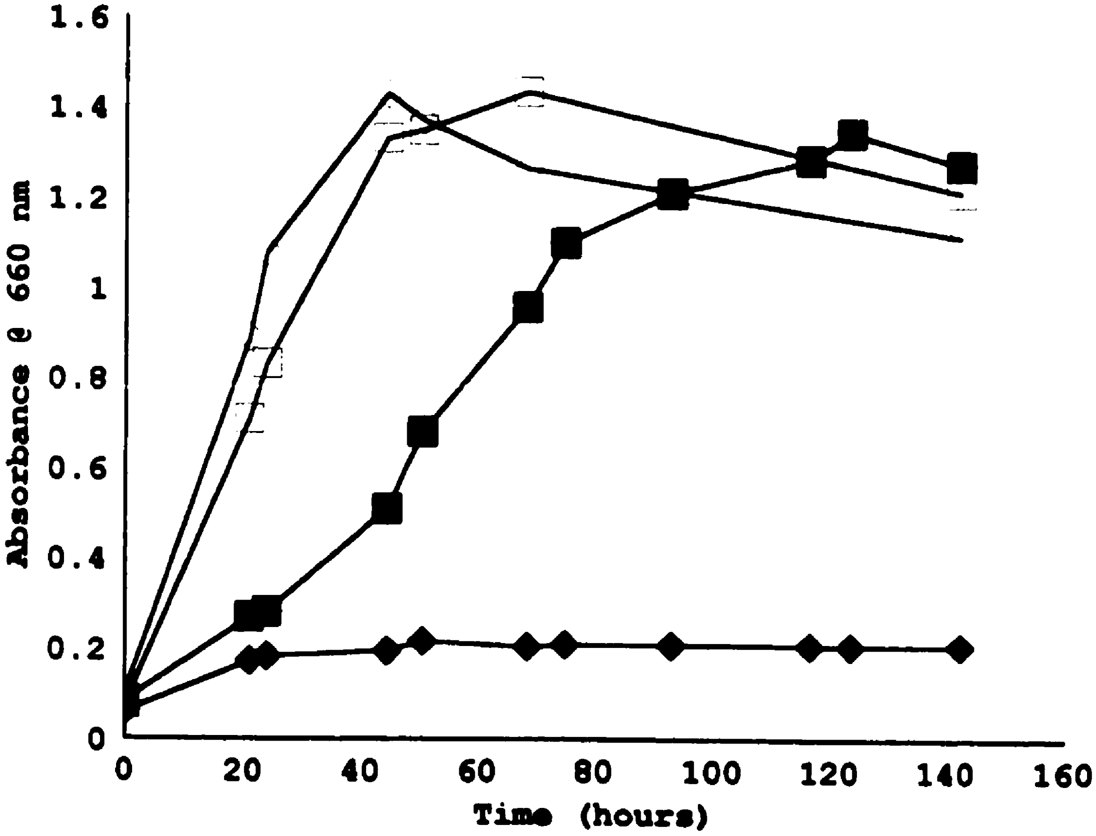

Figure 2.27. The effect of molybdate on the growth of *Desulfovibrio desulfuricans* ATCC 27774 while growing on lactae and nitrate. □ control, ◇ 0.1 mM molybdate, ■ 1.0 mM molybdate, ◆ 10 mM molybdate. Values are the average of triplicates.

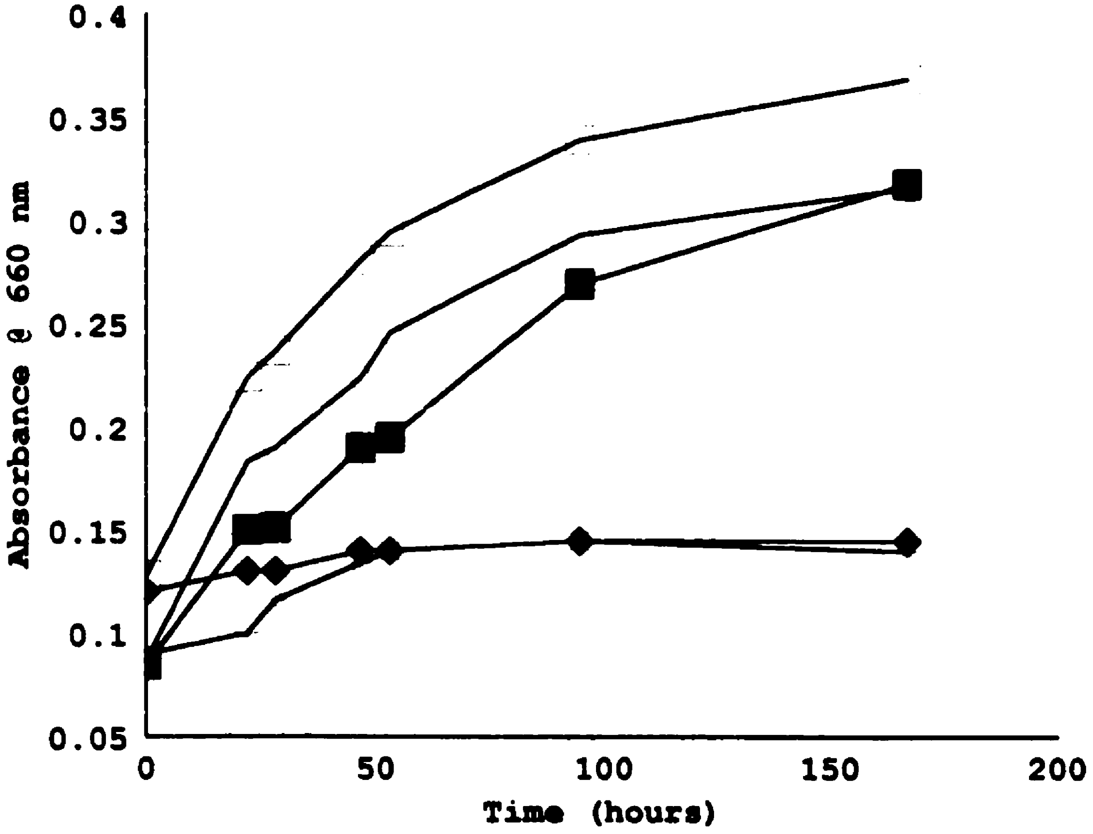

Figure 2.28. The effect of molybdate on the growth of *Desulfovibrio desulfuricans* ATCC 27774 while growing fermentatively on pyruvate. □ control, · 0.01 mM molybdate, ■ 0.1 mM molybdate, ◆ 1.0 mM molybdate, -+- 10 mM molybdate. Values are the average of triplicates.

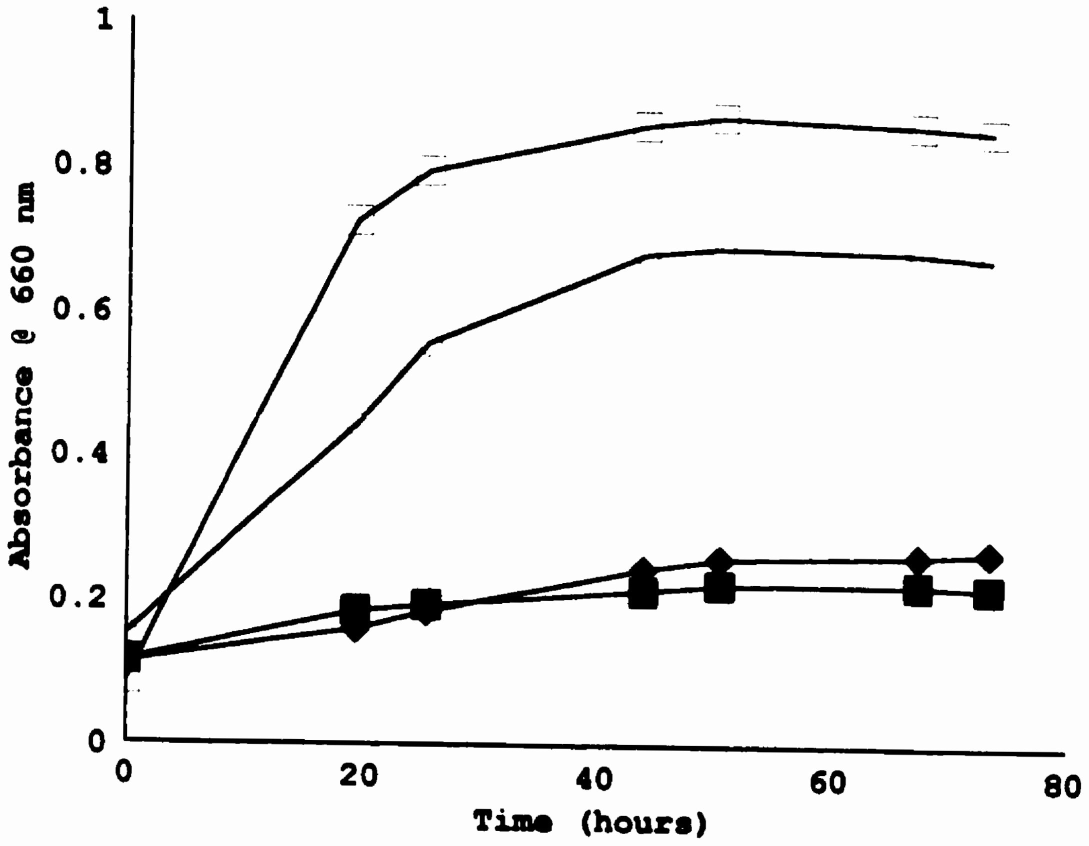

Figure 2.29. The effect of molybdate on the growth of *Desulfovibrio desulfuricans* ATCC 27774 while growing on lactae and sulphate. ☐ control, ◇ 0.1 mM molybdate, ■ 1.0 mM molybdate, ◆ 10 mM molybdate. Values are the average of triplicates.

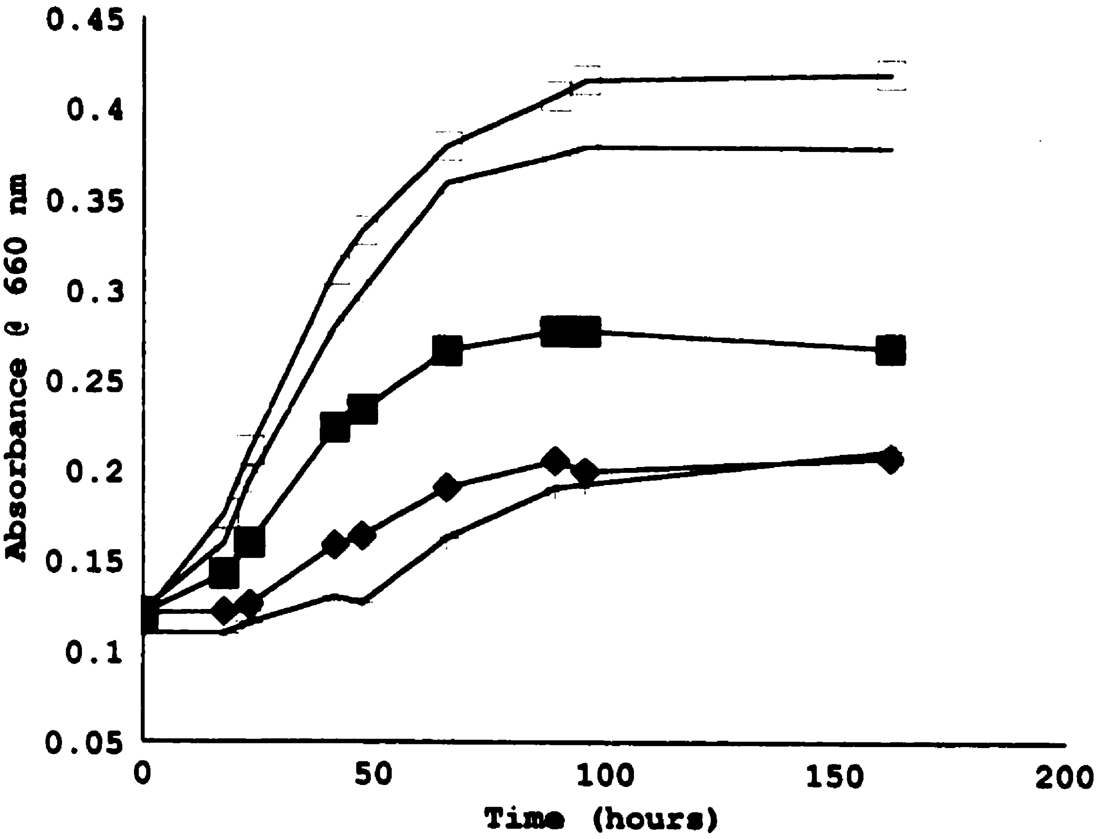

Figure 2.30. The effect of molybdate on the growth of *Desulfovibrio desulfuricans* ATCC 27774 while growing on lactate and thiosulphate. ☐ control, · 0.01 mM molybdate, ■ 0.1 mM molybdate, ● 1.0 mM molybdate, + 10 mM molybdate. Values are the average of triplicates.

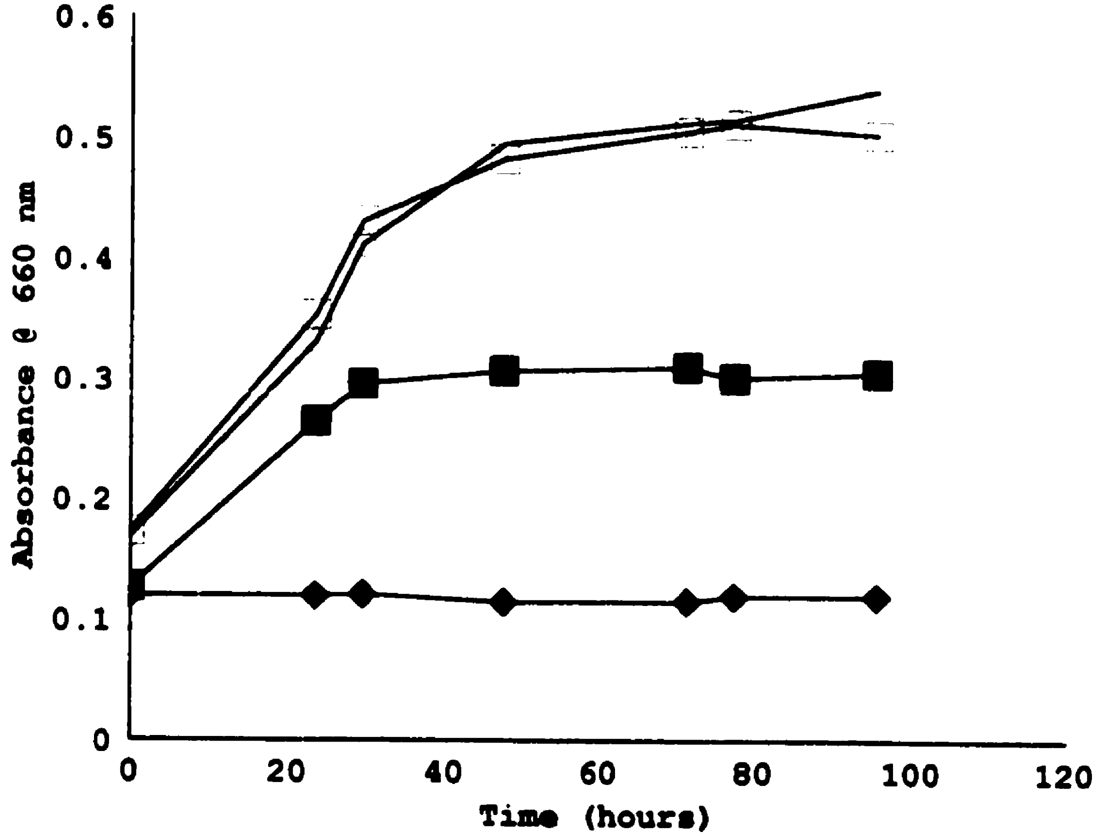

Figure 2.31. The effect of molybdate on the growth of *Desulfovibrio desulfuricans* DSM 642 while growing fermentatively on fumarate. □ control, · 0.1 mM molybdate, ■ 1.0 mM molybdate, ◆ 10 mM molybdate. Values are the average of triplicates.

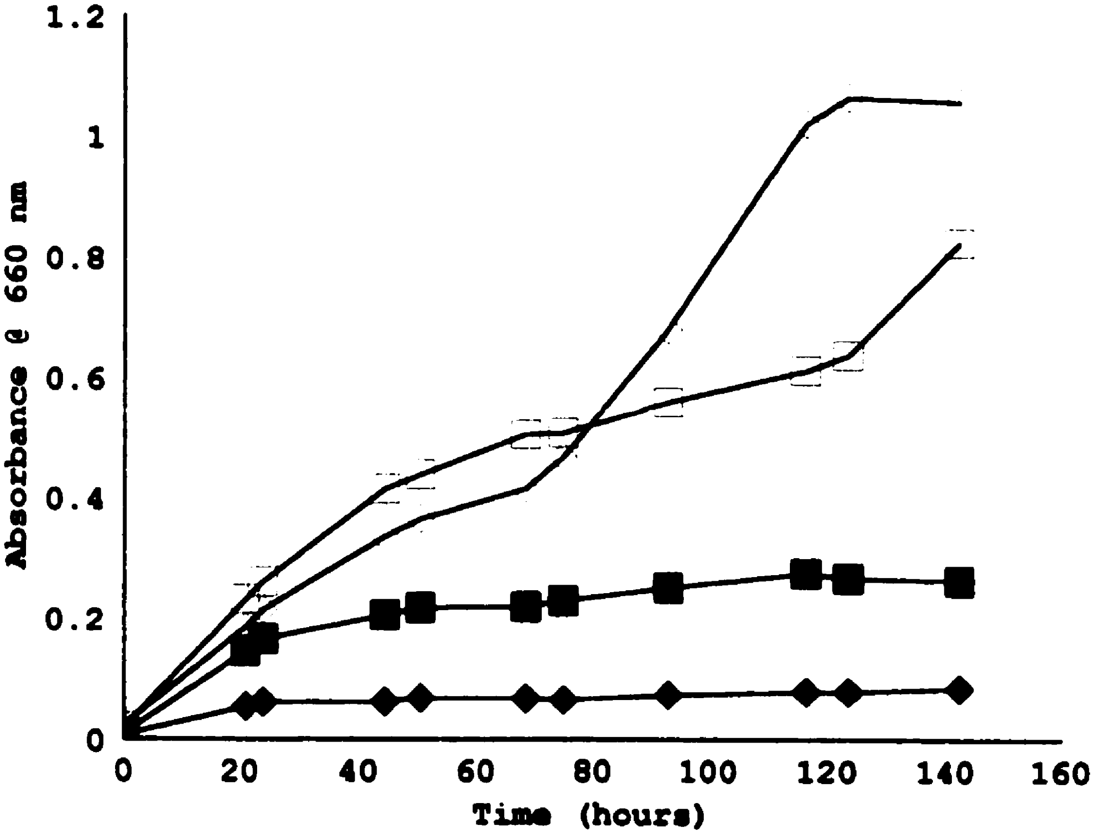

Figure 2.32. The effect of molybdate on the growth of *Desulfovibrio desulfuricans* DSM 642 while growing on lactae and nitrate. □ control, ◇ 0.1 mM molybdate, ■ 1.0 mM molybdate, ◆ 10 mM molybdate. Values are the average of triplicates.

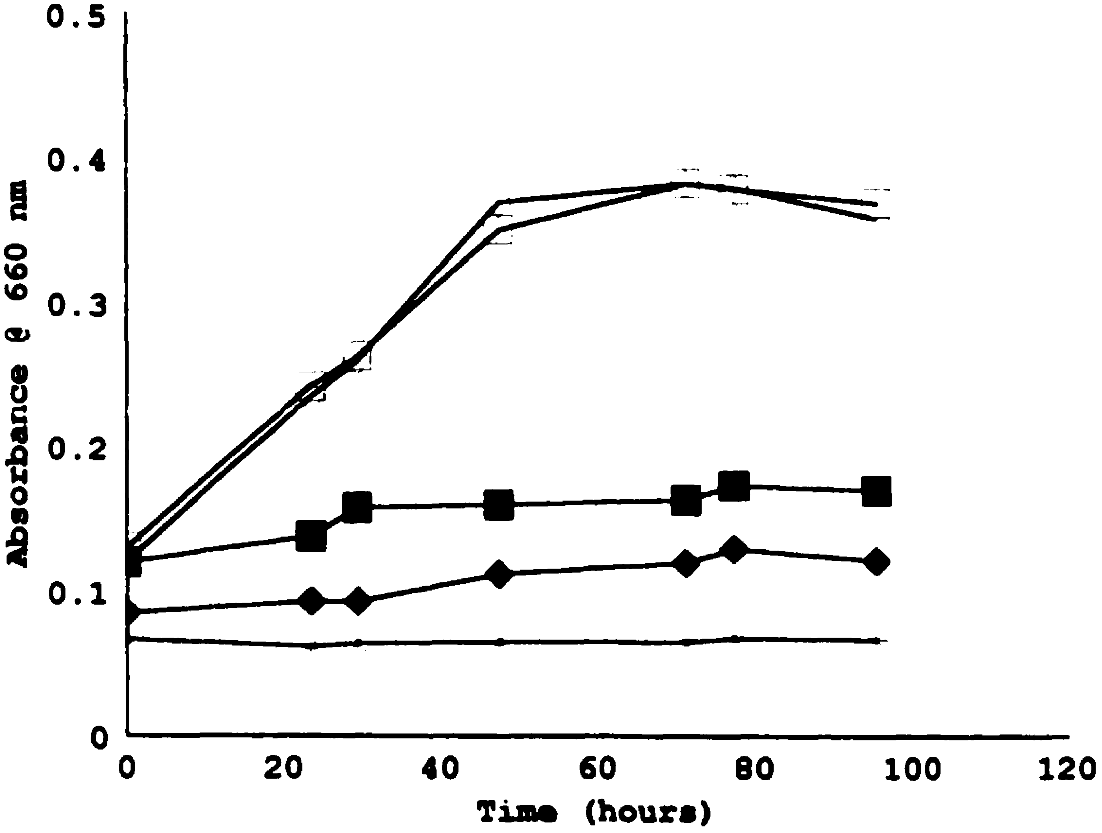

Figure 2.33. The effect of molybdate on the growth of *Desulfovibrio desulfuricans* DSM 642 while growing fermentatively on pyruvate. ☐ control, ◇ 0.01 mM molybdate, ■ 0.1 mM molybdate, ◆ 1.0 mM molybdate, + 10 mM molybdate. Values are the average of triplicates.

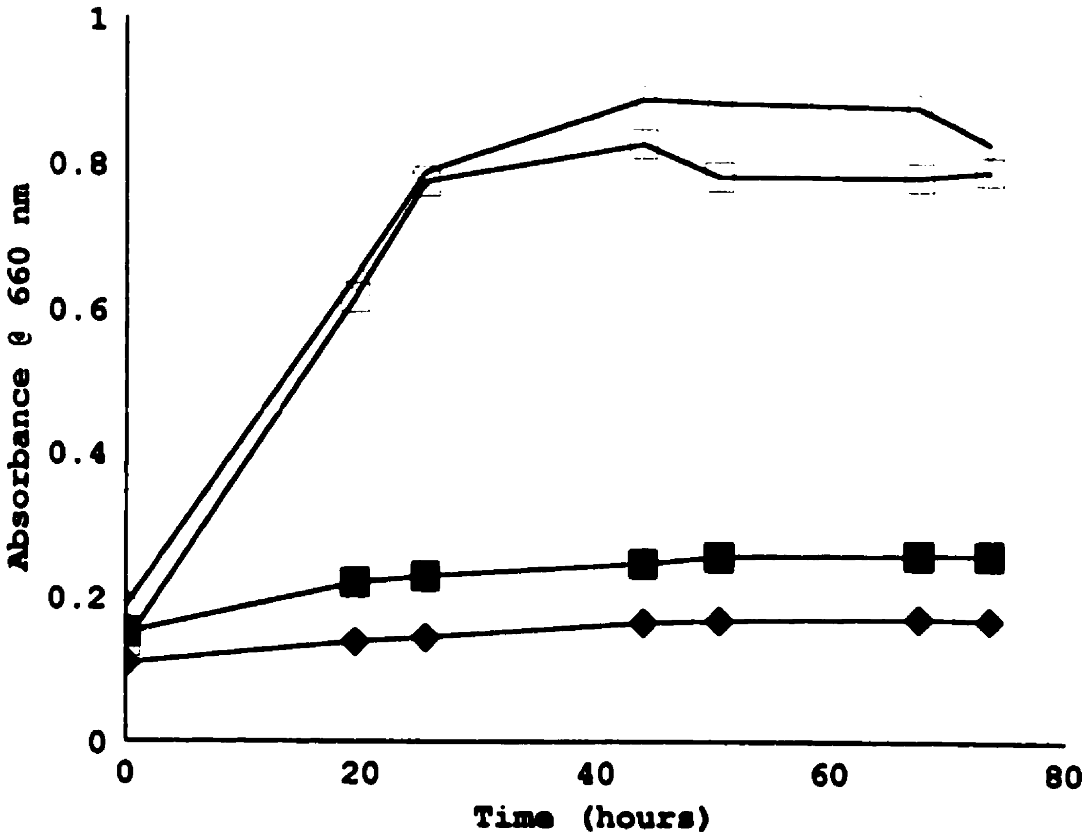

Figure 2.34. The effect of molybdate on the growth of *Desulfovibrio desulfuricans* DSM 642 while growing on lactae and sulphate. □ control, . 0.1 mM molybdate, ■ 1.0 mM molybdate, ● 10 mM molybdate. Values are the average of triplicates.

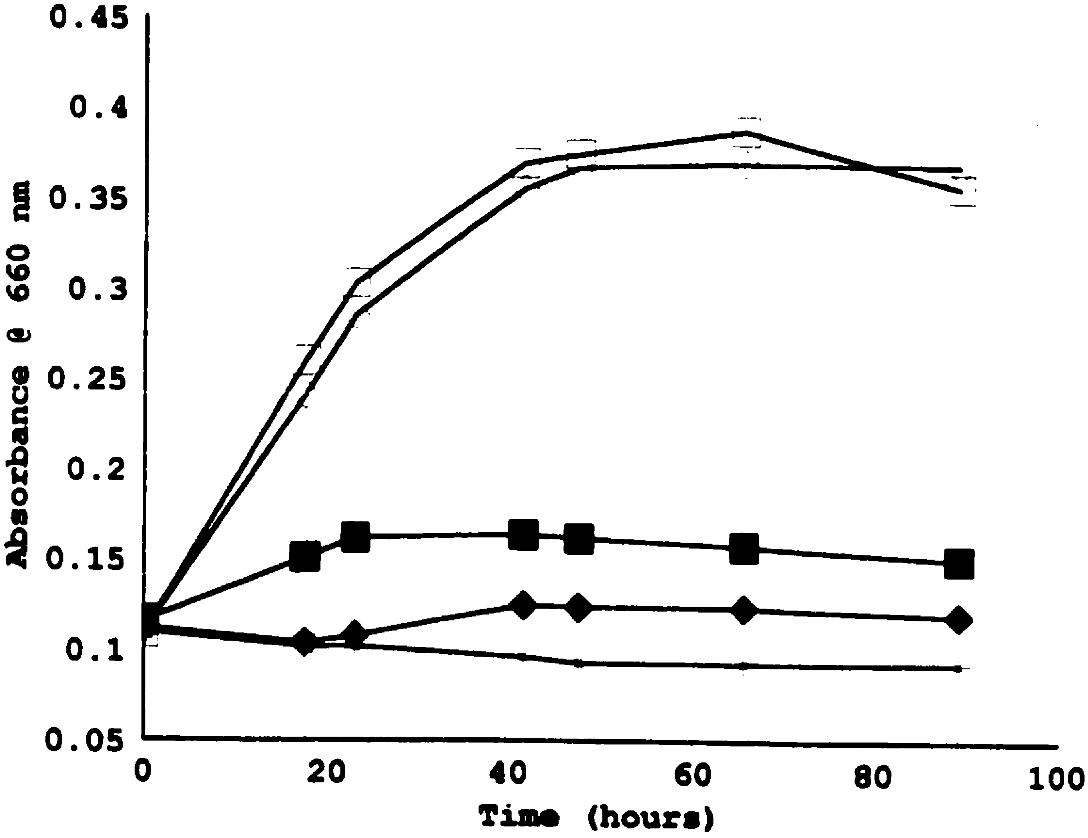

Figure 2.35. The effect of molybdate on the growth of *Desulfovibrio desulfuricans* DSM 642 while growing on lactate and thiosulphate. □ control, ◇ 0.01 mM molybdate, ■ 0.1 mM molybdate, ◆ 1.0 mM molybdate, + 10 mM molybdate. Values are the average of triplicates.

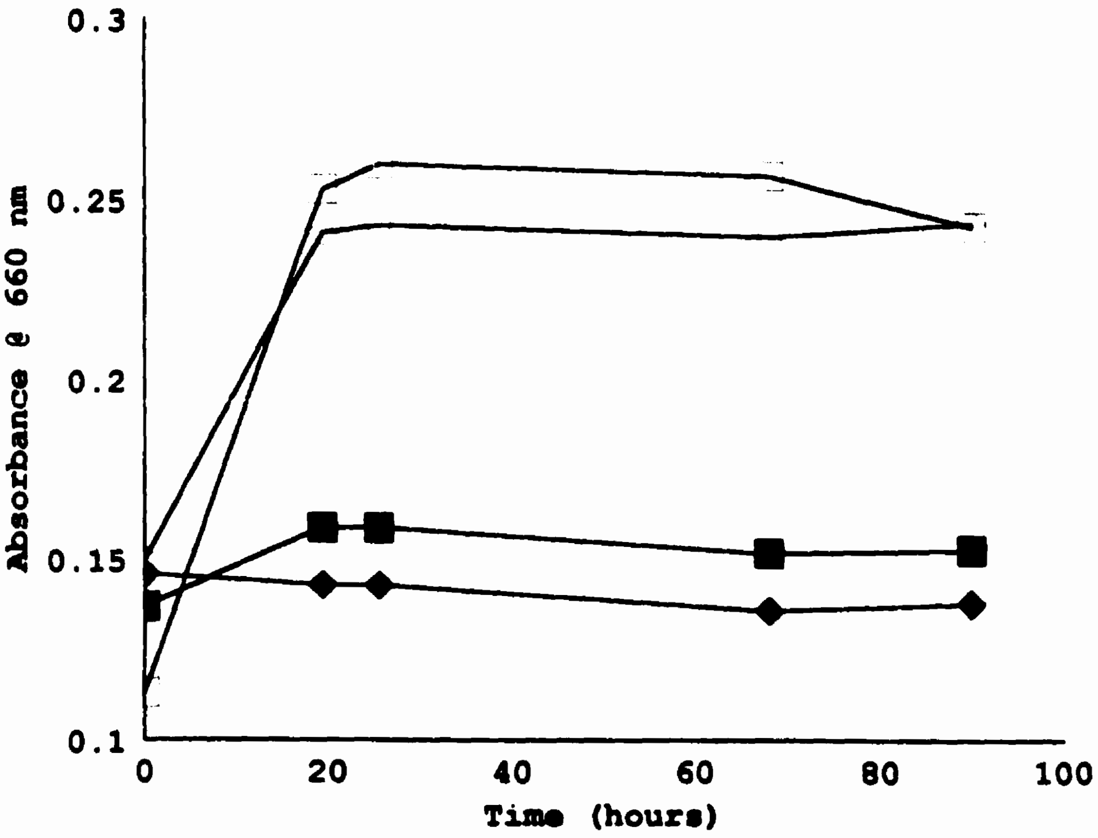

Figure 2.36. The effect of molybdate on the growth of *Desulfovibrio vulgaris* DSM 644 while growing fermentatively on fumarate. □ control, 0.1 mM molybdate, ■ 1.0 mM molybdate, ◆ 10 mM molybdate. Values are the average of triplicates.

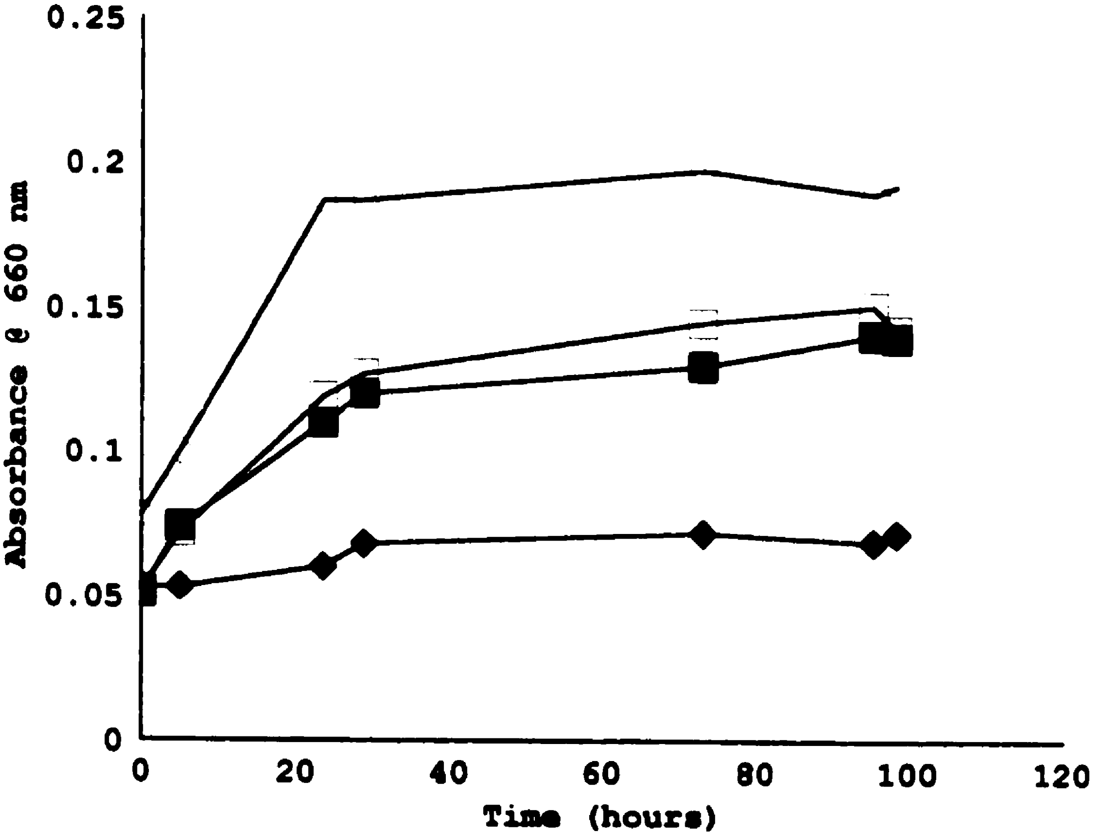

Figure 2.37. The effect of molybdate on the growth of *Desulfovibrio vulgaris* DSM 644 while growing on lactate and nitrate. ☐ control, ◇ 0.1 mM molybdate, ■ 1.0 mM molybdate, ◆ 10 mM molybdate. Values are the average of triplicates.

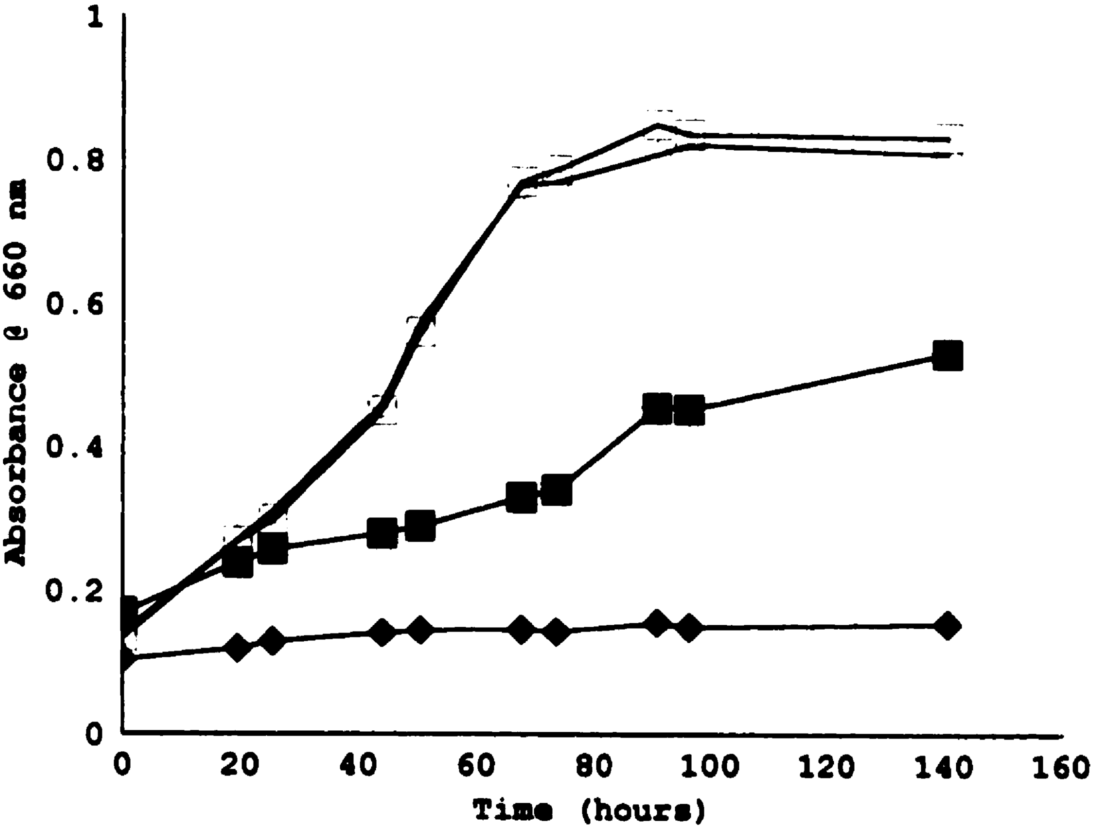

Figure 2.38. The effect of molybdate on the growth of *Desulfovibrio vulgaris* DSM 644 while growing on lactate and sulphate. □ control, ◇ 0.1 mM molybdate, ■ 1.0 mM molybdate, ● 10 mM molybdate. Values are the average of triplicates.

Chapter 3

Survey of organisms for the ability to methylate mercury

Section 3.1 Introduction

Section 3.2 Material and Methods

- i) Mercury-203 experiments
    - a) Organisms surveyed
    - b) Effects of chemical and physical parameters on mercury methylation; pH, substrate type, gas phase composition, nickel concentration, and temperature
    - c) Methyl mercury extraction protocol
- ii) Experiments to determine the ability to methylate at environmentally realistic total mercury concentrations
    - a) Organism tested
    - b) Total and methyl mercury analysis
    - c) Glassware preparation
    - d) Media preparation
    - e) Growth conditions

Section 3.1

Introduction

The ability to methylate mercury has been demonstrated in a wide range of microorganisms, and in a number of laboratories (Gilmour and Henry 1991, Compeau and Bartha 1985, Hamdy and Noyes 1975, Rowland *et al.* 1975, Vonk and Sijpesteijn 1973). Environmentally significant mercury methylation has so far been attributed primarily to the sulphate reducing bacteria (SRB) (Compeau and Bartha 1985). However, early workers, and even some current researchers, suspect that methanogens contribute to environmental mercury methylating activity (Drake *et al.* 1996, Wood *et al.* 1968).

Early work investigating methylation of mercury by bacteria involved the use of high concentrations of inorganic mercury, generally in the form of mercury chloride or mercury nitrate. The majority of methyl mercury work done to date has utilized total mercury concentrations many thousands of times higher than mercury concentrations seen in pristine environments. This includes work with pure cultures of bacteria, and sediment, soil and water investigations. The concentrations used have ranged from as low as 50 ng/ml to as high as 50,000 ng/ml (Zhang and Planas 1994, Hamdy and Noyes 1975). Pristine environments have been reported to have concentrations as low as 0.11 ng/L (Kelly *et al.* 1995). The exception to these investigations is the work of Stordal and Gill (1995) who have developed a method of measuring mercury methylation rates at environmental realistic total mercury concentrations, using high specific activity Hg-203; however, this technique has not been applied to anything other than environmental samples.

It was therefore of interest to conduct a survey of pure cultures of microorganisms to determine if the capability to methylate mercury is common. In the following investigations a sensitive radiochemical technique is employed, allowing for the use of significantly lower concentrations of total mercury than in previous tests of individual species (Furutani and Rudd 1980). The organisms surveyed included a range of taxonomically diverse as well as ecologically significant species. The collection contained methanogens, due to their early implication in mercury methylation (Wood *et al.* 1968), SRB, including organisms previously not tested for methylation and organisms that have been shown capable of mercury methylation (Gilmour unpublished data), an acetogen, which like methanogens have high methylcobalamin concentrations, *Myxococcus xanthus*, due to implications that it is phylogenetically related to the SRB (Devereux *et al.* 1989), as well as species that have been reported by earlier workers to be capable of mercury methylation (Gilmour and Henry 1991, Compeau and Bartha 1985, Hamdy and Noyes 1975, Rowland *et al.* 1975, Vonk and Sijpesteijn 1973).

Current understanding of mercury methylation suggests that anaerobic conditions are favourable for methylating activity (Compeau and Bartha 1984). As mercury complexes strongly to sulphide this calls into question the bioavailability of mercury when sulphide concentrations are high (Compeau and Bartha 1987). Improved anaerobic techniques *ie.* the use of Ti-NTA as a reducing agent, were utilized to reduce the interference of sulphide in regards to mercury bioavailability. Mercury is known to complex strongly with sulphide (Hg-S stability constant $10^{-53.9}$) and much less strongly with NTA (Hg-NTA stability constant $10^{14.6}$) thus making more mercury available for methylation when Ti-NTA is used as a reducing agent (Dyrssen 1989, Martell and Smith 1974).

The identification of organisms that can methylate mercury has been accomplished in the existing literature using gas chromatography and radioisotopic methods. Both of these techniques required the use of high concentrations of inorganic mercury. The more recent technique of Cold Vapour Atomic Fluorescence Spectroscopy (CVAFS) is capable of detecting total and methyl mercury at sub 1 ng/L levels which is currently the most sensitive detection available. It is of interest to determine, by using CVAFS, if organisms identified as being capable of methylating mercury at high concentrations are capable of methylating mercury at or near environmental concentrations.

In early investigations of mercury methylation, methanogens appeared as likely candidates. Mercury methylation was apparently favoured under anaerobic conditions and methanogens are strict anaerobes (Jensen and Jernelov 1969). The transfer of a methyl group to the positively charged mercury ion (Hg^{2+}) requires that the transferred methyl groups carbon atom be negatively charged (carbanion). Methylcobalamin is involved in the transfer of negatively charged methyl groups and methanogens are known to contain high concentrations of methyl cobalamin (Stupperich *et al.* 1990, Stupperich and Krautler 1988).

In later work Compeau and Bartha (1985) proposed sulphate reducing bacteria as the principle methylators of mercury in the environment because molybdate inhibited both sulphate reduction and mercury methylation activity in sediments. Compeau and Bartha (1985) also isolated a *Desulfovibrio desulfuricans* strain which actively methylated mercury. It was later found to contain methylcobalamin, and the enzymes of the acetyl CoA pathway (Berman *et al.* 1990, Choi and Bartha 1993, Choi *et al.* 1994). From isotopic tracer studies following C^{14} labelled metabolites to the methyl group of methyl mercury, the participation of the acetyl CoA pathway in mercury methylation was established, in their isolate (Berman *et al.* 1990, Choi *et al.* 1994). Since their isolation of a mercury methylating strain of SRB, other workers have isolated or identified many more SRB species capable of methylating mercury (Gilmour and Henry 1991).

Interestingly not all SRB are capable of mercury methylation, and methylation of mercury is not exclusive to one genus nor is it shared among strains of the same species. As discussed in Chapter 1, Section 1.7, Choi and Bartha (1993) found the presence of a 40-kDa protein associated with the corrinoid of *Desulfovibrio desulfuricans* LS. If this 40-kDa protein is in fact required for mercury methylation, than its absence in other organisms would explain their inability to methylate mercury, it does not however, address the question of why only certain SRB would possess this enzyme, and why methanogens or acetogens would not.

In this chapter a survey of microorganisms for the ability to methylate mercury was conducted on a group of organisms containing, SRB, non-SRB eubacterial species, methanogens and an acetogen. The ability to methylate at environmentally realistic concentrations of total mercury was also investigated to determine if the organisms capable of mercury methylation at total mercury concentrations many fold higher than those seen in the environment, are actually capable, and thus potentially responsible for mercury methylation in the environment. The effect of culture conditions such as, pH, substrate, gas phase, temperature, and nickel concentration, were investigated to determine their effect on mercury methylation. Changes in pH, substrate, and temperature, have been shown to affect mercury methylation in experiments involving sediments and pure cultures (Regnell 1994, Regnell and Tunlid 1991, Steffan *et al.* 1988, Korthals and Winfrey 1987, Xun *et al.* 1987). Some SRB demonstrate chemolithotrophic growth involving H_2 (Seitz and Cypionka 1986). In this respect they compete with methanogens for H_2, and generally out compete the methanogens for the H_2 (Abram and Nedwell 1978). Growth of SRB with and without H_2 available in the gas phase may affect the metabolic activities of SRB, and may therefore affect mercury methylation. Nickel is a required element in the enzyme, carbon monoxide dehydrogenase (CODH), which is an essential component of the acetyl CoA pathway. The acetyl CoA pathway is a proposed pathway for the methylation of mercury in *Desulfovibrio desulfuricans* LS, supplementing growth media with additional nickel may effect expression of CODH such that changes in mercury methylation may result. Cobalamin has been shown to mediate mercury methylation in extracts of methanogenic bacteria and has been shown to be involved in mercury methylation by *Desulfovibrio desulfuricans* LS. Cobalamin concentrations may provide evidence of a trend among organisms that can and cannot methylate mercury.

Section 3.2

Materials and Methods

i) Mercury-203 Experiments

a) Organisms surveyed

Following are the organisms surveyed for the ability to methylate mercury: *Acetobacterium woodii* DSM 1030, *Bacillus megaterium* ATCC 14581, *B. subtilis* ATCC 6051, *Desulfobulbus propionicus* DSM 2032, *Desulfotomaculum ruminus* DSM 2154, *Desulfovibrio baarsii* DSM 2075, *D. desulfuricans* ATCC 27774, *D. desulfuricans* DSM 642, *D. desulfuricans* DSM 1924, *D. vulgaris* DSM 644, *Desulfovibrio* sp. B-203, *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* MP-180, *E. coli* XL-1 Blue, *Lactobacillus plantarum* ATCC 8014, *Methanosarcina barkeri* DSM 804, *Methanosphaera stadtmanae* DSM 3091, *Methanospirillum hungatei* DSM 1101, *Pseudomonas fluorescens* 493, *Rhodospirillum rubrum* ATCC 11170, *Staphylococcus aureus* 96, *Streptococcus faecalis* 97. As indicated in appendix C, the organisms were grown on a variety of media, and where applicable, under aerobic and anaerobic conditions. Surveyed organisms were exposed to two concentrations of mercury, a low concentration of 74 ng per ml and high concentration of 5000 ng per ml mercury in the form of $HgCl_2$.

Assays for the methylation of mercury were conducted in two ways. For those organisms demonstrating slow growth, and yielding low amounts of biomass (methanogens and SRB), a pre-concentration of cells prior to the addition of mercury was performed. This was an attempt to amplify any mercury methylating capabilities, making detection of methyl mercury easier. For organisms growing quickly and producing high biomass yields (non-SRB eubacterial species), a pre-concentration of cells was not performed.

The pre-concentration procedure consisted of growing the organism in batch culture in 6 to 8, 1 litre reagent bottles in 500 ml of the appropriate growth medium. The cultures were harvested in an anaerobic chamber and dispensed to centrifuge bottles, which had a nominal capacity of 400 ml and were equipped with an O ring seal capable of maintaining anaerobic conditions. The supernatant was poured off and the pellets resuspended in a small volume of fresh media, and then recombined. To the resuspended cells enough fresh media was added to bring the total volume up to 260 ml, this was mixed by shaking and dispensed in aliquots of 65 ml to four 100 ml nominal capacity Wheaton glass serum bottles. The bottles were sealed with butyl rubber stoppers and removed from the anaerobic chamber. Upon removal the bottles had aluminum crimp tops affixed. To the samples a 100 µl addition of working stock mercury/mercury 203 (see appendix B for formulation) solution was added via a hamilton micro syringe, immediately after which two samples were acid killed to stop metabolic activity, by the addition of 5 ml of 4 N HCl administered via BD plastic syringe and 22 gauge needle through the butyl rubber stopper. Samples were incubated for 48 hours, after which the live samples were acid killed by the addition of 5 ml of 4 N HCl administered again via plastic BD syringe and 22 gauge needle. Samples were allowed to sit no less than 10 minutes and no longer than 25 minutes before being assayed for methyl mercury.

For cultures not requiring a pre-concentration of cells a 1 ml innoculum of a mid log phase culture was used to innoculate 6 serum bottles containing 65 ml of the appropriate medium. To four of the innoculated serum bottles a 500 µl addition of a sterile mercury/mercury 203 working stock solution was added. To two of the four bottles a 5 ml addition of 4 N HCl was added to stop biological activity. All 6 samples were then incubated at the appropriate temperature for the particular organism being surveyed (see appendix C) for 24 hours. After incubation a 500 µl addition of a sterile mercury/mercury 203 was made to the two samples not previously recieving the addition. Immediately after the mercury/mercury 203 addition biological activity in these two samples was stopped by the addition of 5 ml 4 N HCl. Samples were analyzed for methyl mercury via the protocol detailed in c) Methyl mercury extraction protocol.

b) Effects of chemical & physical parameters on mercury methylation; pH, substrate type, gas phase composition, nickel concentration, and temperature.

For the following experiments methylation assays were conducted in a fashion similar to that for the survey portion, discussed above, for cells not requiring a pre-concentration step. All cultures were incubated until the end of log phase and a final mercury concentration of 230 ng per ml was used. Protein content was determined via the protein-dye binding method of Bradford (Bradford 1976). Samples were analyzed for methyl mercury via the protocol detailed in c) Methyl mercury extraction protocol. *Desulfovibrio* sp. B-203 was chosen as the experimental organism for all the following experiments. *Desulfovibrio* sp. B-203 grows well fermentatively on both fumarate and pyruvate, and actively methylates mercury, thus it is a suitable organism on which to conduct a variety of methylation experiments. For all experiments except the effect of temperature experiments, cultures were grown a minimum of one subculture under the experimental conditions.

To determine the effect of pH on mercury methylating ability, *Desulfovibrio* sp. B-203 was grown in 65 ml of Postgate's medium D (see appendix A for formulation) buffered to pH values of 5.75, 7.5 and 8.75, with citric acid, 4-morpholinepropanesulfonic acid (MOPS), and 2-(cyclohexylamino)ethanesulfonic acid (CHES), respectively.

To determine the effect of substrate on mercury methylation ability *Desulfovibrio* sp. B-203 was grown in 65 ml of Postgate's medium D with pyruvate, or fumarate as the carbon source (see appendix A for formulation).

To determine the effect of gas phase composition on mercury methylating ability, *Desulfovibrio* sp. B-203 was grown in 65 ml of Postgate's medium D with N_2 and H_2 as the respective gas phases (see appendix A for formulation).

To determine the effect of the addition of nickel on mercury methylating ability *Desulfovibrio* sp. B-203 was grown in 65 ml of Postgate's medium D with and without the addition of nickel beyond that supplied in the mineral elixir used in media preparation (see appendix A and B for formulation) to a final concentration of 0.08 mM.

To determine the effects of temperature on mercury methylating activity, *Desulfovibrio* sp. B-203 was grown in six 1 litre reagent bottles containing 500 ml Postgate's medium (see appendix A and B for formulation and preparation) to mid-log phase. Cells were harvested in an anaerobic chamber into O-ring capped centrifuge bottles. Centrifuge bottles were removed from the anaerobic chamber and centrifuged 4,225 x g for 15 minutes. After centrifuging the bottles were placed back in the anaerobic chamber, supernatant discarded and pellets resuspended in 200 ml of fresh medium D. The 200 ml suspension was then dispensed in 25 ml aliqouts to eight 100 ml nominal capacity serum bottles closed with butyl rubber stoppers. Samples were then injected with a 500 µL mercury/mercury 203 addition, at which time 5 ml time zero samples were drawn and placed in screw cap test tubes with 1 ml concentrated HCL. The samples in duplicate were then placed at 4°, 20°, 30°, and 37° C. Subsequent samples were then drawn at 2, 4, and 6 hours. Samples were analyzed for methyl mercury content via the following protocol.

c) Methyl mercury extraction protocol

The assay technique used is a modification of the method of Furatani and Rudd (1980). Modifications for use with bacterial cultures were made to allow the technique to be used for the detection of methyl mercury in bacterial cultures.

After the acid addition the contents of the serum bottle was poured into a separatory funnel and 2 ml of 0.5 N copper sulfate solution was added. This was shaken and 10 ml of an acidic 3 M NaBr solution (110 ml concentrated H_2SO_4 to reach a total volume of 1 L.) was added, shaken again and allowed to stand for 30 minutes. After 30 minutes 20 ml of toluene (HPLC grade) was added and the separatory funnel was shaken for 5 minutes and allowed to stand for 30 minutes. The lower aqueous layer was drained. The funnel contents were allowed to stand for 10 minutes more and any remaining aqueous phase was drained. Approximately 1-2 grams anhydrous $NaSO_4$ was added to the separatory funnel and gently swirled and allowed to stand for 5 minutes. The contents of separatory funnel was poured into a 15 ml test tube, stoppered and centrifuged for 10 minutes at 834 x g. From the centrifuged test tube 10 ml of the clear toluene was pippetted into a 30 ml test tube and 5 ml of 2.5 mM $Na_2S_2O_3$ in 20% ethanol solution was added, shaken and allowed to stand for 5 minutes. Four millilitres of the thiosulphate layer (bottom) was pippetted into a 7 ml glass test tube with a scintered glass stopper. To this 1 ml of 3M KI was added and shaken. One millilitre of benzene (HPLC grade) was added and shaken for 5 minutes, and then allowed to stand for 5 minutes. Five hundred microlitres of the benzene layer was pippetted into 10 ml BCS scintillation cocktail. The samples were counted in a scintillation counter with an open count window.

ii) Experiments to determine the ability to methylate at environmentally realistic total mercury concentrations

a) Organism tested

Desulfobulbus propionicus was the organism selected for testing. It has been shown in previous work to methylate mercury while growing fermentatively on lactate in Fermentative Freshwater Widdel (FFW) medium (C.C. Gilmour, unpublished data). FFW medium did not present interference problems with the CVAFS analysis for total and methyl mercury, as did Postgate's medium D which is the growth medium for *Desulfovibrio* sp. B-203, hence the selection of *Desulfobulbus propionicus* for this experiment.

b) Total and methyl mercury analysis

Samples were analyzed for total and methyl mercury concentrations via CVAFS at Flett Research Ltd. Winnipeg, MB. Canada.

c) Glassware preparation

All glassware used in the following experiment was vigorously scrubbed with Sparkleen detergent and rinsed with tap water, triple rinsed with concentrated HCl acid, triple rinsed with in house reverse osmosis (RO) conditioned water then filled with a saturated EDTA solution and allowed to sit overnight. The glassware was then triple rinsed with RO water, inverted over paper towels and allowed to dry. All butyl rubber stoppers were soaked overnight in a saturated EDTA solution and rinsed with RO water.

d) Media preparation

Fermentative freshwater Widdel medium (Widdel and Pfennig 1981) was used as the growth substrate. The medium was prepared (see appendix A for formulation), dispensed and bottled in 500 ml amounts in 1 L Wheaton reagent bottles under a laminar flow hood to reduce dust contamination. Media was degassed as described in appendix A and autoclaved. All media and solutions were allowed to sit in direct sunlight for one week prior to use to allow for possible photodegradation of any endogenous methyl mercury. All chemicals were reagent grade and the mercury occurring as a contaminant in the reagents acted as the source of Hg for the methylation experiments. The water used in making the media was from a Milli Q ultra pure filtration system.

e) Growth conditions

Immediately prior to use, media was reduced with Ti-NTA (see appendix B for formulation and usage). All cultures were inoculated with a 10% inoculum from a single late log phase culture that had been subcultured twice into medium prepared as described in the previous section. Cultures were then incubated at 37° C for 72 hours in the dark. At the end of incubation, cultures were acidified with 10 ml of concentrated HCl to stop biological activity and then frozen until time of analysis.
(see appendix D for discussion of pitfalls of low level Hg work)

iii) Cobalamin Experiments

a) Cobalamin Assay

A commercially available radio immuno-assay (SimulTRAC-S RADIOASSAY KIT VITAMIN B_{12} [^{57}Co]/FOLATE [^{125}I] Becton Dickinson) was used for the quantitative determination of cobalamin. The assay was conducted as per the instructions provided with the assay kit.

b) Organisms tested

The following organisms were assayed for cobalamin content; *Desulfobulbus propionicus* DSM 2032, *Desulfotomaculum ruminus* DSM 2154, *Desulfovibrio desulfuricans* strains DSM 1924, DSM 642, ATCC 27774, *Desulfovibrio* species ND 132, and B-203, *Desulfovibrio vulgaris* DSM 644, *Escherichia coli*, *Methanosarcina barkeri* DSM 804 strain Fusaro, *Methanosphaera stadtmanae* DSM 3091, and *Methanospirillum hungatei* DSM 1101 strain GP1. Of the above named organisms, *Desulfobulbus propionicus*, *Desulfovibrio* sp. B-203 and ND 132, are all capable of mercury methylation. Where applicable the SRB were grown fermentatively and on sulphate. Protein content was determined via the Bradford protein assay (Bradford 1976).

Section 3.3

Results and Discussion

i) Methylation survey

Of the 22 organisms tested for mercury methylating ability at both a low and high concentration of mercury, only two organisms displayed mercury methylating ability. Both of these, *Desulfobulbus propionicus* and *Desulfovibrio* sp. B-203, have previously been shown by other researchers to be capable of mercury methylation. Of the 20 organisms that did not show the capacity to actively methylate mercury several were species that in previous work by other researchers had shown the capacity to methylate mercury. None of the three methanogens tested were capable of methylating Hg. This agrees with earlier work demonstrating methylation of mercury by cell extracts of methanogens but not by intact cells (Wood *et al.* 1968). This remains a curious point in that methanogens have high concentrations of methylcobalamin, have been implicated by other workers of being involved in Hg methylation, have cell extracts capable of methylation yet in whole cells are unable to methylate.

Of the SRB tested only *Desulfobulbus propionicus* and a *Desulfovibrio* sp. B-203 were capable of methylating mercury. Two members of the genus *Desulfovibrio* that were tested, namely *D. desulfuricans* and *D. baarsii*, failed to demonstrate mercury methylation. Considering that other members of this genus have demonstrated mercury methylation and in fact strains of *D. desulfuricans* have demonstrated mercury methylation, it can be seen that methylation is not a wide spread trait and appears to be very strain specific.

ii) Experiments achieving ultra-low total mercury concentrations and utilizing CVAFS detection of methyl mercury

Table 3.1 shows the results of the low level mercury methylation experiments conducted with *Desulfobulbus propionicus*. As can be seen *D. propionicus* is capable of methylating mercury at extremely low total mercury

Table 3.1: Methylation of mercury at low total mercury concentrations by *Desulfobulbus propionicus* (DSM 2032)

Total Mercury Concentration ng/L in FFW medium	Concentration of Hg ng/L as CH_3Hg^+
13.80 ± 2.64*	0.55 ± 0.15*
4.0% Total Hg methylated	

* Replicates of 7

CH_3Hg^+ content of FFW medium was consistently less than 0.03 ng/L concentrations. In previous work several researchers have reported total mercury concentrations in freshwater systems of 0.5 ng/L for Vandercook Lake, Wisconsin, to 2.2 to 16.3 ng/L for various sites at the Experimental Lakes Area, Ontario, Canada (Kelly *et al.* 1995, Zillioux *et al.* 1993). Both these sites are considered to be pristine environments. The total mercury concentration of 13.80 ng/L achieved in this experiment falls within the range of concentrations seen for pristine freshwater systems found in the US and Canada. This is believed to be the first reporting of mercury methylation by a pure bacterial culture at environmentally realistic total mercury concentrations.

This demonstration of mercury methylation at ultra low levels of total mercury, approaching and indeed reaching naturally occurring levels, lends support to the argument that SRB play a significant role in mercury methylation in the environment by demonstrating that they can indeed methylate mercury at concentrations found in nature. It also validates the work utilizing mercury-203 in higher concentrations demonstrating mercury methylation, by showing the same organism methylating mercury over a range of concentrations extending in the lower range to environmental concentrations.

iii) Effects of physical parameters on mercury methylation; pH, substrate, gas phase, nickel and temperature

Due to the convenience of working with mercury-203 when conducting mercury methylation experiments requiring extensive manipulation of the growth medium, the mercury-203 protocol was chosen to examine the effects of pH, gas phase, nickel concentration and temperature on mercury methylation. The mercury-203 protocol eliminates the need for extensive and laborious cleaning and handling procedures required to achieve total mercury concentrations seen in the CVAFS experiments. Also the risk of accidental mercury contamination is removed when utilizing the mercury-203 protocol as experimental mercury additions are many times greater than accidental contamination sources (see appendix D for more detailed discussion of accidental contamination).

The effects of pH on mercury methylation can be seen in table 3.2. The cells were grown at the indicated pH values. Slightly more mercury is methylated at a pH of 5.75, then at 7.5, and very little mercury is methylated at a pH of 8.75. However, when corrected for total biomass represented by total protein content, the amount of mercury methylated per unit of biomass is the same for all three pH conditions, indicating that mercury methylation is related to total growth yield of the methylating strain.

Table 3.2: The effects of physical parameters on mercury methylation by *Desulfovibrio* sp. B-203; pH, substrate, nickel concentration, and gas phase composition

The effect of pH on mercury methylation

pH	% added Hg methylated	CH_3Hg^+/ml (ng)	prot./ml (µg)	CH_3Hg^+/µg prot. (ng)
5.75	17.6 ± 1.7	43.7 ± 4.3	237.5 ± 4.9	0.18 ± 0.02
7.50	12.3 ± 2.1	30.5 ± 5.2	163.0 ± 4.2	0.19 ± 0.03
8.75	3.3 ± 1.7	8.1 ± 4.2	46.4 ± 2.5	0.17 ± 0.09

The effect of carbon source on mercury methylation

Sub	% added Hg methylated	CH_3Hg^+/ml (ng)	prot./ml (µg)	CH_3Hg^+/µg prot. (ng)
fum	13.1 ± 0.01	32.2 ± 0.5	170.0 ± 3.9	0.19 ± 0.01
pyr	5.7 ± 0.85	14.1 ± 2.1	90.0 ± 2.1	0.16 ± 0.02

The effect of nickel concentration mercury methylation

Ni	% added Hg methylated	CH_3Hg^+/ml (ng)	prot./ml (µg)	CH_3Hg^+/µg prot. (ng)
0.008 mM	10.0 ± 3.8	24.8 ± 9.4	189.0 ± 2.4	0.13 ± 0.05
0.08 mM	17.0 ± 1.5	42.2 ± 3.8	189.5 ± 2.5	0.22 ± 0.02

The effect of gas phase on mercury methylation

Gas Phase	% added Hg methylated	CH_3Hg^+/ml (ng)	prot./ml (µg)	CH_3Hg^+/µg prot. (ng)
H_2	12.9 ± 0.4	29.7 ± 1.0	175.0 ± 5.7	0.17 ± 0.01
N_2	10.8 ± 4.2	31.6 ± 12.3	237.5 ± 4.6	0.13 ± 0.05

Sub = Substrate
fum = Fumarate
pyr = Pyruvate

The effect of nickel on the ability of *Desulfovibrio* sp. B-203 to methylate mercury can be seen in table 3.2. It is apparent that a small increase in nickel content in the medium results in an increase in mercury methylation. This holds true when corrected for biomass. This suggests that nickel has some effect on the physiology of mercury methylation. The proposed mechanism of methylation for *Desulfovibrio desulfuricans* LS is via the acetyl CoA pathway of which the nickel requiring enzyme carbon monoxide dehydrogenase (CODH) is an essential component. The additional nickel may relieve some limitation in the full expression or functioning of this enzyme, in *Desulfovibrio* sp. B-203.

The effect of temperature on mercury methylation can be seen in Fig. 3.1. The figure shows the rate of mercury methylation over a range of temperatures. It can be seen that mercury methylation occurs over the entire temperature range tested. It can also be seen that the rate of mercury methylation is controlled by the temperature. This indicates that mercury methylation does not turn off at the lower temperature range examined, but responds to temperature effect as a physiological process would be expected.

The data in Table 3.2. show that substrate has an effect on the amount of mercury methylated in each culture. Yet when the amount of mercury methylated is correlated to

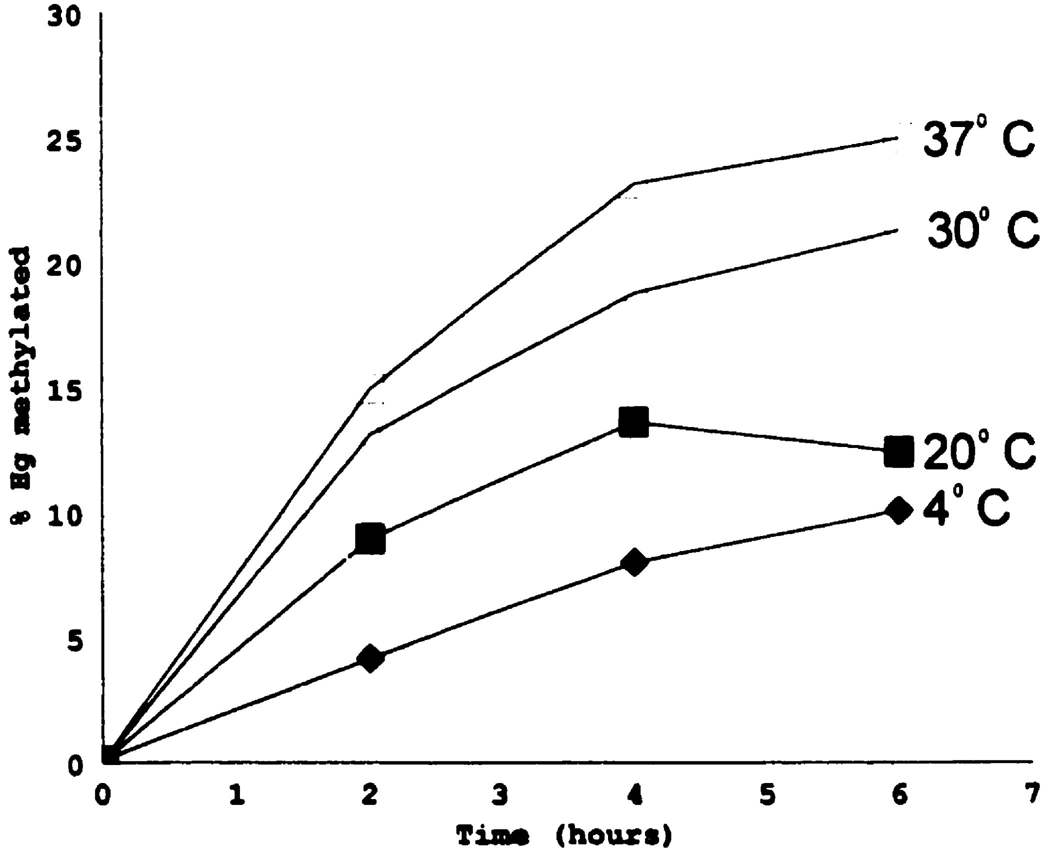

Figure 3.1 The effect of temperature on mercury methylation.

total protein content it is apparent that on a mass per mass basis, mercury methylation is the same, again showing the relationship between bacterial growth rate and mercury methylation.

The effect of gas phase composition on mercury methylation can be seen in Table 3.2. Similar amounts of methyl mercury are seen under both conditions as is similar protein content, indicating similar growth rates for both conditions. This further demonstrates that mercury methylation is controlled by growth rate.

iv) Cobalamin experiments

In Table 3.3 are the cobalamin values for the organisms tested. Although the number of organisms tested is far from exhaustive it is apparent that methanogens indeed have considerably higher cobalamin content as measured by the radio-immuno assay. The results also suggest that those SRB capable of mercury methylation appear to have higher cobalamin content then those that cannot methylate mercury. Increased cobalamin content correlating positively with mercury methylation lends support to the involvement of the acetyl CoA pathway as the mechanism of mercury methylation. A larger range of both mercury methylating and non mercury methylating SRB would be required to draw a definitive conclusion.

Table 3.3: Cobalamin content of selected bacteria

Organism	Cobalamin content (ng/g protein)	
Desulfobulbus propionicus DSM 2032	lactate	93,876
	sulphate	10,589
Desulfotomaculum ruminus DSM 2154	fumarate	425
	sulphate	996
Desulfovibrio desulfuricans DSM 1924	fumarate	2,088
	sulphate	938
Desulfovibrio desulfuricans DSM 642	fumarate	1,987
	sulphate	956
Desulfovibrio desulfuricans DSM 27774	fumarate	1,198
	sulphate	1,411
Desulfovibrio sp. B-203	fumarate	29,383
	sulphate	88,742
Desulfovibrio desulfuricans ND-132	sulphate	42,057
Desulfovibrio vulgaris DSM 644	fumarate	960
	sulphate	711
Escherichia coli MP 180		3,460
Methanosarcina barkerii DSM 804		1,541,290
Methanosphaera stadtmanae DSM 3091		1,058,206
Methanospirillum hungatei DSM 1101		840,341

Assay values are the average of triplicates. Standard deviation of all cobalamin measurements <10%. For all SRB sulphate indicates growth with sulphate as the terminal electron acceptor, lactate and fumarate indicate fermentative growth. All other organisms were grown on media particular to each species. See appendics for media formulations.

Differences in cobalamin content occurred for individual SRB when grown fermentatively or in the presence of sulphate; however, no pattern is apparent in the data.

Section 3.4

Conclusion

i) Mercury methylation survey

It is apparent from the survey data that the ability to methylate mercury is strain specific. Amongst both the SRB and the other eubacterial species surveyed, several species that have previously been shown by others to methylate mercury failed to demonstrate this capacity in this survey.

ii) CVAFS experiments

From the data shown it is seen that *Desulfobulbus propionicus* has the capacity to methylate mercury when mercury is present in exceedingly low concentrations. This indicates that it is possible for *D. propionicus* to contribute to methyl mercury production in pristine environments. Since mercury methylation occurred while *D. propionicus* was growing fermentatively this suggests that a correlation between sulfate reduction and mercury methylation need not exist. Finally, with the indication that one species of SRB is capable of mercury methylation at low total mercury concentrations it is not unreasonable to expect other mercury methylating SRB (*ie.* mercury methylation at high mercury concentrations) capable of mercury methylation at environmentally realistic total mercury concentrations. If so this would add further support to the claim that SRB are environmentally significant methylators of mercury.

iii) Mercury-203 experiments

The rate of mercury methylation appeared to be unaffected under the variety of conditions tested when normalized to cell biomass. Figure 3.2 shows the relationship between cell biomass as represented by protein content and methyl mercury concentration. It is readily apparent that cell biomass dictates the amount of methyl mercury that will be produced. Again from Figure 3.2 and the data in Table 3.2 it is apparent that the ratio of mercury methylated to protein content was similar for all of these various experimental conditions. This would be consistent with the concept of mercury methylation being an incidental consequence of normal metabolism. This is further supported by the mercury methylation experiments that have shown mercury methylation over a range of temperatures. As temperature affects the rate of metabolic activity, it is also seen to affect the rate of mercury methylation.

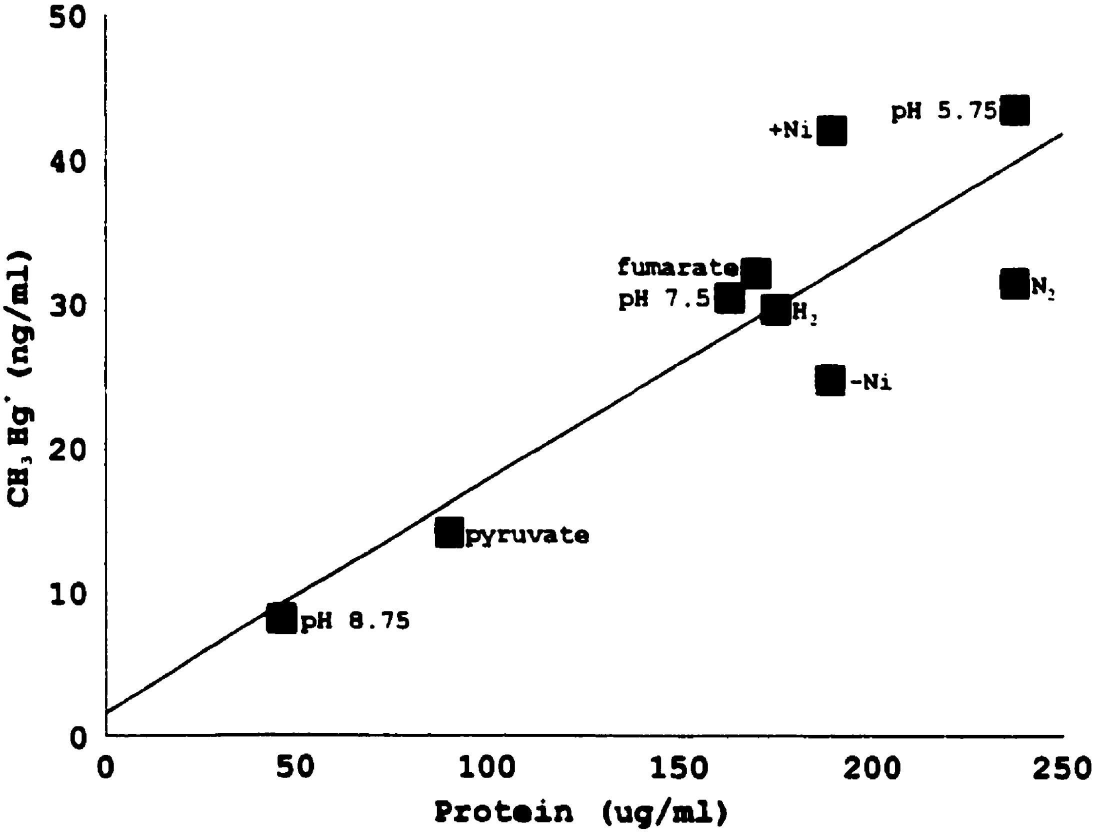

Figure 3.2. Relationship between cell biomass as represented by protein content, and methyl mercury concentration

iv) Cobalamin experiments

It is apparent that methanogens have higher cobalamin content then SRB. Among the SRB there is an indication that mercury methylating strains may have higher cobalamin content then non mercury methylating strains. This may give support to the proposed role cobalamin has in mercury methylating activities of the SRB. Namely, that those SRB capable of methylating mercury have a significantly higher cobalamin content than non mercury methylating strains. This does not address the situation presented by methanogens which have significantly higher cobalamin content yet have not demonstrated mercury methylation in intact cells but only in cell extracts (Wood *et al.* 1968).

v) Summary of conclusions

From the body of work in this chapter it can be seen that mercury methylation is not wide spread among the surveyed organisms. Indeed it is only seen in the SRB, and among the SRB only select species and or strains were capable of mercury methylation. Of those that were capable of mercury methylation, one was shown to methylate mercury over a wide range of mercury concentrations. This was the first demonstration that a pure culture can methylate mercury at the extremely low concentrations of mercury typical of pristine natural environments, further supporting the role of SRB as significant mercury methylators in the environment. From experiments conducted using mercury-203 it is apparent that changes in physiology had little direct effect on mercury methylation other than by affecting growth, which positively correlated with mercury methylation. This would suggest that in the environment any changes to a system that result in increased growth of microbial communities would then result in increased production of methyl mercury.

Examination of cobalamin concentrations among mercury methylating and non mercury methylating SRB showed a trend of higher cobalamin concentrations in mercury methylators than in non mercury methylators. This tends to support the role of cobalamin as the donor of the carbanion methyl group to mercury.

Chapter 4

Conclusion

Section 4.1 Thesis conclusions

Section 4.2 Suggestions for the direction of future work

Section 4.1

Thesis conclusions

The study of bacterial mercury methylation poses a number of interesting challenges. The first of these challenges is in resolving the data present in the already existing body of work on the subject.

Two major focus areas have developed with respect to research in mercury methylation. One area is research conducted on pure cultures of bacteria capable of mercury methylation. The other is research on undefined natural populations of bacteria, mainly in lake sediments, demonstrating mercury methylation activity.

The pure culture work has identified a number of bacterial species capable of mercury methylation, while the work with natural populations, either in soil, sediment or water, have established some of the parameters that affect mercury methylation in the environment.

One of the most challenging aspects of research on mercury methylation is reconciling the information available from the pure culture and natural population work. Extracts of methanogenic bacteria were shown to methylate mercury yet whole cells of methanogens were unable to methylate mercury (Wood *et al.* 1968). Early experiments with the undefined bacterial population found in aquaria sediment initially established the bacterial nature of mercury methylation (Jensen and Jernelöv 1969). These same experiments showed that mercury methylation was favoured under anaerobic conditions (Jensen and Jernelöv 1969). Later experiments showed a variety of pure cultures of bacteria were capable of mercury methylation, confirming the ability of bacteria to methylate mercury.

With the knowledge that mercury methylation is favoured under anaerobic conditions and through the use of molybdate, it has been shown that SRB are significant methylators of mercury (Compeau and Bartha 1985). This has led to the isolation on a number of pure cultures of SRB that can methylate mercury (Gilmour and Henry 1991, Compeau and Bartha 1985).

From the work conducted in the previous chapters of this thesis a number of conclusions can be made. The first portion of the research dealt with the specificity of molybdate as an inhibitor of SRB. Three significant conclusions can be drawn from this work. The first was that for a wide variety of eubacterial species molybdate proved to be non-inhibitory at concentrations that are indeed inhibitory to SRB. So it would seem that molybdate is indeed a specific inhibitor of SRB.

The second significant conclusion is drawn from the evidence that two methanogens are inhibited at concentrations lower than those commonly used to inhibit SRB. This would indicate that the use of molybdate to isolate the activities of SRB from other bacteria should be carefully considered and not assumed to be an absolute. This is particularly applicable to methanogens, as SRB and methanogens share a similar habitat. This is further supported by the work of Wolin and Miller (1980) demonstrating that a strain of *Ruminococcus albus*' hydrogenase activity is also inhibited at molybdate concentrations lower than what has been used to inhibit SRB in the past. Thus, there is still some question about the current attribution of mercury methylation in the environment to SRB alone. There is no doubt that some SRB do indeed methylate mercury. Attributing the bulk of mercury methylating activity to the SRB is based on the use of molybdate to suppress their activity and the resultant decrease in mercury methylation was directly attributed to the inhibition of the SRB. Since molybdate also inhibits some methanogens and cell free extracts of methanogens have been shown to methylate mercury the use of molybdate alone is not sufficient to rule out the participation of methanogens in mercury methylation.

The third significant conclusion that can be drawn from the work with molybdate is that it inhibits SRB growing fermentatively, something previously thought not to occur. This indicates that a mechanism of inhibition different than what has been proposed for the inhibition of SRB by molybdate is obviously in operation.

From the data generated in the mercury methylation study it is apparent that mercury methylation is closely tied to overall microbial mass which is a function of the growth rate and changes that affect biomass production correspondingly change the amount of mercury methylated. This would suggest that mercury methylation is catalyzed by a constitutive enzyme, or at least an enzyme independent of the nature of bioenergetic substrate used but affected by the quantity of substrate because that affects growth rate.

Cobalamin content in the SRB is significantly lower than in the methanogens yet no known methanogens methylate mercury. Of the SRB tested those that are capable of mercury methylation also had the highest cobalamin content of all the SRB. Perhaps indicating its involvement in mercury methylation.

From the data derived from the low level mercury methylation work it is apparent that *Desulfobulbus propionicus* is capable of mercury methylation at extremely low total mercury concentrations. Concentrations that are within the range of that seen for natural unperturbed environments.

It is clear that a number of questions remain in regards to microbial methylation of mercury. In eubacterial mercury methylators, other than the SRB, is the mercury methylation rate dependant on the growth rate, as seen with the SRB?

Section 4.2

Suggestions for the direction of future work

While answering certain questions, the current work stimulates the asking of even more questions. The survey of organisms conducted in this work did not find mercury methylators outside of the SRB; however, several of the species tested have been shown by others to be capable of mercury methylation. It must be remembered that the early work looking at non-SRB eubacterial species used extremely high concentrations of total mercury as compared to this study. This work and others indicates that the ability to methylate mercury is strain specific, and therefore until many more strains of many more species are tested for mercury methylating ability it is difficult to clearly determine the extent of mercury methylating ability. Identification of non-SRB strains would allow investigation of mercury methylation in something other than an SRB. This would allow for a comparison of mercury methylating abilities and provide answers to the questions; i) Are mercury methylators other than SRB capable of mercury methylation at concentrations typical of pristine natural environments? ii) What physiological factors affect rates of mercury methylation, or is mercury methylation simply influenced by growth rate alone? iii) In a larger and more diverse survey of mercury methylating and non mercury methylating SRB do the trends seen in cobalamin content and the ability to methylate mercury hold true? iv) Are there trends in cobalamin levels amongst SRB and non-SRB mercury methylators? v) Does mercury methylation amongst SRB and non-SRB mercury methylators occur via the same metabolic process or by a variety of processes? *ie.* Does a mercury methylating enzyme, as suggested by Choi and Bartha (1993) really exist and if so is it the means of mercury methylation for all organisms capable of mercury methylation or do other modes of mercury methylation operate? All of the proceeding questions, if answered, would greatly aid our understanding of the phenomenon of mercury methylation.

Appendix A

Media

The following is a list of the components of the media used as growth substrate for the microorganisms examined in the previous work.

i) Yeast peptone nutrient broth, contains per litre, 10 g yeast extract, 10 g peptone, 1 ml resazurin solution. Gas phase N_2. Final pH 7.0.

ii) Postgate's medium D (Postgate 1984), contains per litre, 0.5 g KH_2PO_4, 1.0 g NH_4Cl, 0.1 g $CaCl_2*2H_2O$, 1.6 g $MgCl_2*6H_2O$, 1.0 g yeast extract, 0.004 g $FeSO_4*7H_2O$, 3.7 g fumaric acid, 10 ml vitamin supplement, 10 ml mineral elixir, 0.5 ml titanium-NTA, 1 ml resazurin solution. Gas phase N_2. Final pH 7.4.

iii) Fermentative Freshwater Widdel (Widdel and Pfennig 1981), contains per litre, 3 ml sodium lactate (60% solution) 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.5 KCl, 0.15 g $CaCl_2*2H_2O$, 1 g NaCl 0.4 g $MgCl_2*6H_2O$, 2.5 g $NaHCO_3$, 1 ml mineral elixir, 1 ml vitamin supplement, 0.5 ml tungstenate solution, 0.5 ml selenate solution, 250 µl vitamin B-12 solution, 50 µl DL pantothenate solution, 1 ml resazurin solution. Gas phase 80:20 N_2:CO_2. Final pH 7.5.

iv) Peptone and glucose nutrient broth (Vonk and Sijpesteijn 1973), contains per litre, 10 g peptone, 10 g glucose, 4 g K_2HPO_4, 1 g KH_2PO_4, 1 ml resazurin solution. Gas phase N_2. Final pH 7.0.

v) *Acetobacterium woodii* medium (Medium 135, DSM catalogue), contains per litre 1.0 g NH_4Cl, 0.33 g KH_2PO_4, 0.45 g K_2HPO_4, 0.1 g $MgSO_4{*}7H_2O$, 10 ml mineral elixir, 10 ml vitamin supplement, 2.0 g yeast extract, 10.0 g fructose, 10.0 g $NaHCO_3$, 0.5 g cysteine-HCl, 1 ml resazurin solution. Gas phase 80:20 N_2:CO_2. For autotrophic growth fructose is omitted and gas phase is changed to 80:20 H_2:CO_2. Final pH 8.2.

vi) Pfennig's freshwater *Desulfovibrio baarsii* medium (Jansen *et al.* 1984), contains per litre, 3.4 g sodium formate, 3.0 g Na_2SO_4, 0.2 g KH_2PO_4, 0.25 g NH_4Cl, 1.0 g NaCl, 0.4 g $MgCl_2{*}6H_2O$, 0.5 g KCl, 0.15 g $CaCl_2{*}2H_2O$, 2.5 g $NaHCO_3$, 1 ml mineral elixir, 8 µL Na_2SeO_3 solution, 12 µL Na_2WO_4 solution, 1 ml resazurin solution. Gas phase 80:20 N_2:CO_2. Final pH 7.4.

vii) *Rhodospirillum rubrum* medium (Medium 1308 ATCC catalogue), contains per litre, 1.0 g yeast extract, 0.5 ml ethanol, 1.0 g di-sodium succinate, 5.0 ml 0.1% ferric citrate (aqueous solution), 0.5 g KH_2PO_4, 0.4 g $MgSO_4{*}7H_2O$, 0.4 g NaCl, 0.4 g NH_4Cl, 0.05 g $CaCl_2{*}2H_2O$, 0.5 g sodium ascorbate, 1 ml mineral elixir, 1 ml resazurin solution. Gas phase N_2. Final pH 6.0.

viii) *Methanococcus thermolithotrophicus* medium (Sparling and Daniels 1986), contains per litre, 0.31 g KCl, 0.45 g NH_4Cl, 0.04 g $CaCl_2{*}H_2O$, 0.25 g K_2HPO_4, 0.25 KH_2PO_4, 11.7 g NaCl, 10 ml of a 2.5 M $MgCl_2{*}6H_2O$ anaerobic sterile stock solution, added after autoclaving, 0.16 g Na_2CO_3, 20.7 g sodium formate, 9.76 g 2-(*N*-morpholino)ethanesulfonic acid

(MES), 15.12 g piperazine-*N*,*N*'-bis(2-ethanesulfonicacid) (PIPES), 11.9 g *N*-2-hydroxyethyl-piperazine-*N*'-2-ethanesulfonic acid (HEPES), 1 ml selenate solution, 1 ml tungstate solution, 10 ml mineral elixir, 1 ml resazurin solution. Gas phase N_2. Final pH 5.9.

ix) *Methanosarcina barkerii* medium (Müller *et al.* 1986), contains per litre, 0.35 g K_2HPO_4, 0.23 g KH_2PO_4, 0.5 g NH_4Cl, 0.5 g $MgSO_4*7H_2O$, 0.25 g $CaCl_2*2H_2O$, 2.25 g NaCl, 0.002 g $FeSO_4*7H_2O$, 10 ml vitamin supplement, 1 ml mineral elixir, 2 g yeast extract, 2 g tryptone, 0.85 g $NaHCO_3$, 10 ml of a 40% methanol solution (), 1 ml resazurin solution. Gas phase 80:20 N_2:CO_2. Final pH 6.8.

x) *Methanosphaera stadtmanae* medium (Sparling *et al.* 1993), contains per litre, 2.0 g tryptone, 2.0 g yeast extract, 0.3 g K_2HPO_4, 2.8 g KH_2PO_4, 0.3 g $(NH_4)SO_4$, 0.61 NaCl, 0.13 g $MgSO_4*7H_2O$, 0.08 g $CaCl_2*2H_2O$, 0.5 g sodium acetate, 1.7 g $NaCO_3$, 1.0 g NH_4Cl, 15 ml vitamin supplement, 15 ml mineral elixir, 10 ml of a 40 % methanol solution (added from an anaerobic sterile stock after autoclaving), 0.5 ml Na_2SeO_3 solution, 1 ml resazurin solution. Gas phase 80:20 N_2:CO_2. Final pH 7.0.

xi) *Methanospirillum hungatei* medium (Daniels *et al.* 1986), contains per litre, 0.3 g NaCl, 0.064 g $MgCl_2*6H_2O$, 0.4 g NH_4Cl, 0.42 g sodium acetate, 0.82 g K_2HPO_4, 0.064 g $CaCl_2*2H_2O$, 1.0 g $NaCO_3$, 10 ml mineral elixir, 10 ml vitamin supplement, 1 ml resazurin solution. Gas phase 80:20

H_2:CO_2. Final pH 7.0.

xii) *Myxococcus xanthus* medium (Medium 791, ATCC catalogue), contains per litre, 10.0 g casitone, 0.5 g $MgSO_4$*$7H_2O$, 0.017 g KH_2PO_4, K_2HPO_4. Final pH 7.6.

All culture media were autoclaved at 121° C for 20 minutes. All culture media, except *Myxococcus xanthus* ATCC culture medium 791, were degassed via the Balch-Wolfe technique (Balch and Wolfe 1976). All SRB, methanogen and acetogen cultures for the molybdate survey were reduced with a 1% v/v addition of a 200 mM sodium sulphide solution. All SRB, methanogen and acetogen cultures for mercury methylation experiments were reduced with 0.2% v/v addition of a Ti-NTA solution.

Appendix B

Solutions and Amendments

The following is a list of solutions and amendments used in the preceding experiments.

i) Vitamin supplement (Wolin *et al.* 1963), contains per litre, 10.0 mg pyridoxine-HCl, 5.0 mg riboflavin, 5.0 mg thiamine, 5.0 mg nicotinic acid, 5.0 mg p-aminobenzoic acid, 5.0 mg lipoic acid, 2.0 mg biotin, 2.0 mg folic acid, 1.0 mg cyanocobalamin.

ii) Mineral elixir (Belay *et al.* 1984), contains per litre, 2.02 g trisodium nitriloacetate, 0.21 g $FeCl_3*6H_2O$, 0.20 g $CoCl_2*6H_2O$, 0.10 g $MnCl_2*4H_2O$, 0.10 g $ZnCl_2$, 0.05 g $CaCl_2*2H_2O$, 0.05 g $CuSO_4*2H_2O$, 0.05 g $NaMoO_4*2H_2O$, 0.10 g $NiCl_2*6H_2O$.

iii) Resazurin solution contains 25 mg resazurin per 100 ml distilled water.

iv) Tungstenate solution is a 1 mM solution of $Na_2WO_4*2H_2O$ in distilled water.

v) Selenate solution is a 1 mM solution of Na_2SeO_4 in distilled water.

vii) Harvesting buffer contains per litre, 6.8 g KH_2PO_4 and 0.37 g $MgCl_2$ adjusted to pH 7.5, degassed, placed under an N_2 gas phase and autoclaved.

iix) Vitamin B_{12} solution is 0.1 g vitamin B_{12} in 100 ml distilled water.

ix) DL calcium pantothenate solution is 0.01 g DL calcium pantothenate in 100 ml distilled water.

x) Fumarate sulphate solution contains per litre, 4.64 g fumarate and 0.14 g Na_2SO_4, degassed, placed under an N_2 gas phase and autoclaved.

xi) Lactate sulphate solution contains per litre, 7.4 ml of a 60% sodium lactate solution and 0.14 g Na_2SO_4, degassed, placed under an N_2 gas phase and autoclaved.

xii) Pyruvate sulphate solution contains per litre, 4.44 g pyruvate and 0.14 g Na_2SO_4, degassed, placed under an N_2 gas phase and autoclaved.

xiii) Mercury/mercury 203 working stock solutions used for the survey for the ability to methylate mercury, contained 240.5 µg Hg, with a specific activity of 50 µCi, in 5.0 ml total volume, and 16,250 µg Hg, with a specific activity of 250 µCi, in 5.0 ml total volume, generating a final mercury concentration of 74 ng/ml and 5000 ng/ml, respectively, when 100 µl addition is used. Mercury/mercury 203 working stock solution used for determination of factors affecting mercury methylating activity contained 1495 µg Hg, with a specific activity of 200 µCi, in 50.0 ml total volume, generating a final Hg concentration of 230 ng/ml when a 500 µl addition is used (This stock is filter sterilized before use).

xiv) Ti-NTA solution was prepared via the method of Moench and Zeikus (1983) and had a final concentration of 25 mM Ti and 100 mM NTA.

Appendix C

Growth Conditions

The following are the growth conditions for the organisms used in the thesis research:

Acetobacterium woodii DSM 1030 incubation temperature 30° C, medium DSM #135 with and without fructose.

Bacillus subtilis ATCC 6051 incubation temperature 30° C, media yeast peptone nutrient broth, and peptone glucose nutrient broth.

Bacillus megaterium ATCC 14581 incubation temperature 30° C, media yeast peptone nutrient broth, and peptone glucose nutrient broth.

Desulfobulbus propionicus DSM 2032 incubation temperature 37° C, medium fermentative freshwater Widdel.

Desulfotomaculum ruminus DSM 2154 incubation temperature 30° C, medium Postgate's medium D with fumarate.

Desulfovibrio baarsii DSM 2075 incubation temperature 30° C, medium Pfennig's freshwater medium.

Desulfovibrio desulfuricans DSM 642 incubation temperature 30° C, medium Postgate's medium D with fumarate.

Desulfovibrio desulfuricans DSM 1924 incubation temperature 30° C, medium Postgate's medium D with fumarate.

Desulfovibrio desulfuricans ATCC 277724 incubation temperature 30° C medium Postgate's medium D with fumarate.

Desulfovibrio desulfuricans ND-132 incubation temperature 30° C medium Postgate's medium C.

Desulfovibrio vulgaris DSM 644 incubation temperature 30° C, medium Postgate's medium D with fumarate.

Desulfovibrio sp. B-203 incubation temperature 30° C, medium Postgate's medium D with fumarate.

Enterobacter aerogenes ATCC 13048 incubation temperature 37° C, media yeast peptone nutrient broth, and peptone glucose nutrient broth.

Escherichia coli MP 180 incubation temperature 37° C, media yeast peptone nutrient broth, and peptone glucose nutrient broth.

Escherichia coli XL-1 Blue incubation temperature 37° C, media yeast peptone nutrient broth, and peptone glucose nutrient broth.

Lactobacillus plantarum ATCC 8014 incubation temperature 30° C, media yeast peptone nutrient broth, and peptone glucose nutrient broth.

Methanococcus thermolithotrophicus DSM 2095 incubation temperature 65° C.

Methanosarcina barkeri strain Fusaro DSM 804 incubation temperature 37° C.

Methanosphaera stadtmanae DSM 3091 incubation temperature 37° C.

Methanospirillum hungatei strain GP1 DSM 1101 incubation temperature 37° C.

Pseudomonas fluorescens 493' incubation temperature 30° C, media, yeast peptone nutrient broth, and peptone glucose nutrient broth with KNO_3.

Rhodospirilum rubrum ATCC 11170 incubation temperature 37°

C, medium, ATCC # 1308.

Staphylococcus aureus 96' incubation temperature 30° C, media yeast peptone nutrient broth, and peptone glucose nutrient broth.

Streptococcus faecalis 97' incubation temperature 30° C, media yeast peptone nutrient broth, and peptone glucose nutrient broth.

Appendix D

CVAFS Pitfalls in Development

The major obstacle encountered when attempting to work with ultra-low concentrations of mercury (*ie.* those approaching sub 15 nanogram per litre concentrations) is that of contamination. Possible contamination arises from a variety of sources and in the scope of this work identifying the contribution of each of the sources is prohibited by the cost of analysis. A second difficulty when dealing with low level mercury experiments is found in the undetermined reactions of mercury with components of the microbial growth medium, glassware, and butyl rubber stoppers. Any residual mercury that remains bound to the culture vessels after cleaning can be a possible source of contamination if it desorbs into the culture media. Also binding of mercury from the culture medium to the glassware or stopper of the culture vessel can remove mercury from the culture media. The exact extent of mercury binding or desorption from the glassware is beyond the extent of this research project. This potential binding or desorption of mercury into the culture medium is really only of concern when dealing with near environmental or ultra low total mercury concentrations. When dealing with mercury concentrations used in radio isotope based experiments contamination by residual mercury or loss of added mercury via sorption to the culture vessel does not pose a problem due the high concentrations used.

Following is some of the preliminary development data used in developing the low level methylation experiments. Table D.1 shows the extreme range of mercury concentrations found in samples that were not treated to any special cleaning or handling procedures but rather were treated in the routine manner used for materials in radio isotope experiments. As well the results show the effects of sulphide addition on mercury concentration. Obviously negative mercury concentrations are an impossibility. The negative value seen for mercury concentration thus indicates a limitation in the assay procedure when dealing with high sulphide concentrations. This can pose a serious concern when developing or conducting assays involving anaerobes that either require anaerobic conditions achieved with the use of sulphide or produce sulphide as a by-product of their metabolism. It must be noted that the source of the mercury in the four samples is undetermined. It may represent mercury actually present in the water (both Milli Q and glass distilled), it may represent mercury in the reagents used in making the media, it may be contamination from the glassware and stoppers, it may have been introduced from some unknown source during sample collection, or some combination of the above. It is readily apparent that ordinary cleaning procedures are insufficient to produce reliable low total mercury concentrations.

The second problem area encountered in developing low level mercury protocols is the undetermined reactions and

Table D.1: Preliminary results showing range of mercury concentrations in various water sources and media

Sample	Hg concentration
Milli Q water	376.18 ng/L
Glass distilled water	91.15 ng/L
Postgate's medium D	15.45 ng/L
Postgate's medium D plus sulphide	-1.25 ng/L

Table D2: Developmental data detailing methyl mercury concentrations and spike recovery rates in both inoculated and uninoculated Postgate's medium D SRB culture media

Bottle Identifier	Sample Type	Methyl Hg concentration ng/L	Spike Recovery
1	A	0.028	8.7
2	A	0.046	8.7
3	B	0.049	8.7
4	B	0.019	8.7
5	B	0.00	8.7
6	C	0.10	8.7
1	D	0.001	82.86
2	D	0.00	82.86
3	D	0.00	82.86
4	E	0.00	4.81
5	E	0.031	4.81
6	E	0.0362	4.81
7	F	0.007	4.81
8	F	0.056	4.81
9	F	0.011	4.81
d	G	0.018	4.81
e	G	0.014	4.81

A. *Desulfovibrio* sp. B-203 frozen after inoculation.
B. *Desulfovibrio* sp. B-203 grown to the end of log phase.
C. *Desulfovibrio* sp. B-203 grown to mid-point of log phase then treated with molybdate 30 mM final concentration.
D. *Desulfovibrio desulfuricans* DSM 1924 frozen after inoculation.
E. *Desulfovibrio desulfuricans* DSM 1924 grown to the end of log phase.
F. *Desulfovibrio desulfuricans* DSM 1924 grown to mid-point of log phase then treated with molybdate 30 mM final concentration.
G. Uninoculated Postgate's medium D with fumarate.

sorption/desorption mercury undergoes with components of the medium and the culture vessel itself. This is apparent in table D1 for the sample containing sulphide in regards to total mercury. The undetermined reactions methyl mercury can undergo with the culture medium can call results into question as can be seen in the data presented in table D2. The range of methyl mercury value for replicate samples and the low to extremely low spike recovery rates, indicate that the data is unreliable. Obviously with such low spike recovery rates true methyl Hg concentrations cannot be known. The cause of the low spike recovery rates is unknown, but appears only with Postgate's medium D. This prevents any easy comparison of low level mercury methylation abilities between the two mercury methylating SRB species present in the survey population.

Appendix E

Pertinent Characteristics of Organisms Examined in Thesis Research

Table E1: Some pertinent characteristics of the organisms examined in various aspects of the thesis research

Footnotes to table:

1. Shown by other researchers to methylate mercury.
2. Members of the genus have been shown by other researchers to methylate mercury.
3. Tested for mercury methylation in this work.
4. Species has been shown by other researchers to be inhibited by molybdate while respiring sulphate.
5. Species has been shown by other researchers not to be inhibited by molybdate while growing fermentatively.
6. Tested for molybdate inhibition in this work.
7. Tested for methylcobalamin content in this work.

[A] Department of Microbiology, University of Manitoba culture collection.

[B] Designation assigned by C. Gilmour, undeposited culture

[C] A particular form of anaerobic respiration involving H_2 as electron donor and CO_2 as the terminal electron acceptor. In the case of the methanogens, it can also involve some simple organic compounds such as, methanol, formate, methyl mercaptan, acetate, and methylamines.

[D] Anaerobic respiration via nitrate.

[E] Aerobic respiration in the dark.

[F] Anaerobic respiration in the light.

Organism	Oxygen Tolerance	Metabolism	Add Info
Acetobacterium woodii DSM 1030	strict anaerobe	anaerobic respiration fermentative	3,6
Bacillus megaterium ATCC 14581	aerobe anaerobe	aerobic respiration anaerobic respiration	1,2,3
B. subtilis ATCC 6051	aerobe anaerobe	aerobic respiration anaerobic respiration	2,3,6
Desulfovibrio baarsii DSM 2075	strict anaerobe	anaerobic respiration	2,3,6
Desulfovibrio sp. ND-132	strict anaerobe	anaerobic respiration	1,2,7
Desulfovibrio sp. B-203[B]	strict anaerobe	anaerobic respiration fermentation	1,2,3, 4,7
D. desulfuricans DSM 1924	strict anaerobe	anaerobic respiration fermentation	2,3,4, 6,7
D. desulfuricans DSM 642	strict anaerobe	anaerobic respiration fermentation	2,3,4, 6,7
D. desulfuricans ATCC 27774	strict anaerobe	anaerobic respiration fermentation	2,3,4, 6,7
D. vulgaris DSM 644	strict anaerobe	anaerobic respiration fermentation	2,3,4, 5,6,7
Desulfobulbus propionicus DSM 2032	strict anaerobe	anaerobic respiration fermentation	1,3,6, 7
Desulfotomaculum guttoideum DSM 4024	strict anaerobe	anaerobic respiration fermentation	6
Dm. ruminus DSM 2154	strict anaerobe	anaerobic respiration fermentation	3,4,5, 6,7
Enterobacter aerogenes ATCC 13048	facultative aerobe	aerobic respiration fermentation	1,2,3, 6
Escherichia coli MP 180 and XL-Blue	facultative aerobe	aerobic respiration fermentation	1,2,3, 6,7
Lactobacillus plantarum ATCC 8014	aerotolerant anaerobe	fermentation	2,3,6
Methanococcus thermolithotrophicus DSM 2095	strict anaerobe	anaerobic respiration[C]	6
Methanosarcina barkeri DSM 804	strict anaerobe	anaerobic respiration[C]	3,6,7
Methanosphaera stadtmanae DSM 3091	strict anaerobe	anaerobic respiration[C]	3,6,7
Methanospirrillum hungaeti DSM 1101	strict anaerobe	anaerobic respiration[C]	3,6,7
Myxococcus xanthus DK 1050	strict aerobe	aerobic respiration	6
Pseudomonas fluorescens 493[A]	aerobe anaerobe	aerobic respiration anaerobic respiration[D]	1,3,6
Rhodospirrillum rubrum ATCC 11170	facultative phototroph	aerobic respiration[E] anaerobic respiration[F]	3,6
Staphylococcus aureus 96[A]	facultative anaerobe	fermentation aerobic respiration	2,3,6

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