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CHAPTER 1.31

The Colorless Sulfur Bacteria

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The name "the colorless sulfur bacteria" has been used since the time of Winogradsky to designate prokaryotes that are either able, or believed to be able, to use reduced sulfur compounds (e.g., sulfide, sulfur and organic sulfides) as sources of energy for growth. Today, it is known that this group comprises a very heterogeneous collection of bacteria, many of which have little or no taxonomic relationship to each other. The colorless sulfur bacteria play an essential role in the oxidative side of the sulfur cycle (Fig. 1). Like all of the element cycles, the sulfur cycle has an oxidative and a reductive side, which, in most ecosystems, are in balance. However, this balance does not always exist, and accumulations of intermediates such as sulfur, iron sulfides, and hydrogen sulfide are often found. On the reductive side, sulfate (and sometimes elemental sulfur) functions as an electron acceptor in the metabolic pathways used by a wide range of anaerobic bacteria, leading to the production of sulfide. Conversely, on the oxidative side of the cycle, reduced sulfur compounds serve as electron donors for anaerobic, phototrophic bacteria or provide growth energy for the extremely diverse group of (generally) respiratory colorless sulfur bacteria. Common oxidation products of sulfide are elemental sulfur and sulfate (Fig. 1). The adjective "colorless" is used because of the lack of photopigments in these bacteria, although it should be noted that colonies and dense cultures can actually be pink or brown because of their high cytochrome content. This chapter will concentrate on the colorless sulfur bacteria, while the sulfate reducers and phototrophs will be discussed in 13 and 24.

There is a wide range of different types of colorless sulfur bacteria with very diverse morphological, physiological and ecological properties and with equally diverse environmental requirements. Table 1 lists the genera that have traditionally been regarded as colorless sulfur bacteria (part A), as well as genera containing species originally not classified as such that have now been shown to be able to obtain energy from the oxidation of reduced sulphur compounds (part B). As will be discussed later, the apparent similarity of the metabolic pathways for sulfur oxidation disguises a high level of variation in these pathways indicating that the diversity among the colorless sulfur bacteria is probably due to convergent rather than divergent evolution. In addition to inorganic sulfur compounds, some species can also gain energy from the oxidation of other inorganic compounds such as hydrogen or ferrous iron. As well as differences in substrate range, there is also some variation in electron acceptor usage. Although most colorless sulfur-oxidizing bacteria require oxygen, a few are able to grow anaerobically using nitrogen oxides (e.g., nitrate) as their terminal electron acceptor during denitrification. One or two species (of the genus *Acidianus*) are capable of anaerobic metabolism by the reduction of sulfur (Segerer and Stetter, 1989), during which organic compounds or hydrogen serve as electron donors. *Thiobacillus ferrooxidans* is known to be able to reduce ferric iron under anaerobic conditions (Sugio et al., 1985). A somewhat exotic example of a sulfate reducer that might also be considered to be a colorless sulfur bacterium is *Desulfovibrio sulfodismutans*, which can grow anaerobically by the disproportionation of thiosulfate to sulfate and sulfide (Bak and Pfennig, 1987). Some of the reactions that generate energy from inorganic reduced sulfur compounds using oxygen and nitrate as electron acceptors are shown in Table 2.

In the following sections, we will first discuss the physiology of the colorless sulfur bacteria, since physiology forms the basis of their present taxonomy, and then treat the taxonomy in the following section. This will be followed by a discussion of the habitats of the colorless sulfur bacteria, including artificial habitats, and finally some applications of their use. The chapter concludes with a brief section on the role of the colorless sulfur bacteria in the natural sulfur cycle, together with a description of the tech-

This chapter was taken unchanged from the second edition.

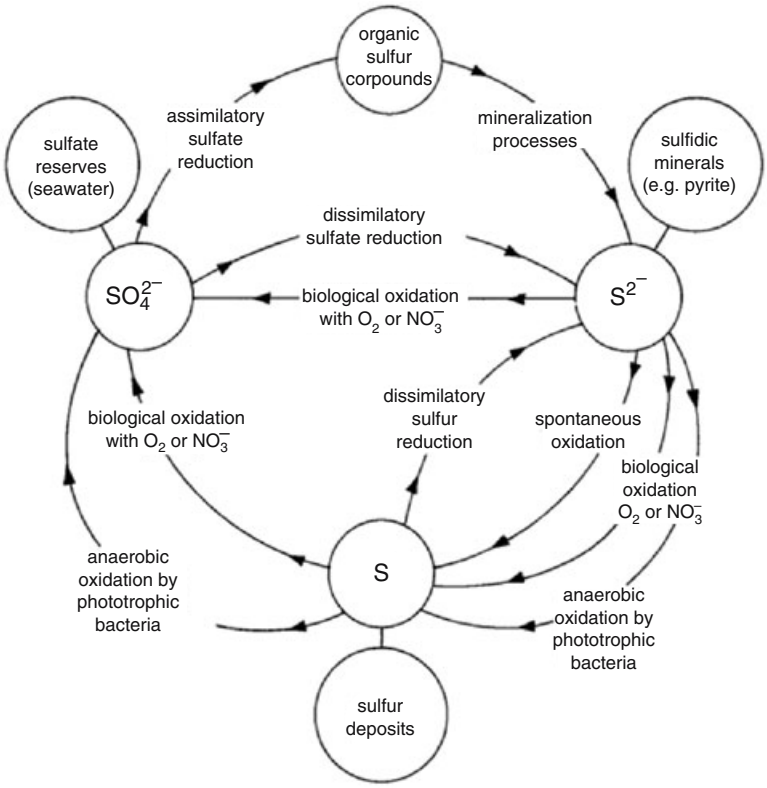

Fig. 1. The sulfur cycle. The colorless sulfur bacteria are involved primarily in those steps in which S^{2-} and S are oxidized with O_2 or NO_3^-. (Adapted from Bos and Kuenen, 1983).

Table 1. Genera of the colorless bacteria traditionally recognized as being capable of growth on reduced sulfur compounds and their environmental parameters.

	pH requirement		Thermal requirement		Anaerobic growth		
Genus	Neutrophilic	Acidophilic	Mesophilic	Thermophilic	Denitrifier	S^0/Fe^{3+} as electron acceptor	Symbiont
A. Traditional colorless sulfur bacteria							
Thiobacillus	+[a]	+	+	+	+	V	+
Thiomicrospira	+	–	+	–	+	–	?[b]
Thiosphaera	+	–	+	–	+	–	–
Sulfolobus	–	+	–	+[χ]	–	–	–
Acidianus	–	+	–	+[χ]	–	+	–
Thermothrix	+	–	–	+	+	–	–
Thiovulum[d]	+	–	+	–	–	–	–
Beggiatoa	+	–	+	–	–	+	–
Thiothrix	+	–	+	–	–	–	–
Thioploca[d]	+	–	+	–	–	–	–
Thiodendron[d]	+	–	+	–	–	–	–
Thiobacterium	+	–	+	–	–	+	–
Macromonas	+	–	+	–	–	+	–
Achromatium[d]	+	–	+	–	–	+	–
Thiospira[d]	+	–	+	–	–	–	–
B. Other bacteria capable of growth on reduced sulfur compounds							
Paracoccus	+	–	+	–	+	–	–
Hyphomicrobium	+	–	+	–	–	–	–
Alcaligenes	+	–	+	–	+	–	–
Pseudomonas	+	–	+	–	+	–	–
Hydrogenobacter	+	–	–	+	–	–	–

+, example known to exist; –, example unknown; V, variable.
16S rRNA analysis indicates a possible relationship.
Hyperthermophilic archaebacterium.
Axenic cultures are not available.

Table 2. Examples of the reactions used by the colorless sulfur bacteria to gain energy for growth.

$H_2S + 2O_2 \rightarrow H_2SO_4$
$2H_2S + O_2 \rightarrow 2S^0 + 2H_2O$
$2S^0 + 3O_2 + 2H_2O \rightarrow 2H_2SO_4$
$Na_2S_2O_3 + 2O_2 + H_2O \rightarrow Na_2SO_4 + H_2SO_4$
$4Na_2S_2O_3 + O_2 + 2H_2O \rightarrow 2Na_2S_4O_6 + 4NaOH$
$2Na_2S_4O_6 + 7O_2 + 6H_2O \rightarrow 2Na_2SO_4 + 6H_2SO_4$
$2KSCN + 4O_2 + 4H_2O \rightarrow (NH_4)_2SO_4 + K_2SO_4 + 2CO_2$
$5H_2S + 8KNO_3 \rightarrow 4K_2SO_4 + H_2SO_4 + 4N_2 + 4H_2O$
$5S^0 + 6KNO_3 + 2H_2O \rightarrow 3K_2SO_4 + 2H_2SO_4 + 3N_2$

niques available for the measurement of their activities.

Physiology

The great diversity of colorless sulfur bacteria is also reflected in their physiology. This will come as no surprise if we remember that the group encompasses archaebacteria as well as eubacteria, and that the latter group is also very diverse, including common pseudomonads and organisms that might be considered "colorless blue green bacteria," such as species of *Beggiatoa*.

Most of our knowledge of the physiology of these organisms comes from the study of the relatively limited number of bacteria, such as the thiobacilli, that can be grown relatively easily in the laboratory. This is particularly true of our understanding of the biochemistry of sulfur metabolism and, to a lesser extent, of carbon metabolism.

Although the biochemistry of the oxidation of sulfur compounds has received much attention over the last few decades, the pathways involved were not well understood. This was due, in particular, to the fact that the research was formulated around the hypothesis that there would be a single unifying enzymatic pathway for the oxidation of all reduced sulfur compounds. However, it is now clearly established that this is not the case. For example, the facultatively autotrophic *Thiobacillus versutus* and the obligately autotrophic *T. tepidarius* use two entirely different pathways (Fig. 2a and b). It should be noted that not only do the enzymes and electron carriers differ, but their localization in the membranes of the two species appears to be different. This is, of course, important for the mechanism behind the generation of a proton motive force (PMF) in these organisms. Little is known of the electron carriers involved in reverse electron transport for the production of reducing power during autotrophic growth, but all available evidence indicates that the PMF is the driving force for this process. For an extensive review of the state of the art, the reader is referred to Kelly (1988b).

In most obligate and facultative autotrophs, the Calvin cycle serves as the route for carbon dioxide fixation. This is true, for example, for species from the genera *Thiobacillus*, *Thiomicrospira*, *Thiosphaera*, and *Beggiatoa*. Some other species, including those from *Sulfolobus* and *Hydrogenobacter*, possess a carbon dioxide fixation pathway based on a reductive Calvin cycle (Segerer and Stetter, 1989).

Energy and Carbon Sources or Electron Donors

It has been common practice to subdivide the colorless sulfur bacteria in terms of their physiological type as defined mainly by their carbon metabolism. Table 3 defines these physiological types, which will be discussed briefly below. It should be remembered that some genera or species have not been studied in pure culture, and it is not yet known to which of the physiological groups they belong.

Obligate Chemolithotrophs. These highly specialized bacteria require an inorganic source of energy and obtain their cell carbon from the fixation of carbon dioxide. As mentioned above, except in the case of the archaebacteria (which use a reductive carboxylic cycle [König and Stetter, 1989]), the colorless sulfur bacteria do this by means of the Calvin cycle (e.g., Schlegel, 1981). The citric acid cycle in these bacteria seems to be incomplete, and its enzymes probably serve a purely biosynthetic function. Despite their label as "obligate" autotrophs, it has been shown that many of these species actually can use small amounts of exogenous carbon compounds as a supplementary carbon source (Kuenen and Veldkamp, 1973; Matin, 1978), or can even ferment endogenous organic storage compounds such as glycogen (Beudeker et al., 1981; Kuenen and Beudeker, 1982), but these are both secondary metabolic activities, the organisms being primarily dependent on a lithotrophic energy source and carbon dioxide for autotrophic growth. Many *Thiobacillus* species, at least one species each from *Sulfolobus* and *Hydrogenobacter*, and all of the known species of *Thiomicrospira* fall into this group.

Facultative Chemolithotrophs. These bacteria can grow either chemolithoautotrophically with an inorganic energy source and carbon dioxide, or heterotrophically with complex organic compounds providing both carbon and energy, or mixotrophically. Mixotrophy is the simultaneous use of two or more different metabolic pathways for energy and carbon (Gottschal and Kuenen, 1980). In the laboratory, mixotrophic growth is most easily observed

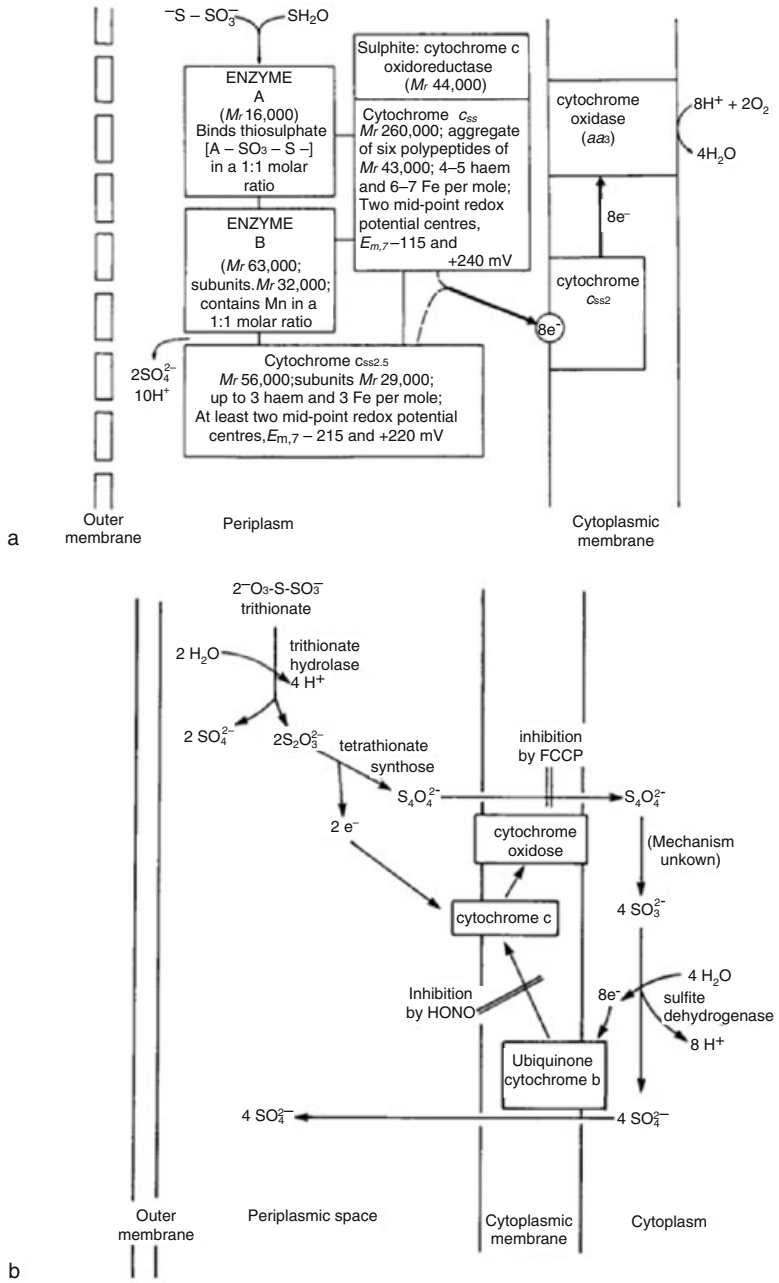

Fig. 2. Pathways of oxidation of reduced sulfur compounds in two different organisms. (a) The periplasmic thiosulfate-oxidizing system of *Thiobacillus versutus* as proposed by Kelly (1988a). The enzyme complex does not produce or metabolize polythionates such as tetrathionate. Thiosulfate is oxidized to sulfate without the formation of sulfur or other intermediates. Thiosulfate metabolism is initiated by its binding to enzyme A. In subsequent steps, sulfate is produced and released, while electrons are finally transferred to an aa_3-type of cytochrome oxidase. (b) The periplasmic and cytoplasmic metabolism of trithionate, thiosulfate, and tetrathionate by *Thiobacillus tepidarius* as proposed by Kelly (1988b). In contrast to the system shown in part a, tetrathionate appears to be an intermediate in the oxidation of both thiosulfate and trithionate. After an initial hydrolysis of trithionate, yielding thiosulfate and sulfate, the thiosulfate is oxidized to tetrathionate. Available evidence indicates a periplasmic location of these systems. Tetrathionate is believed to be transported into the cell and then oxidized to sulfite in the cytoplasm by an unknown mechanism. Sulfite dehydrogenase is responsible for the final oxidation to sulfate, in which cytochrome *b* may be involved. FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; HQNO, 2-heptyl-4-quinolinol-1-oxide.

during continuous culture on limiting mixtures of substrates. The term mixotrophy usually designates simultaneous growth on a mixture of autotrophic and heterotrophic substrates (e.g., on thiosulfate and acetate). However, the simultaneous use of any mixture of substrates requiring (partially) separate metabolic pathways or enzymes, and thus might produce diauxie or biphasic growth in batch culture (e.g., glucose and lactose, succinate and glucose, iron and sulfur, hydrogen and sulfide, acetate and lactate), could be considered as mixotrophy.

Table 3. Classification of the different physiological types of colorless sulfur bacteria.[a]

	Carbon source		Energy source	
Physiological type	Inorganic	Organic	Inorganic	Organic
Obligate chemolithotroph	+[b]	–	+	–
Facultative chemolithotroph (mixotroph)	+	+	+	+
Chemolithoheterotroph	–	+	+	+
Chemoorganoheterotroph (heterotroph)	–	+	–	+

Commonly used synonyms for chemolithotroph include chemolithoautotroph, autotroph, chemoautotroph, and lithotroph.
+, used by the group; –, not used.

Some of the thiobacilli, *Thiosphaera pantotropha*, *Paracoccus denitrificans* (Friedrich and Mitrenga, 1981), and certain *Beggiatoa* species (Nelson and Jannasch, 1983) are typical examples of organisms able to grow on mixtures of reduced sulfur compounds and organic substrates. To some extent, the phototrophic sulfur-oxidizing bacteria might also be considered members of this group since most, if not all, of them are able to grow chemolithoautotrophically and mixotrophically on reduced sulfur compounds in the dark (Kuenen et al., 1985).

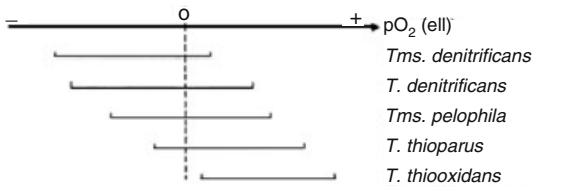

Fig. 3. A "spectrum" showing the response of five different species of colorless sulfur bacteria to redox. The position of each line indicates the range of conditions of redox under which the organism can grow. *T.*, *Thiobacillus*; *Tms.*, *Thiomicrospira*. (Based on Timmer ten Hoor, 1977.)

Chemolithoheterotrophs. This little-known group of bacteria is characterized by an ability to generate energy from the oxidation of reduced sulfur compounds, but which cannot fix carbon dioxide. Until recently, *Thiobacillus perometabolis* was considered to be a member of this group, but it is now known that under certain conditions, it can grow autotrophically (Katayama-Fujimura et al., 1984). However, unnamed chemolithoheterotrophic species have been isolated, and a few strains of *Thiobacillus* have been well characterized (Tuttle et al., 1974; Gommers and Kuenen, 1988). Some *Beggiatoa* strains may also belong in this group (Larkin and Strohl, 1983). As is clear from the example of *T. perometabolis*, careful testing under a variety of conditions is necessary in order to discriminate chemolithoheterotrophs from the facultative autotrophs as well as from the sulfur-oxidizing heterotrophs.

Sulfur-Oxidizing Chemoorganoheterotrophs. Some heterotrophic bacteria can oxidize reduced sulfur compounds, but do not appear to derive energy from them. However, they may benefit from the reaction by the detoxification of metabolically produced hydrogen peroxide (e.g., some species of *Beggiatoa*, *Macromonas*, *Thiobacterium*, and *Thiothrix*) (Larkin and Strohl, 1983; Dubinina and Grabovich, 1984). The oxidation of thiosulfate to tetrathionate by many heterotrophic bacteria that do not seem to gain energy from the reaction is well documented (Tuttle and Jannasch, 1972; Tuttle et al., 1974; Mason and Kelly, 1988).

Electron Acceptors for Aerobic and Anaerobic Growth

Oxygen is universally used among the colorless sulfur bacteria, although the degree of aerobiosis that can be tolerated by different species varies. The response of some of the colorless sulfur bacteria to redox can be demonstrated by means of a "spectrum" as shown in Fig. 3.

Various colorless sulfur bacteria have different ways of growing or surviving anaerobically. One of the best studied is the use of nitrate or nitrite as a terminal electron acceptor, whereby the nitrogen oxides are reduced to nitrogen, a process termed denitrification. This will be discussed in detail in Chapter 23, but a brief consideration of the nitrate-reducing colorless sulfur bacteria is appropriate here.

The denitrifying species tend to be neutrophilic (Table 4), but not necessarily mesophilic,

Table 4. Examples of the neutrophilic, mesophilic species capable of autotrophic growth on reduced sulfur compounds.

	Autotrophy		Denitrification	
Species	Obligate	Facultative	To NO_2–	To N_2
Thiobacillus thioparus	+[a]	–	+	–
T. neapolitanus	+	–	–	–
T. denitrificans	+	–	+	+
T. novellus	–	+	–	–
T. versutus	–	+	+	+
T. intermedius	–	+	–	–
T. perometabolis	–	+	–	–
T. delicatus	–	+	+	–
T. thyasiris	–	+	+	+
Thiomicrospira pelophila	+	–	–	–
Tms. denitrificans	+	–	+	+
Tms. crunogena	+	–	–	–
Thiosphaera pantotropha	–	+	+	+
Beggiatoa sp. (marine)	–	+	–	–
Beggiatoa sp. (freshwater)	–	+	+	+

+, property present; –, property absent.

since at least one of the thermophiles (*Thermothrix thiopara*) can denitrify. A few (e.g., *Thiobacillus thioparus*) can only reduce nitrate to nitrite and require the presence of a nitrite-reducing bacterium for anaerobic growth (Table 4). Strictly speaking, of course, the latter reaction is not truly denitrification, but since the reaction still serves for electron acceptance and survival under anaerobic conditions, these species are appropriately included here.

There are two known obligately chemolithotrophic sulfur bacteria that carry out complete denitrification to nitrogen. *Thiobacillus denitrificans* is relatively versatile in being able to grow under fully aerobic conditions with oxygen, and under fully anaerobic conditions with nitrate or nitrite (Aminuddin and Nicholas, 1973; Ishaque and Aleem, 1973). *Thiomicrospira denitrificans* is more fastidious. It grows well anaerobically with nitrate or nitrite, but can only use oxygen for growth if its concentration is kept extremely low (i.e., below the detection level of normal oxygen electrodes) (Timmer ten Hoor, 1975). These obligate autotrophs are far more efficient at anaerobic (denitrifying) growth on reduced sulfur compounds than the facultative species. Of the latter, only *Thiosphaera pantotropha* has been, thus far, found to retain its sulfur-oxidizing potential under denitrifying conditions, but its μ^{max} while doing so is extremely low (approx. 0.015 h^{-1}) compared with those of *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* (0.06 h^{-1}). Other facultatively autotrophic bacteria lose their sulfur-oxidizing capacity in anaerobic cultures, but are still able to denitrify using organic compounds, or even hydrogen. Among these are *Thiobacillus versutus* and *Paracoccus denitrificans* (Taylor and Hoare, 1969; Friedrich and Mitrenga, 1981). Sulfide-dependent reduction of nitrate to N_2 by *Beggiatoa* tufts has recently been shown using ^{15}N-labelled nitrate (Sweerts et al., 1990).

Of the two sulfur-oxidizing genera of archaebacteria, *Sulfolobus* species appear to be the more dependent on oxygen, although some have been shown to use ferric iron and molybdate as electron acceptors under microaerobic conditions (Brock and Gustafson, 1976; reference is not an exact matchBrierly, 1982). Members of the genus *Acidianus* are able to grow under anaerobic conditions, by using hydrogen as the electron donor and sulfur as the acceptor, thus making these bacteria both sulfur-oxidizing and sulfur-reducing, depending on the conditions (Segerer and Stetter, 1989). Nelson and Castenholz (1981) have reported that some *Beggiatoa* species carry out an anaerobic reduction of intracellularly stored sulfur, using organic compounds such as acetate as electron donors. The ability of these organisms to oxidize sulfide to sulfur under aerobic conditions and then to reverse this reaction anaerobically would permit them to optimally profit from their habitat, where aerobic and anaerobic conditions frequently alternate. They may also actively migrate between aerobic and anaerobic zones.

Even apparently obligately aerobic strains may have mechanisms allowing them to survive during anaerobiosis for a limited length of time. Thus, *Thiobacillus neapolitanus*, a species normally considered to be obligately respiratory, has been shown to be able to ferment internal reserves of polyglucose when confronted with anoxic conditions (Beudeker et al., 1981). As mentioned in the introduction, *T. ferrooxidans* can use ferric iron as an electron acceptor, although it is not yet clear whether this is linked to energy generation.

Ecophysiology as a Function of pH, Temperature and Nutrient Availability

Colorless sulfur bacteria have been found growing at pH 9.0 and pH 1.0, at 4°C and 95°C, and at dissolved oxygen concentrations ranging from air-saturation to anaerobic levels (Table 1). It is obvious that a combination of physical, chemical, and (eco)physiological factors will suit the ecological niche of the organism within a particular microbial community. A number of these will be considered here.

pH Range and Effects. The pH ranges of some of the colorless sulfur bacteria are surveyed in Table 1, and examples of neutrophilic and acidophilic species are listed in Tables 4 and 5. Within these ranges, of course, species often have different pH optima. The outcome of competition for a substrate at different pH values will therefore be dictated to a large extent by the pH optima of the competing bacteria. Thus, Kuenen et al. (1977) found that at pH values above 7.5, *Thiomicrospira pelophila* dominated

Table 5. Characteristics of acidophilic, mesophilic species capable of growth on reduced sulfur compounds and/or iron.

	Autotrophy		Utilization of	
Species	Obligate	Facultative	Sulfur	Iron
Thiobacillus ferrooxidans	+	–	+	+
T. thiooxidans	+	–	+	–
T. albertis	+	–	+	–
T. acidophilus	–	+	+	–
Leptospirillum ferrooxidans	+	–	–[a]	+

[a] Also negative on other sulfur compounds, can use the iron in pyrite.

thiosulfate-limited chemostat cultures, whereas when the pH was below 6.5, *Thiobacillus thioparus* was able to outcompete the other for thiosulfate. At intermediate pH values, the outcome of the experiments was not reproducible, with varying levels of the two populations. Apparently, the substrate affinities of the two species were so similar that other, less well-controlled variables (e.g., iron concentration, minor amounts of wall growth, etc.) became important for the outcome of the competition. Similar pH effects have been observed in the competition between *T. versutus* and *T. neapolitanus* (Smith and Kelly, 1979).

The colorless sulfur bacteria that grow at neutral to slightly alkaline pH values are found in marine and freshwater sediments, soils, and wastewater treatment systems, to name but a few sources. As can be seen from Table 4, representatives of almost all of the genera fall within this group. Many of them have specialized in growth in the gradients where (anaerobic) sulfide-containing zones come into contact with air or oxygen-containing water and will be discussed in the section on gradients. Some colorless sulfur bacteria are extreme acidophiles, able to grow at pH values as low as 1. As Table 5 shows, the group includes mesophilic obligate and facultative autotrophs (e.g., *T. ferrooxidans* and *T. acidophilus*, respectively). The acidophilic colorless sulfur bacteria are abundant in locations such as acid mine-drainage water, and it is therefore interesting that many of them are also able to oxidize (and gain energy from the oxidation of) metals such as iron. Thus, *T. ferrooxidans* is able to grow "mixotrophically" on the iron and sulfur components of pyrite (Arkestein, 1980) or on mixtures of ferrous iron and tetrathionate, gaining energy from the iron and sulfur oxidizing reactions (Hazeu et al., 1986, 1988). There have been a few reports of facultatively heterotrophic growth by *T. ferrooxidans* (e.g., Shafia and Wilkinson, 1969; Lundgren et al., 1964). However, it has since been shown that most of the *T. ferrooxidans* cultures available from culture collections were contaminated with acidophilic facultative autotrophs and heterotrophs (Harrison, 1984), including *T. acidophilus* and *Acidiphilium cryptum*, and it is now generally accepted that *T. ferrooxidans* is an obligate autotroph.

It has frequently been assumed that *T. ferrooxidans* is one of the key species active in pyrite oxidation. In order to assess its likely significance for pyrite oxidation during coal desulfurization, Muyzer et al. (1987) used antibodies raised against *T. ferrooxidans* for an immunofluorescent assay of slurries made from coal from different sources. Unsterilized and sterilized coal samples were inoculated with *T. ferrooxidans*, with a mixed culture of pyrite-oxidizing bacteria from a coal-washing installation, and a mixture of the two. Despite the fact that a DNA-fluorescent stain indicated abundant microbial life in all of the slurries, the only sample in which a significant *T. ferrooxidans* population was detected was the control, which had been sterilized and then inoculated with the pure culture of *T. ferrooxidans*. It appears that in all other cases, other strains (which might include such species as *T. thiooxidans*, *Leptospirillum ferrooxidans*, or *Acidiphilium cryptum*, to name but a few) were able to successfully out-compete *T. ferrooxidans* for a niche in the consortium.

Temperature As pointed out at the beginning of this section, colorless sulfur bacteria can be found growing at temperatures ranging from 4–95°C. However, the majority of the well-studied species are mesophilic. Although it is evident that the majority of natural environments are suitable for the growth of mesophiles, the diversity of the thermophilic organisms is likely to be much larger than suggested by Table 6, particularly in view of the recent discoveries of new thermophilic species among the colorless sulfur bacteria and other metabolic groups. Thus, it is clear that the species discussed in this section should be regarded as indicative rather than definitive. As most of the examples discussed elsewhere in this chapter will be taken from mesophilic bacteria, most of this section will be dedicated to consideration of the thermophiles.

Thermophilic bacteria are generally associated with waters that have been geothermally heated. These range from warm springs, used for bathing since Roman times, through solfataras to submarine hydrothermal vents (e.g., Caldwell et al., 1976; le Roux et al., 1977; Jannasch, 1985). As can be seen from Table 6, the bacteria in this group can be subdivided into two groups, the moderate thermophiles (generally eubacteria),

Table 6. Characteristics of moderately and extremely thermophilic species capable of growth on reduced sulfur compounds.

Species	Autotrophy		Temperature range (°C)
	Obligate	Facultative	
Thiobacillus tepidarius	+	–	20–52
T. aquaesulis	–	+	30–55
Thermothrix thiopara	–	+	72
Sulfolobus acidocaldarius	–	+	60–85
Sulfolobus sp. HVS	+	–	60–95
Acidianus infernus	+	–	60–95
A. brierleyi	–	+	60–95

which grow over the range 45–55°C, and the extreme thermophiles (generally archaebacteria), some of which can grow at temperatures approaching 100°C.

Neutrophilic species make up the moderately thermophilic group. One neutrophile, *Thermothrix (Tx.) thiopara* has a higher optimum growth temperature (72°C). This facultative autotroph was found in neutral (pH 7.0), hot (74°C) springs (Caldwell et al., 1976; Brannan and Caldwell, 1980), where it forms macroscopic streamers as well as microscopic mats on the tufa. The streamers occur at the sulfide: oxygen interface (Caldwell et al., 1983), and the key role that oxygen plays in their development was demonstrated by means of a very simple experiment during which the surface of the hot spring was covered by a sheet of plastic to restrict entry of oxygen from the air. As a result of this, the dissolved oxygen dropped to 0.1 mg l^{-1} from 3 mg l^{-1}, but other parameters such as pH and temperature were unaffected. The *Tx. thiopara* streamers then disappeared from their accustomed positions and reappeared at the edges of the sheet, where the sulfide:oxygen gradient had been reestablished.

The acidophilic archaebacteria of the genera *Sulfolobus* and *Acidianus* represent the colorless sulfur bacteria among the hyperthermophiles. These genera include both obligately and facultatively autotrophic species. They are frequently found in association with sulfidic ores such as pyrite, chalcopyrite, and sphalerite. It has been suggested that the failure to find *Sulfolobus* species around hydrothermal vents, where *Acidianus* does occur, is due to the low salt tolerance of *Sulfolobus* species. *Acidianus* species can tolerate NaCl concentrations of up to 4% (Stetter, 1988). Of course, with growth temperatures between 60–95°C, these strains seem almost "moderate" in comparison to the growth temperatures of the sulfur-reducing *Pyrobaculum* and *Pyrodictium* species (74–110°C).

Nutrient Availability and Ecological Niches. Of the physiological types shown in Table 3, the obligate and facultative chemolithotrophs are the best known, having been the most extensively studied in pure and mixed cultures (e.g., Kelly and Kuenen, 1984; Kuenen, 1989; Kelly and Harrison, 1989; Kuenen et al., 1985; Kuenen and Robertson, 1989a, 1989b). One of the most important environmental parameters affecting the selection of these bacteria in freshwater environments was found by Gottschal and Kuenen (1980) to be the relative turnover rates of inorganic and organic components in the available substrates (Fig. 4). Thus, if the available substrate in energy-limited systems is wholly or predominantly inorganic, obligate autotrophs such as *Thiobacillus neapolitanus* will normally tend to dominate a community. Similarly, abundant organic substrates will generate communities dominated by heterotrophs. On mixed substrates, facultative autotrophs such as *T. versutus* or chemolithoheterotrophs will appear, depending on the ratio between the two types of substrate. If the substrate supply is predominantly organic, the sulfide-oxidizing heterotrophs or other heterotrophs will appear. This model was put to the test by means of a number of competition experiments in two- and three-membered mixed cultures of representatives from the physiological groups. In addition, a number of enrichment cultures inoculated from natural samples containing representatives of all of the physiological types were obtained. All of the experiments essentially showed that the predicted metabolic type became dominant (for example, see Fig. 5a and b). Although mathematical modelling predicted that in some cases pure cultures of only one metabolic type should be obtained, in practice, satellite populations of the others remained (Fig. 6). Clearly, secondary environmental or experimental conditions (e.g., excretion products such as glycollate, fluctuations in substrate or oxygen concentrations, and growth on the wall of the vessel) can result in deviations from the idealized model. It is obvious that a well-mixed chemostat is a model system that is rather remote from the common natural habitats of colorless sulfur bacteria, such as the sulfide:oxygen gradient in a sediment, and the results obtained can only demonstrate the principle. Moreover, the relative turnover rate of the organic and inorganic substrates is only one of the environmental parameters that determines the success of a particular species. Nevertheless, the use of this model (Fig. 4) has now clarified the situation, a practical consequence

$$H_2S + 2O_2 \rightarrow H_2SO_4$$
$$2H_2S + O_2 \rightarrow 2S + 2H_2O$$
$$2S + 3O_2 + 2H_2O \rightarrow 2H_2SO_4$$
$$Na_2S_2O_3 + 2O_2 + H_2O \rightarrow Na_2SO_4 + H_2SO_4$$
$$4Na_2S_2O_3 + O_2 + 2H_2O \rightarrow 2Na_2S_4O_6 + 4NaOH$$
$$2Na_2S_4O_6 + 7O_2 + 6H_2O \rightarrow 2Na_2SO_4 + 6H_2SO_4$$
$$2KSCN + 4O_2 + 4H_2O \rightarrow (NH_4)_2SO_4 + K_2SO_4 + 2CO_2$$
$$5H_2S + 8KNO_3 \rightarrow 4K_2SO_4 + H_2SO_4 + 4N_2 + 4H_2O$$
$$5S + 6KNO_3 + 2H_2O \rightarrow 3K_2SO_4 + 2H_2SO_4 + 3N_2$$

Fig. 4. A model to describe the selection of different physiological types by the ratio of inorganic to organic substrates supplied in the medium. This model may also hold for complex (semi-natural) systems, where the relative turnover rates of the inorganic and organic compounds (or the ratio between the fluxes of these compounds) would determine the selection of different physiological types. For definitions of the various terms, see Table 3.

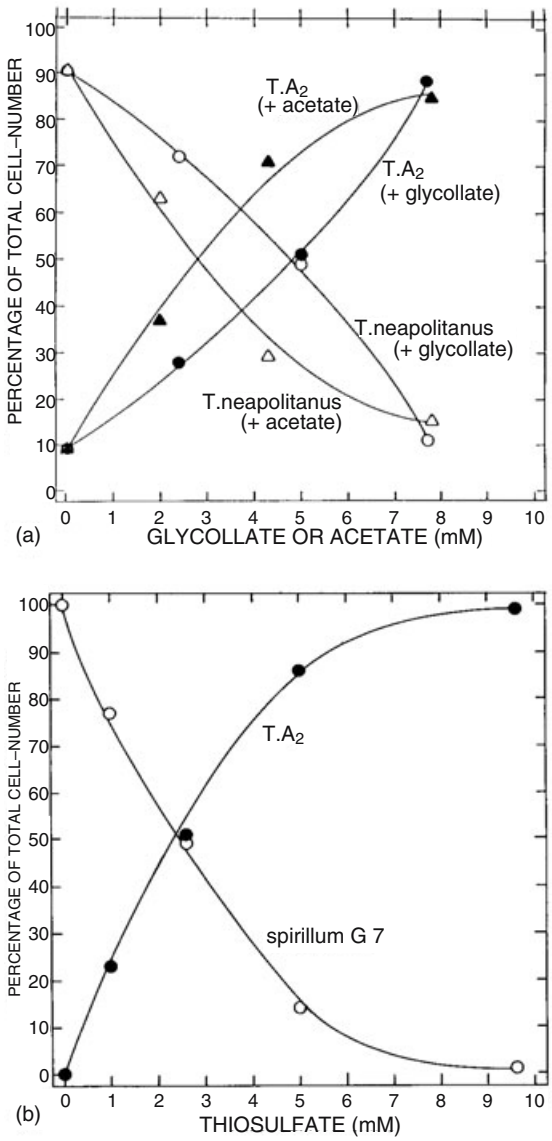

Fig. 5. The effect of organic or inorganic energy sources on competition. (a) The effect of different concentrations of organic substrates on the competition between *Thiobacillus versutus* (T. A_2) and *T. neapolitanus* for growth-limiting thiosulfate in a continuous culture. The influent medium contained 40mM thiosulfate. During growth limitation by thiosulfate, it and the organic additives (where present) were used simultaneously by the mixed culture, and their actual concentrations in the chemostat were below the detection level. The graph shows the ratios of the two species at steady state. Open symbols, *T. versutus*; closed symbols, *T. neapolitanus*; circles, glycollate supplied; triangles, acetate supplied. (b) The effect of thiosulfate on the competition for acetate (10 mM) between *T. versutus* (T. A_2) and a heterotrophic spirillum called G7. For experimental details, see (a). Open symbols, *Spirillum* G7; closed symbols, *T. versutus*. (Based on Gottschal et al., 1979.)

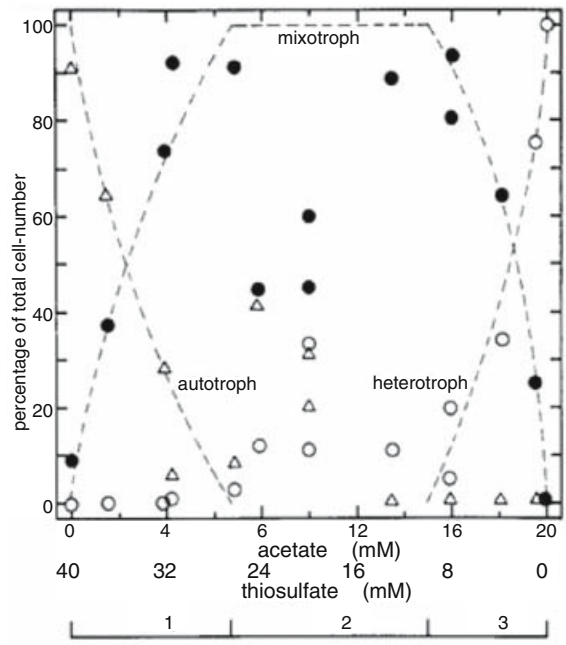

Fig. 6. Competition for acetate and thiosulfate in a chemostat between an autotroph, *T. neapolitanus* (open triangles); a mixotroph, *T. versutus* (closed circles); and a heterotroph, *Spirillum* G7 (open circles). The dotted lines indicate the results predicted from the model, the symbols indicate the actual results. The model held well for the extreme ratios of thiosulfate and acetate. However, although *T. versutus* dominated at intermediate ratios, as predicted, the other two types did not completely disappear. For the experimental details, see Fig. 5a. This model can be used for the selective enrichment of facultative autotrophs in chemostat cultures using an intermediate ratio of acetate and thiosulfate. (Based on Gottschal et al., 1982.)

being that it has shown the way for the selective enrichment of facultatively autotrophic sulfur bacteria from fresh water.

Steady-state conditions are more common in artificial environments than in nature, and therefore in order to test the effect of substrate fluctuations on the selection of the three representative species used in the experiments discussed above (Figs. 5a, 5b, and 6), Gottschal et al. (1981) ran chemostat cultures alternating feeds of acetate and thiosulfate. In two-membered cultures, the mixotrophic *T. versutus* was able to maintain itself on the substrate not used by whichever obligate species was involved, so that both species were subject to alternating periods of growth and starvation. However, in three-membered cultures, the two specialists were able to react more swiftly to the onset of substrate provision because of their constitutive enzymes, while the facultative species, which had to reinduce its autotrophic enzymes each time, disappeared. As with the steady-state experiments, when different mixtures of acetate and thiosulfate alternated, the outcome was deter-

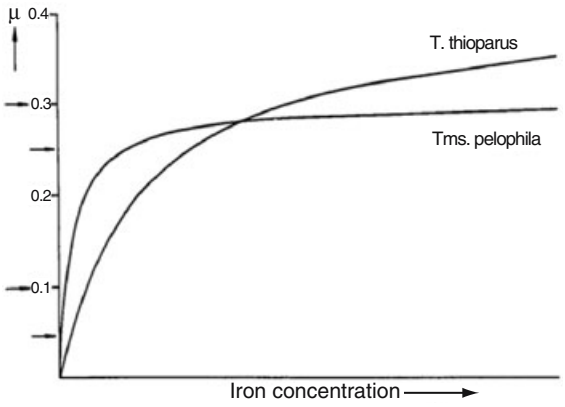

Fig. 7. The specific growth rates (μ) of *Thiomicrospira pelophila* and *Thiobacillus thioparus* as a function of the iron concentration in chemostat cultures at 25°C. The graph was constructed from the results of competition experiments (at the growth rates indicated by the arrows at the y axis). The actual iron concentrations were not determined. (From Kuenen et al., 1977.)

mined by the concentrations involved. Enrichment cultures under this regime yielded a facultative autotroph that was able to avoid the need to induce its carbon dioxide fixation system by accumulating large amounts of PHB during the heterotrophic period.

This work was carried out on aerobic, freshwater chemostat cultures and, as has been discussed in previous reviews (Kelly and Kuenen, 1984; Kuenen, 1989; Kelly and Harrison, 1989; Kuenen et al., 1985), marine enrichments are, for unknown reasons, generally less predictable. For example, mixotrophs did not form the dominant population in thiosulfate/acetate-limited marine cultures (Kuenen et al., 1985). That marine mixotrophs do exist has been shown by the isolation of a facultatively chemolithotrophic marine strain of *T. intermedius* from a thiosulfate-limited culture (Smith and Finazzo, 1981).

Of course, factors other than the availability of electron donors can determine the type of population to be found in any given environment. For example, Kuenen et al. (1977) studied the effect of iron limitation and pH on the outcome of competition between two marine obligate autotrophs, *Thiomicrospira* (*Tms.*) *pelophila* and *Thiobacillus* (*T.*) *thioparus*. As can be seen from Fig. 7, *Tms. pelophila* will dominate mixed cultures of the two species at low iron concentrations, whereas *T. thioparus* will do better when iron is more abundant. One of the characteristics of *Tms. pelophila* is its tolerance of sulfide concentrations high enough to inhibit *Thiobacillus* spp. It has been postulated that sulfide inhibition is caused by the reaction of the sulfide with available iron, forming insoluble ferrous sulfide and thus drastically reducing the concentration of iron available for microbial utilization. If this hypothesis is accurate, the ability of *Tms. pelophila* to grow well at very low iron concentrations would explain its sulfide tolerance.

Taxonomy

Many of the colorless sulfur bacteria were discovered in the early years of microbiology, at a time when scientists were relying mainly on morphological characteristics to identify their organisms, and this fact is still reflected in our approach to their taxonomy. Needless to say, this has caused a certain amount of confusion (see Table 1 for an overview of the genera involved). The problems associated with the identification of some colorless sulfur bacteria have been aggravated because many of the bacteria involved are very specialized (e.g., obligate autotrophs) and, as a consequence, the number of physiological traits that can be screened is limited. This has resulted in relatively trivial features being given undue weight during classification. Taxonomy is a way of establishing identities and relationships in an attempt to create a sense of order among the various forms of life on earth. In ecology, as in other applications of taxonomy, the precise identification of a particular species may not always be as relevant as an accurate description of its physiological characteristics, but the comparison and correlation of data from different sources becomes easier if one can be certain, or even reasonably sure, of the identities of the various bacteria involved. Changes in taxonomic practice largely reflect new developments in available technology as well as improvements in our understanding of which factors indicate relationships, and which are merely resemblances. Taxonomic research into the colorless sulfur bacteria can thus be separated into three distinct, if overlapping phases, which will be discussed sequentially here.

Morphology

The colorless sulfur bacteria, as a group, encompasses rods, spirals, cocci, filamentous cells and archaebacteria, and it comes as no surprise to find that the first of them to be described, *Beggiatoa* (Trevisan, 1842), is also one of the largest. The longest cells reported in the latest edition of *Bergey's Manual* are 50 μm long (Strohl, 1989), but a recent paper described the observation of a marine strain more than 100 μm long (Nelson et al., 1989). Another morphologically distinct genus, *Thiothrix,* was described by Winogradsky in 1888, but it was not until 1904 that Beijerinck described the first of the smaller colorless sulfur bacteria, *Thiobacillus thioparus*.

As may be seen from a survey of the relevant chapters in *Bergey's Manual* a few genera are still, today, based largely on morphological descriptions (e.g., *Thiospira*, *Macromonas*, *Thiovulum*) because pure cultures are either not available, or have only recently been achieved.

In addition to cell size and shape, other morphological details that have been considered important are the appearance of inclusion bodies such as sulfur or poly β-hydroxybutyrate (PHB), number and placement of flagella, colony size, colony form and colony color. One of the dangers associated with too strong a reliance on such features is that all of them can vary depending on the growth conditions. As a single example of this problem, the faculatively autotrophic *Thiosphaera pantotropha* might be considered. When grown autotrophically on thiosulfate, it occurs as small cocci (0.7×0.9 μm), which are generally found singly or in pairs (Fig. 8a). Cultivation in batch culture on rich media in which rapid growth will occur leads to a slightly larger, pleomorphic form (Fig. 8b). In chemostat cultures on mineral medium with acetate, chains of cocci appear. The internal structure of *Thiosphaera pantotropha* also changes with its growth conditions. Thus the normal appearance, with few inclusions, of a Gram-negative organism, which is found during substrate-limited chemostat culture (Fig. 8c), gives way to cells with PHB granules and complex membranous structures (Fig. 8d) when grown under oxygen or nitrogen-limited conditions, or in the presence of hydroxylamine. Cultivation on acetone or propan-2-ol results in the formation of large, crystalline structures (Fig. 8e), while denitrifying growth on sulfide can result in the accumulation of a fine deposit of sulfur in the periplasm (Fig. 8f). The colonial form of this species also varies, with off-white, translucent colonies being produced during growth on mineral medium with acetate or thiosulfate; and larger, thicker, browner colonies being generated during growth on rich media.

Even the obligate autotrophs, which with their more limited range of growth conditions might appear to have less scope for variation, can produce substantial morphological changes. Thus, the number of carboxysomes formed by *Thiobacillus neapolitanus* increases dramatically under CO_2 limitation (Beudeker et al., 1980), and polyglucose inclusions appear under nitrogen limitation (Beudeker et al., 1981).

From all of this, it is clear that while valuable information can be gained from morphological studies on cells or colonies grown under well-defined conditions, this information should be used cautiously and in conjunction with other data. In exceptional circumstances, very distinctive morphology (e.g., in the case of *Beggiatoa* or *Hyphomicrobium*) might be more reliable as an indicator of identity.

Physiological Screening

As more pure cultures became available, it became possible to determine the physiological capabilities of different bacteria, and physiological criteria gradually became an integral part of the taxonomists' armory. For the obligate autotrophs, these might include such tests as optimum pH, growth temperature, ability to denitrify, and (generally very limited) substrate range. In addition to these, the facultative autotrophs are generally subjected to the same range of tests used for heterotrophic bacteria including oxidase, catalase and urease reactions, and the ability to grow on or generate acid from a range of substrates. An extensive study of the *Thiobacillus* species then available resulted in a numerical taxonomy analysis of the genus (Hutchinson et al., 1969) that recognized that "species" such as *Ferrobacillus ferrooxidans* and *Thiobacillus thiocyanoxidans* were actually strains of existing species (*T. ferrooxidans* and *T. thioparus*, respectively). The tests recommended by Hutchinson et al. (1969) for the identification of new *Thiobacillus* species included growth on sulfide, sulfur, thiocyanate, citrate and nutrient broth, the amount of thiosulfate used, sulfur deposition, and the effect of inhibitors such as streptomycin, bacitracin and ampicillin.

In many respects, the range of substrates on which an isolate is tested is defined by the interests of the research group. The reduced sulfur compounds are not included in standard test batteries, and the sulfur-oxidizing abilities of many bacteria are only now being discovered. For example, Friedrich and Mitrenga (1981) tested a number of hydrogen-oxidizing bacteria and found that many of them, including *Paracoccus denitrificans* and some *Alcaligenes* species, were able to grow autotrophically on thiosulfate. Attempts to use thiosulfate as an inhibitor of heterotrophic nitrification by a "*Pseudomonas*" species gave anomalous results until it was realized that the culture was growing mixotrophically, using both the acetate supplied as the primary growth substrate and the thiosulfate added as a possible inhibitor. Subsequent experiments revealed that this "*Pseudomonas*" species was also able to grow autotrophically using reduced sulfur compounds (Robertson et al., 1989).

A problem associated with the use of substrate ranges for taxonomic purposes is that it is difficult to determine how closely related bacteria with the same enzyme system are. Thus, possession of the Calvin cycle enzymes for carbon dioxide fixation or the denitrification pathway enzymes is not considered sufficient grounds for

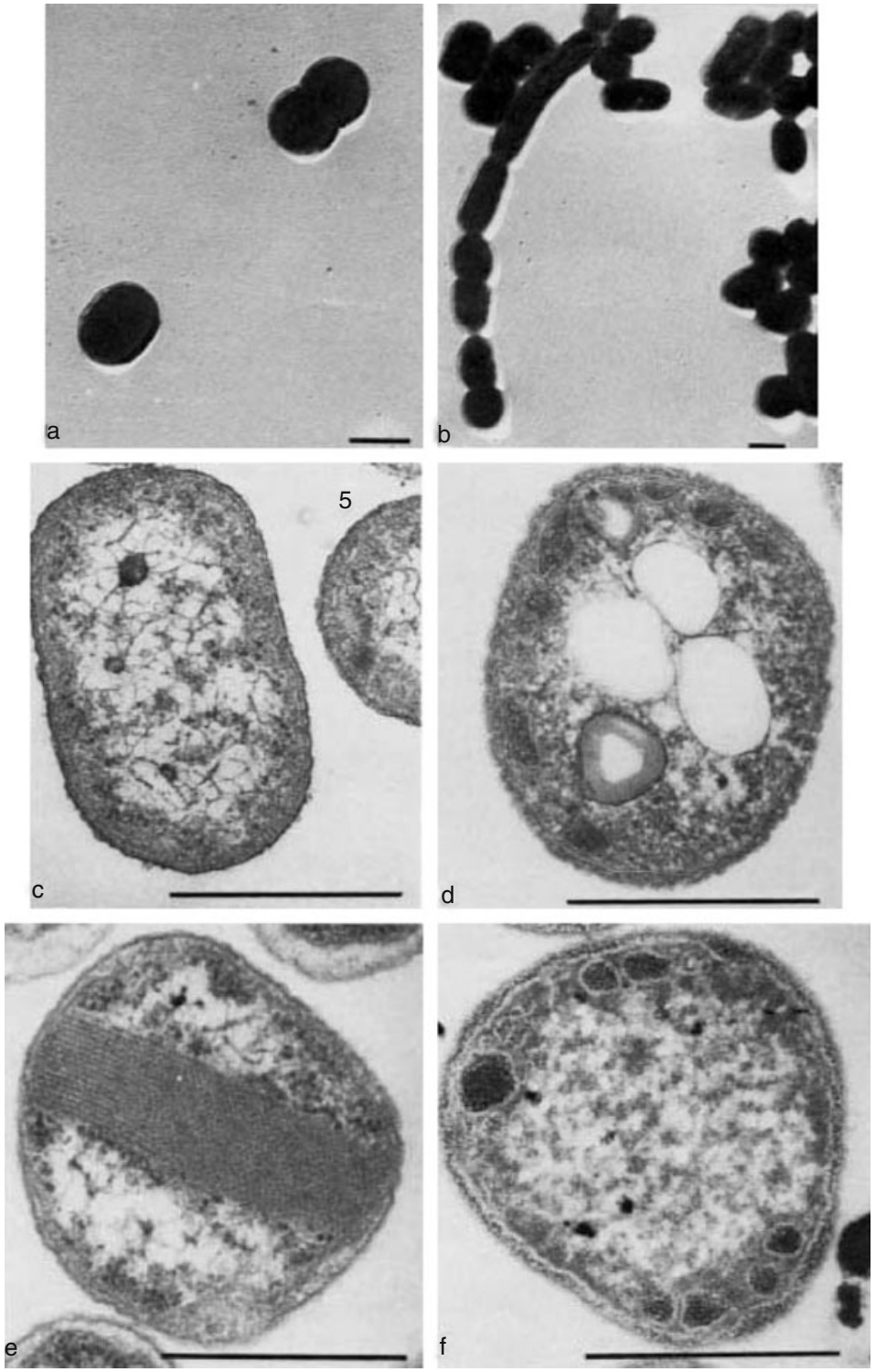

Fig. 8. Variations in the morphology of cells of *Thiosphaera pantotropha* in relation to growth conditions or substrates as seen under the electron microscope. (a) Aerobic, autotrophic growth on thiosulfate, Pt shadowed. (b) Aerobic, heterotrophic growth on a mixture of acetate, fructose, and yeast extract, Pt shadowed. (c) Thin section of cells from an acetate-limited, chemostat-grown culture, stained with ruthenium red to show the membrane structures. (d) Thin section of a cell from an aerobic, acetate-limited chemostat with hydroxylamine, stained with ruthenium red to show the membrane structures. The white bodies are PHB granules. (e) Thin section of an acetone-grown cell showing crystalline inclusions. (f) Thin section of an anaerobic (denitrifying) cell grown on sulfide and stained with silver to show the periplasmic deposits of sulfur. (Fig. 8b from Robertson and Kuenen, 1983b. Fig. 8c from Bonnet-Smits et al., 1988. Fig. 8f, courtesy of H. J. Nanninga. All electron microscopy courtesy of W. Batenberg.) All bars = 0.5 μm.

classifying the relevant bacteria into a single group, and it must be questioned whether the sulfur-oxidizing enzymes are a better indicator, especially since there appears to be several different pathways involved (Kelly, 1988a, 1988b) (see also Fig. 2, above). Certainly, it is recognized that at least one genus, *Thiobacillus*, is very heterogeneous (Kuenen, 1989; Kelly and Harrison, 1989) and will probably require subdivision. It has been suggested that this separation should be made between the obligate and the facultative autotrophs (thus, again on physiological grounds) but, as will be seen in the following section, this is probably not sufficient.

Analytical Techniques

The determination of the GC content of the DNA of bacterial isolates has been used for a long time to determine whether or not strains could be related. It is, to some extent, a negative test because, while widely differing GC values could confirm that two strains were not related, matching GC values do not guarantee that they are the same.

Cellular fatty acid analysis has been used in the taxonomy of the *Thiobacilli* (Agate and Vishniac, 1973; Katayama-Fujimura et al., 1982). Katayama-Fujimura et al. (1982) also included the analysis of ubiquinones and DNA base composition in their study. They initially subdivided the bacteria into groups based on whether they were obligately or facultatively autotrophic, and then on the basis of their possession of menaquinone 8 or 10, (MK-8 or MK-10) and then used the fatty acid analysis to further examine each group. This led to a proposal for the grouping of the different strains, which is shown in Table 7.

Some of the first publications to consider the *Thiobacilli* in relation to other colorless sulfur bacteria involved the phylogenetic analysis of the various species by comparison of their 5S rRNA sequences (Lane et al., 1985; Stahl et al., 1987). This work has now been extended by the use of 16S rRNA analysis (Lane et al., 1990; Oyaizu et al., 1990), and has revealed that there are closer matches between some sulfur-oxidizing bacteria and other apparently unrelated strains such as *Escherichia coli* than between these and other sulfur oxidizers. Table 8 summarizes some of the results from the 5S and 16S rRNA comparisons. The sulfur oxidizing genera *Sulfolobus* and *Acidianus* are archaebacteria and therefore not listed in Table 8.

If the initial separation into obligate and facultative autotrophs employed by Katayama-Fujimura et al. (1982) is removed, it can be seen that the results in Tables 7 and 8 support each other. Thus groups I.1 and I.2 from the menaquinone/fatty acid analysis correspond to group alpha from the 16S rRNA, groups II and III-1 with group beta-1, and groups III-2 and III-3 with beta-2. Of course, the range of bacteria subjected to the menaquinone/fatty analysis was much more limited than that in the 5S and 16S rRNA survey, and more data would be useful. However, such independent agreement must confer additional weight that chemotaxonomy and phylogeny may provide more reliable tools for the classification of these bacteria than physiological or morphological observations.

Habitats

As may be deduced from the range of physiological characteristics discussed above, the colorless sulfur bacteria, in one form or another, are to be found in almost every life-supporting environment where reduced sulfur compounds are found. Because the range of habitats is so wide, the principles underlying the selection of colorless sulfur bacteria in selected situations will be be discussed below. The following section will then deal more generally with the role of the

Table 7. Classification of the *Thiobacillus* species based on analysis of their menaquinone and fatty acid composition.

Autotrophy type	Menaquinone	Hydroxy fatty acid	Species	Group
Facultative	MK-10	None	*T. novellus*	I.1
Facultative	MK-10	3OH 10:0	*T. versutus*	I.1
Facultative	MK-10	3OH 14:0	*T. acidophilus*	I.2
Facultative	MK-8	3OH 10:0	*T. delicatus*	II
Facultative	MK-8	3OH 10:0, 3OH 12:0	*T. perometabolis*	II
Facultative	MK-8	3OH 10:0, 3OH 12:0	*T. intermedius*	II
Obligate	MK-8	3OH 10:0, 3OH 12:0	*T. denitrificans*	III.1
Obligate	MK-8	3OH 10:0, 3OH 12:0	*T. thioparus*	III.1
Obligate	MK-8	3OH 12:0	*T. neapolitanus*	III.2
Obligate	MK-8	3OH 14:0	*T. ferrooxidans*	III.3
Obligate	MK-8	3OH 14:0	*T. thiooxidans*	III.3

MK, menaquinone. The number indicates the number of isoprenoid units. Groupings are as proposed by Katayama-Fujimura et al. (1982).

Table 8. Classification of the colorless sulfur bacteria and examples of apparently related species (group "purple"), also termed *Proteobacteria* (Stackebrandt et al., 1988), as shown by 16S rRNA analysis.[a]

Main group	Subgroup	Species
Alpha	1	*Thiobacillus (T.) acidophilus*, *Acidiphilium rubrum*
	1	*A. cryptum*, *T. novellus*
	2	*Rhodobacter capsulatus*, *T. versutus*
	2	*Paracoccus denitrificans*
Beta	1	*T. denitrificans*, *T. thioparus*
	1	*T. intermedius*, *T. perometabolis*
	1	*Rhodocyclus gelatinosa*
	1	*Vitreoscilla*
	2	*T. tepidarius*, *T. ferrooxidans*
	2	*T. albertis*, *T. thiooxidans*
Borderline		*T. neapolitanus*, *Chromatium vinosum*
Gamma	1	*Thiothrix nivea*, *Riftia symbionts*
	1	*Thiomicrospira pelophila*, *Thiomicrospira* L-12
	1	*Bathymodicius* symbionts
	1	Other symbionts
	1	*Pseudomonas aeruginosa*, *P. putida*
	1	*Beggiatoa alba*, *Beggiatoa* sp.
	2	*Escherichia coli*, *Salmonella*, *Proteus*, *Vibrio*
Delta		*Thiovulum*, *Campylobacter*, *Wollinella*

Atypical strains have been omitted for the sake of simplicity.
Adapted from Lane et al., 1990; and Harrison (1989).

colorless sulfur-oxidizing bacteria in the sulfur cycle, and this discussion of habitats is not intended to be exhaustive.

In natural habitats, the reduced sulfur compounds available tend to be either sulfides (including metallic ores) or sulfur. Thanks to the activities of sulfate-reducing bacteria, especially in anoxic sediments, hydrogen sulfide is very commonly available, and some algal and cyanobacterial mats have been shown to generate organic sulphides (e.g., Andreae and Barnard, 1984). One of the main factors that bacteria growing on hydrogen sulfide have to contend with is the chemical reaction between sulfide and oxygen, and therefore the colorless sulfur bacteria are frequently found in the gradients at the interface between anoxic, sulfide-containing areas and aerobic waters and sediments where, at very low oxygen and sulfide concentrations, they can effectively compete with the spontaneous chemical oxidation reaction. Of course, the rate of chemical oxidation of metal sulfides with oxygen is very low at acid pH levels, so that the acidophilic bacteria need not, therefore, occur predominantly in gradients, as their neutrophilic counterparts must. The same holds for deposits of elemental sulfur, which does not react spontaneously with oxygen at a significant rate. Another habitat in which sulfide-oxidizing bacteria appear to be of some importance is in the complex communities of prokaryotes and eukaryotes around hydrothermal vents, where the sulfide is geologically rather than biologically generated. In the course of research into the life around these vents, it was shown that many invertebrates have symbiotic colorless sulfur bacteria, and this can itself be regarded as a distinct habitat (Cavanaugh et al., 1981). A third example of a type of habitat for these bacteria that is becoming steadily more common is that associated with human activities, largely in connection with waste treatment and industrial leaching of ores for (heavy) metal recovery.

Gradients in Aquatic Systems and Sediments

Sulfide:oxygen gradients occur in stratified water bodies, as well as in soils and sediments. Such gradients can range in size from a few hundred-micrometers-thick in a microbial mat or surface sediment to several meters in a stratified body of water (Sorokin, 1970, 1972; Jørgensen et al., 1979). These gradients can sometimes be distinguished with the naked eye. For example, *Thiovulum* grows as a fine white veil at the interface between sulfide and oxygen (Jørgensen, 1988). Wirsen and Jannasch (1978), studying the effect of the sulfide:oxygen gradient on the formation of these veils in continuous flow cultures, observed that the veils dispersed within minutes of the cessation of the flow of sea water through the culture vessel, and formed again once the flow was resumed, indicating chemotaxis of the swarming form of *Thiovulum* toward critical concentrations of oxygen and sulfide.

The genus *Beggiatoa* contains marine and freshwater species that are typical of life at the aerobic:anaerobic interface. Dense mats of

almost axenic cultures of *Beggiatoa* on sulfide-containing sediments are frequently observed, especially in marine sediments where sulfide production rates can be very high. These mats are characterized by very steep oxygen and sulfide gradients over a few mm (Jørgensen, 1982, 1988). Since *Beggiatoa* oxidizes the sulfide at a very high rate, the overlying aerobic water is effectively "protected" from diffusion of toxic sulfide. The typical conditions for growth in this type of mat have been very difficult to reproduce in the laboratory. Indeed, they are so specialized that it was only recently, when available techniques had improved sufficiently to allow in vitro cultivation on sulfide:oxygen gradients, that the autotrophic potential of marine strains was established unambiguously (Nelson and Jannasch, 1983; Nelson, 1988) (see also Chapter 166). The *Beggiatoa* cells were cultured in closed tubes using a layer of very soft (0.2%) agar over a sulfide-containing plug of harder (1.5%) agar, thus allowing the formation of an upward sulfide gradient. Diffusion from a headspace containing air contributed a downward oxygen gradient. The *Beggiatoa* colony grew as a "plate" that was less than 1 mm thick at the point where the two gradients overlapped. The very rapid oxidation of sulfide allowed the organisms to maintain an extremely low concentration of the two substrates. As a result, chemical oxidation of sulfide was insignificant. For example, the turnover time for sulfide and oxygen was only 3 seconds in *Beggiatoa* gradients, whereas the half life of these two substances in sterile controls was about 20 min. Enzyme analysis and the fixation of $^{14}CO_2$ by these cells confirmed that they were capable of autotrophic growth. The situation regarding freshwater strains is not so clearcut. Schmidt et al. (1987) showed sulfide oxidation rates for a freshwater strain comparable to those obtained with the marine strain discussed above, but further experimentation is necessary in order to establish whether energy for growth can be derived from the reaction.

Another well-known place where gradients occur is within phototrophic mats. Jørgensen and des Marais (1986) studied the zonation around a cyanobacterial mat growing in a hypersaline pond and found that a band of *Beggiatoa* occurred 1.5 mm below the cyanobacteria. The photosynthetic activity of the cyanobacteria generated sufficient oxygen to produce an oxygen peak with a maximum of 1mM at the cyanobacterial band. A steep downward gradient of oxygen overlapped a sulfide gradient at the point where the *Beggiatoa* were growing. In an earlier study, Jørgensen (1982) described the diurnal changes in the sulfide and oxygen gradients and the microbial community to be found in a sulfuretum (a microbial mat in which the total turnover of inorganic and organic compounds is heavily dominated by the sulfur cycle) on the surface of a sediment. It was observed that the mixture of cyanobacteria, phototrophic sulfur bacteria, and *Beggiatoa* was stratified, and that the relative positions of the the three populations among the strata were governed by the level of photosynthetically generated oxygen (Fig. 9). During the night, when the oxygen had been depleted and the oxygen boundary extended to the surface of the sediment, the phototrophic *Chromatium* was found at the surface. However, once photosynthesis began, with the onset of daylight, oxygen began to build up in the sediment, and the *Chromatium* followed the sulfide boundary down, remaining within the anaerobic part of the sediment. The *Beggiatoa* population tended to move with the sulfide:oxygen interface, except during the night when this was in the stagnant water above the surface of the sediment. As *Beggiatoa* is only motile by means of a gliding action, it is restricted to the solid phase.

Other conspicuous colorless sulfur bacteria such as *Thiothrix*, *Thioploca*, and *Archromatium* have all been encountered as typical organisms in such gradients. Furthermore, mixed cultures of *Thiobacillus*-like bacteria sampled from sulfide:oxygen gradients and showing active sulfide-dependent carbon dioxide fixation clearly exhibit chemotaxis toward the interface when transferred to artificial sulfide:oxygen gradients in the laboratory (J. G. Kuenen, unpublished observations).

Hydrothermal Vents

An interesting extension of the model for the selection of freshwater colorless sulfur bacteria discussed above is to be found in the results of research on the mesophilic bacterial communities found around the different hydrothermal vents (Jannasch, 1985, 1988). These vents are a result of the movements of the tectonic plates of the earth's crust. Seawater penetrates deep under the sea floor and is heated geothermally, reaching temperatures as high as 1,200°C. Under these conditions, it reacts with and dissolves various reduced chemicals before being forced to the surface again as hydrothermal fluid, which contains sulfide, CO_2, and methane, as well as various metals and hydrogen. The type of vent that occurs depends very much on the overlying geology, and can be at least partially separated into "bare lava" and "warm" systems. In the bare lava vents, the pressurized hydrothermal fluid reaches the surface of the sea floor at temperatures around 350°C. As it issues from the vents, it reacts with chemicals in the sea water, forming precipitates that often accumulate as "chim-

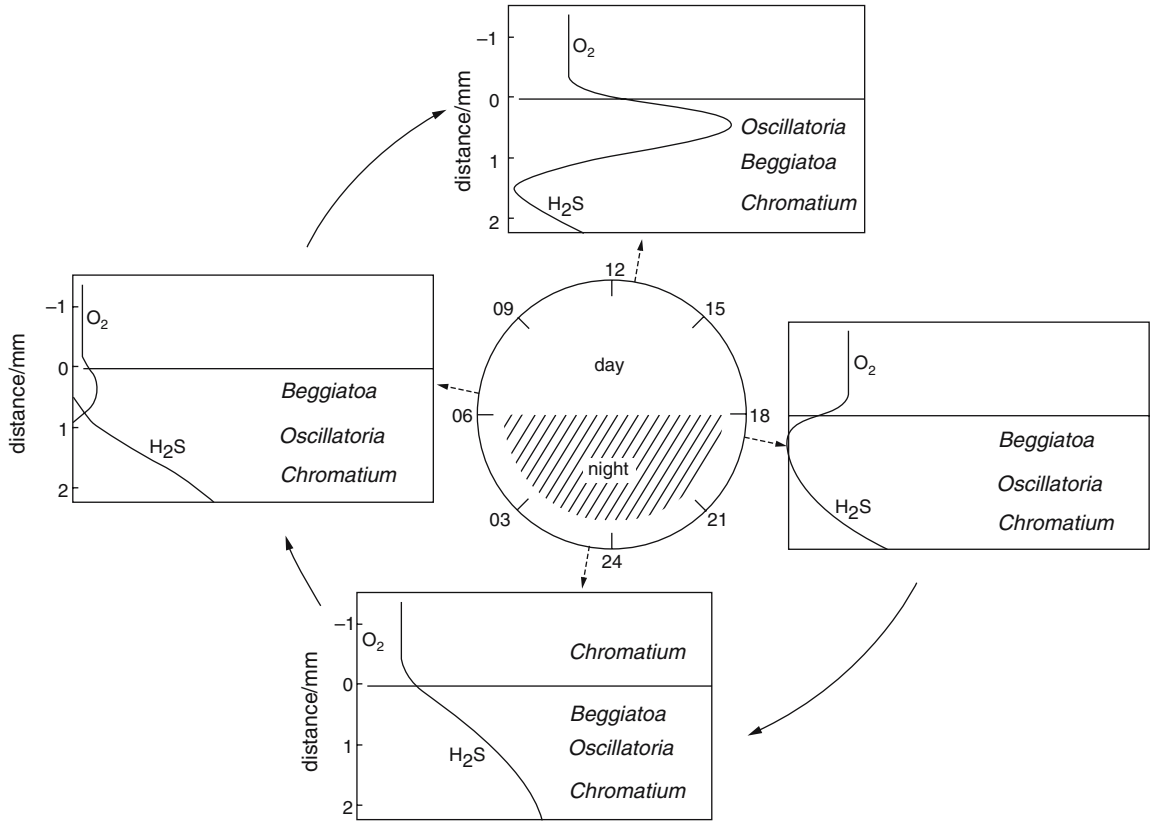

Fig. 9. Diurnal cycle of oxygen and sulfide distribution and of microbial zonation in a marine sulfuretum. The zero line in each box indicates the interface between the sediment and the overlaying water phase. The dominant genera at each stratum are indicated in each box. Diatoms were primarily seen among the *Oscillatoria.* In addition to diurnal changes in light, oxygen, and sulfide, another important factor was that the *Beggiatoa* which are gliding bacteria could not move out of the sediment, whereas *Chromatium,* which is also motile, was able to move into the water phase above. From Jørgensen (1982).

neys." Because the formation of metal sulfides gives the fluid issuing from these chimneys the appearance of smoke, they have become known as "black smokers." The "warm" vents, on the other hand, are the result of the hydrothermal fluid percolating through sediments on its way to the surface, and the solution tends to be much cooler (<25°C) and to have substantial organic content when it reaches the sea water. The waters around these vents support dense communities of bacteria able to grow on the geothermally generated reduced compounds. Thus far, obligately autotrophic sulfur bacteria, especially *Thiomicrospira* species (Ruby et al., 1981; Ruby and Jannasch, 1982), have been found associated with the areas around black smokers where the organic turnover is relatively low, while facultative autotrophs such as *Beggiatoa* appear to dominate around the sedimented vents where the organic turnover is much higher (Jannasch, 1988). Indeed, until recently, it was believed that *Beggiatoa* mats rarely exceeded 1 mm in thickness (Nelson, 1988). However, observations at the Guaymas Basin hydrothermal vents, where the hydrothermal fluid in some areas percolates though 400 m of sediment before reaching the surface, revealed mats of *Beggiatoa* up to 60 cm thick (Nelson et al., 1989). The communities around these vents appeared to be made up of three strains of *Beggiatoa* that had widely differing cell widths (115–122 μm, 40–42 μm, and 24–32 μm). The narrowest of these dominated, almost to the point of a monoculture, in the thickest layers, which were apparently associated with colonies of the vestimentiferan tube worms (see "Symbiosis," below). In addition to sulfide concentration and temperature, the authors suggest that organic excretion compounds from the worms may be an important factor in the development of these mats.

Symbiosis

Geologists studying areas of volcanic activity on the sea bed (1,800–3,700 meters below the surface) were surprised to find that not only were there dense, free-living bacterial populations associated with the vents, but that these perma-

nently dark areas were also occupied by an extensive community of invertebrates (and also, in some areas, fish), most of which were previously unknown (Corliss et al., 1979). Despite the density of the bacterial community, it was difficult to see how a food chain based entirely on suspended bacteria as a source of prey could support the considerable population of very large tube worms, clams and other invertebrates. Investigation of the anatomy of the tube worms (*Riftia pachyptilia*) revealed that they do not have an alimentary tract, but instead possess a large (more than half the weight of the worm) body of tissue, the trophosome, which is very rich in blood vessels. Examination of this tissue under the electron microscope revealed that it also contained a dense, intracellular community of bacteria (Cavanaugh et al., 1981; Cavanaugh, 1983a). The trophosome had already been shown to contain the enzymes necessary for chemoautotrophic growth on reduced sulfur compounds. These enzymes did not occur elsewhere in the tissues of the worm (Felbeck, 1981; Felbeck et al., 1981) and were presumably derived from the bacteria. It appears that this is an example of prokaryotic:eukaryotic symbiosis in which the tube worms rely on organic compounds excreted by the bacteria (see Chapter 215). The blood of the tube worms carries sulfide as well as oxygen from the gills to the trophosome, and has a special sulfide-binding protein that prevents sulfide toxicity. Endosymbionts have also been found in the giant white clams (*Calyptogena magnifica*), among other vent fauna and are not limited to sulfide-oxidizing bacteria, since methylotrophs have also been found (Jannasch, 1988) (see also Chapter 18). As yet, successful attempts to produce cultures of the symbiotic colourless sulphur bacteria have not been reported, but 5S and 16S rRNA analysis has indicated a relatively close relationship with members of the genus *Thiomicrospira* (Lane et al., 1985, 1990), which, as mentioned above, is one of the best-represented genera among the free-living bacterial community at the vents (Ruby et al., 1981; Ruby and Jannasch, 1982; Jannasch, 1988).

Once the occurrence of endosymbiotic bacteria in the animals of the hydrothermal vents had been accepted, many more occurrences were recognized in more mundane locations, including sewage outfalls and sulfide-rich sediments (Southward, 1986; Dando and Southward, 1986). Many of the animals associated with symbionts resemble *Riftia pachyptila* in that they completely lack a mouth and digestive system, whereas others may have only small guts and feeding appendages (Cavanaugh, 1983a, 1983b). Although not all of them have a specialized organ like the trophosome, many endosymbionts do appear to be associated with the gills of the eukaryotic host. For example, intracellular colorless sulfur bacteria have been found in the gill tissues of bivalves such as *Solemya velum* (Cavanaugh, 1983b) and *Thyasyris flexuosa* (Wood and Kelly, 1989). The recent description of a novel *Thiobacillus* species, *T. thyasiris*, (Wood and Kelly, 1989) from the gill tissue of *Thyasyris flexuosa* is probably the first report of the isolation of one of these symbionts. Whether all symbionts are capable of free-living growth (albeit with possible complex nutritional requirements), or whether some are so adapted to their symbiotic way of life that they are no longer capable of independent growth, remains to be seen.

A recent publication (Smith et al., 1989) has illustrated the effect that a localized deposit of organic material in an otherwise oligotrophic environment can have on the indigenous community. The skeleton of a 20 meter-long whale at a depth of 1,240 meters on the sea bed in the Santa Catalina basin was not only covered with mats of *Beggiatoa* resembling *Beggiatoa gigantea*, but it also supported six metazoan species, at least four of which are known in other locations to contain endosymbionts. As well as vent species (*Vesicomya gigas* and *Calyptogena pacifica*), others organisms known from anoxic sediments (*Lucinoma annulata*) and rotting wood (*Idasola washingtonia*) were also observed. None of these prokaryotic or eukaryotic species had been observed in this area before. It was found that the pore water under the skeleton contained around 20 μM sulfide, and the samples of whale bone that were recovered were found to be rich in oil and smelled strongly of sulfide. It would appear from the apparent ages of some of the molluscs present that a single whale carcass is sufficient to support these sulfide-dependent communities for several years.

Artificial Habitats and Application of Sulfur Bacteria

Artificial environments, such as the bioreactors used for industrial wastewater treatment, have provided habitats for bacteria that impose selective parameters not necessarily found in nature. Thus, substrates tend to be more abundant and conditions are generally more stable than in most natural situations. Two categories of artificial habitat where colorless sulfur bacteria are particularly important are wastewater treatment bioreactors and those associated with various leaching activities. Examples of other artificial habitats include industrial sulfur deposits or dumps, mining operations that expose sulfidic ores or sulfur to water or air, coal storage sites, and, last but not least, systems (including sewage

treatment plants) containing various amounts of reduced sulfur compounds.

Waste Treatment

Reduced sulfur compounds can occur in industrial wastes in a variety of forms and from a variety of sources. Thus, sulfide is an inevitable by-product of sulfate reduction associated with methanogenesis (if the effluent from which the methane is being generated contains significant amounts of sulfate) and the oil and gas industries. Thiosulfate and thiocyanate make up a substantial amount of the chemical content of photographic processing waste, and some papermaking processes generate both inorganic and organic sulfides. Of course, the amount of reduced sulfur compounds generated from industrial processes pales into insignificance when the quantity generated from animal wastes is considered, and research into methods of dealing with this is currently underway.

Reduced sulfur compounds present a problem both environmentally, because of their toxicity, and socially, because of their odor. If large amounts of sulfide are released into natural waters, this can result in oxygen depletion, either because of the oxygen demand for biological oxidation or, in the absence of suitable bacteria, by spontaneous chemical oxidation. Many water treatment plants impose surcharges for the treatment of such effluent, and there is obviously considerable pressure on companies to treat their effluent on the site. There are both chemical and physical methods of removing hydrogen sulfide from effluent; these include the use of ion-exchange resins, absorption with aqueous or organic solvents, and chemical oxidation (Gommers, 1988). Many of these simply transfer the problem to another waste stream or involve expensive or complex processes, and they are all expensive, especially for the removal of the last traces of sulfidic compounds.

Colorless sulfur bacteria occur in many sewage treatment systems and, in fact, are inadvertantly used to oxidize reduced sulfur compounds in the waste water. In some cases, this can lead to problems, such as the "bulking" caused by *Thiothrix*. The deliberate use of the biological treatment of sulfide-containing waste using colorless sulfur bacteria has attracted considerable attention of late. The end products (sulfur or sulfate) are not hazardous, and sulfate can be discharged directly into the sea or into brackish estuaries (which already are so high in sulfate that the discharge is insignificant). Moreover, biological treatment systems can be based on existing reactor designs (e.g., fluidized and packed bed reactors) and require very little in the way of new technology.

Another advantage of a biological process is that it can be combined with the treatment of other problems in an effluent. For example, the effluent of a methane reactor will contain ammonia in addition to sulfide. If the ammonia is then converted to nitrate or nitrite by aerobic, nitrifying bacteria, the resulting effluent can then be recycled to provide the electron acceptor for a sulfide-oxidizing reactor immediately after the methane reactor. The microbiological investigation of such a sulfide-oxidizing, denitritrifying reactor revealed the presence of large numbers of facultatively autotrophic colorless sulfur bacteria, which could oxidize sulfide to sulfate while reducing nitrate to nitrogen gas (Robertson and Kuenen, 1983a). In addition to the removal of nitrogen compounds, other advantages associated with the use of denitrifying bacteria rather than aerobic ones include lower production of both biomass and acid.

Combined Sulphide Oxidation and Denitrification. A denitrifying, sulfide-oxidizing reactor system was patented by a Dutch company, Gist brocades, for the post-treatment of effluent from methane-producing reactors (Patent number E.P.A.0051 888). Studies on a laboratory-scale model of this reactor, running on artificial waste water, revealed that sulfide (2–3 kg S/m^3·day), acetate (4–6 kg S/m^3·day) and nitrate (5 kg S/m^3·day) were all effectively removed (Gommers et al., 1988a). The rate-limiting step in the reactor proved to be the oxidation of sulfur to sulfate and, under most loads, the biomass had an overcapacity for both the oxidation of sulfide to sulfur and the conversion of acetate (Gommers et al., 1988b). During experiments in which nitrate depletion occurred, it became evident that in the absence of nitrate, at least one member of the bacterial community was able to reduce any available sulfur, thus illustrating the need for careful monitoring of the electron donor:electron acceptor ratios in such reactors (Gommers et al., 1988b).

The facultatively autotrophic species *Thiosphaera pantotropha* was isolated from a denitrifying, sulfide-oxidizing fluidized bed reactor that was supplied with approximately equivalent amounts of organic and inorganic substrates (Robertson and Kuenen, 1983b), and it initially appeared that the selection of a facultative bacterium would lend support to the model described for the ecological niches of aerobic, fresh-water sulfur-oxidizing bacteria (Fig. 4). However, subsequent attempts to isolate obligate autotrophs from a laboratory-scale model of this system that was being fed with an exclusively inorganic feed also resulted in the isolation of facultative autotrophs (M. Verbeek, W. Bijleveld, L. A.

Robertson, and J. G. Kuenen, unpublished observations). As yet, it is not clear whether obligate autotrophs were present in the inoculum, or the isolation techniques employed were inadequate for any obligate autotrophs present (although they were adequate for the cultivation of known obligate autotrophs), or whether growth in a biofilm in this type of reactor poses an additional selective pressure that favors facultatively autotrophic bacteria. Work has shown that a number of sulfide oxidizers from a wastewater system required cultivation on special membrane filters with sulfide gas before isolated colonies could be obtained (G. C. Stefess, R. de Schrijver, and J. C. de Bruyn, unpublished observations).

The same basic idea, that of using denitrifying colorless sulfur bacteria, was employed in a method proposed by Sublette and Sylvester (1987) for removing H_2S from gas streams by passing them through a reactor containing *Thiobacillus denitrificans*. The bacteria were first immobilized by co-culturing with floc-forming heterotrophs after the authors demonstrated that the presence of the heterotroph had no effect on the sulfide oxidation rate of *T. denitrificans*.

Removal of Sulfide as Elemental Sulfur. As already mentioned, sulfate-containing effluents can be discharged into the sea without significantly increasing the sulfur budget. However, the same is not true if the effluent is discharged into a body of fresh water. To overcome this problem, recovery as elemental sulfur, an intermediate in the oxidation of sulfide to sulfate, would be more appropriate. Research has shown that certain *Thiobacillus*-like bacteria are more inclined to produce sulfur than other species, and that both the dissolved oxygen and the sulfide concentration play an important part in determining whether sulfur or sulfate is the primary end product during sulfide oxidation. Both electron acceptor limitation and high sulfide loads favor sulfur production (Stefess and Kuenen, 1989). A pilot plant based on this principle, using a mixed bacterial biofilm reactor to treat the effluent from a paper mill, is being developed in the Netherlands (Buisman, 1989).

Removal of Organic Sulphides. A problem frequently encountered during the alkaline pulping of wood is the production of organic sulfides, such as methyl mercaptan and dimethyl sulfide. Alkaline pulping is done in order to improve the yield and quality of pulp derived from conifers to be used primarily in the manufacture of paper. Organic sulfides are toxic at even lower concentrations than hydrogen sulfide and have a very low threshold odor. Despite their toxicity, it has proved possible to grow bacteria on high concentrations of organic sulfides by using substrate-limited chemostats (Suylen et al., 1986; Kanagawa and Kelly, 1986; Smith and Kelly, 1988a, 1988b, 1988c). That the ability to oxidize these compounds may be widespread is suggested by the observation that the dominant organism in one set of experiments was a *Hyphomicrobium* species that was later shown to be able to grow as a facultative chemolithotroph on organic sulfur compounds in pure culture (Suylen and Kuenen, 1986; Suylen et al., 1986), whereas the key organism in the other series was a strain of *Thiobacillus thioparus*, an obligate autotroph (Kanagawa and Kelly, 1986). Immobilized cells of *T. thioparus* strain TK-m have now been successfully used on the laboratory scale to deodorize gases containing methyl mercaptan, dimethyl sulfide, dimethyl disulfide, and hydrogen sulfide (Kanagawa and Mikami, 1989; Tanji et al., 1989).

All of the colorless sulfur bacteria mentioned thus far are beneficial in wastewater treatment. However, in oxidation tanks fed with sulfide-containing waste water, the filamentous *Thiothrix* species can cause problems because they are associated with the phenomenon known as "bulking"; this occurs when bacterial aggregations that usually settle easily become loose and flocculent. This can result in blockages or loss of the biomass from the reactor.

Leaching-Associated Activities

Acidophilic bacteria are used in the recovery of metals from poor ores by leaching, and their potential use in the desulfurization of coal is currently being studied. To some extent, coal desulfurization and microbial leaching are the same process, in that in both cases sulfidic ores are oxidized, using similar organisms. However, the desired end products are different, and they are thus generally discussed separately. The aim of coal desulfurization is to produce a solid product (coal) that is as free of sulfur (including sulfur-containing precipitates) as possible, and it is therefore necessary to convert reduced sulfur compounds to soluble forms. In leaching, it is metal recovery that is important, and the presence of jarosite ($M{\cdot}Fe_3(SO_4)_2OH_5$, where M is a monovalent cation such as Na^+ or K^+) and other precipitates in the solid waste is not relevant (although it may constitute an environmental problem around the leaching heaps).

Bacterial Leaching. Bacterial leaching is used in the recovery of metals from ores that are too poor for conventional metallurgical extraction

methods (Tuovinen and Kelly, 1972; Brierley and Lockwood, 1977; Brierley, 1982). Combinations of *T. ferrooxidans* and either *T. thiooxidans* or *T. acidophilus* (previously called *T. organoparus*) and *Leptospirillum ferrooxidans* have been associated with the degradation of pyrite (FeS_2) and chalcopyrite ($CuFeS_2$). The leaching reactions may involve the direct bacterial oxidation of the sulfide ores with oxygen and/or an indirect process during which ferric ions produced by the bacterial oxidation of ferrous iron are used to chemically oxidize the sulfide ores. The ferric ions are thereby reduced to ferrous iron, which, in turn, can be recycled by the bacteria. During this process, other metallic ions such as cupric copper dissolve. Other metals that have been extracted using processes that involve bacteria include zinc, uranium, lead, gold, molybdenum, and, especially, copper.

Dump leaching operations, which are frequently used to extract copper, can be fairly primitive, involving the creation of ore dumps, often in valleys or old open pit mines. As water percolates through the heaped rocks, bacterial activity releases the metals into solution. This solution is then collected in catch basins, the metals recovered, and the liquids recycled to the top of the dump. A somewhat better controlled system is known as heap leaching. During this process, the ore-bearing rocks are crushed to promote contact with the acidified water, and the heaps are built on impermeable bases that prevent seepage into the soil beneath. Aeration systems can be built into the heaps. It is to be expected that as mineral reserves become depleted, and it becomes economically attractive to extract even small amounts of metals in poor ores and spoilage heaps, technological improvements will increase the efficiency of microbial leaching processes and, perhaps, lessen their environmental impact. For a full review of bacterial leaching, the reader is referred to reviews such as Brierley (1982) and the volume edited by Ehrlich and Brierley (1990).

Coal Desulfurization. Research into the use of the pyrite-oxidizing abilities of bacteria, such as *Thiobacillus ferrooxidans* and *Sulfolobus* species, for the removal of sulfur compounds from coal before it is burned, thus reducing sulfur emission into the atmosphere, has been carried out at a number of centers in the last decade. It has been shown that such a process could be effective, especially for low-sulfur coals, using consortia of mesophilic bacteria (Bos et al., 1988; Bos and Kuenen, 1990). Laboratory studies have shown that an optimal process requires two steps. First, a mixed-flow inoculation step, where a fairly dense population of bacteria already growing on pyrite can be brought into contact with fresh, finely ground coal at a pH suitable for growth (around pH 1.8). This inoculation step would then be followed by the use of plug-flow reactors, where the bulk of the pyrite oxidation would take place. At the end of the process, the process water can be recirculated, as can some of the biomass-bearing coal particles, to serve as the inoculum for the fresh coal. A plant design, involving a cascade of Pachuca tanks (Fig. 10), was devised for this type of system (Bos et al., 1988). Pachuca tanks (in their simplest form, an inverted cone with aeration at the narrowest point, at the bottom of the tank) are particularly suitable for this type of process because the upflow of air into the tanks not only provides the bacterial community with the oxygen and carbon

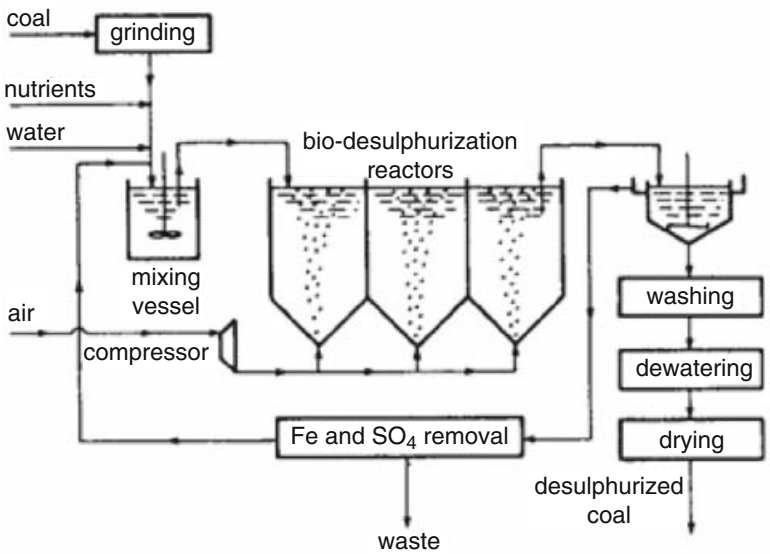

Fig. 10. Simplified scheme for the microbiological desulfurization of coal in Pachuca tanks (bio-desulfurization reactors). After grinding and mixing with water, the coal slurry contains particles less than 100 μm in diameter at a concentration of 20% (w/v). At this particle size, virtually all of the pyrite crystals become accessible to microbial leaching. The total leaching process requires about 10 days. More than 95% of the inorganic sulfur is removed, but little or no organic sulfur is degraded.

dioxide necessary, but it also keeps the slurry well mixed, without any need for complex and expensive stirring mechanisms.

Corrosion

Together with the sulfate-reducing bacteria, many of the sulfuric-acid-producing bacteria, and in particular the acidophiles, have been implicated in many corrosion problems. Indeed a strain of *T. thiooxidans* isolated from a corroding concrete pipe was originally known as *T. concretivorans* (i.e., concrete-eating). In sewage pipes with an aerobic headspace, sulfide may be produced in the anaerobic water phase and then be transferred to the film of water on the aerobic part of the pipe where it may be oxidized to sulfuric acid. In order to dissolve the carbonates in concrete, the pH need only be below 5.0–5.5 and such a pH can be generated by either neutrophilic or acidophilic bacteria. The activities of the acidophiles may also be responsible for steel pipe corrosion (Kuenen and Bos, 1988) as well as many of the pollution problems associated with the acid run-off from mine spoil heaps. These environmental problems are not only associated with the low pH of the water, but also with the toxic concentrations of heavy metals that they may contain. In addition, acidic water containing ferric sulfate may generate precipitates of jarosite, and these can block drainage pipes and cover stream and river sediments.

The Role of Colorless Sulfur-Oxidizing Bacteria in the Sulfur Cycle

Although much is known about the physiology and occurrence of colorless sulfur bacteria, less is known about the quantitative aspects of their activity in nature. Many of the reasons for this are difficulties commonly associated with field work (e.g., heterogeneous samples, unstable gradients, low concentrations of substrates), and are therefore outside the scope of this chapter, but a few difficulties are uniquely associated with the colorless sulfur bacteria.

Commonly used methods for estimating the activity of sulfur-oxidizing bacteria in the field include cell counts, oxidation of (radiolabelled) substrate (sulfide, thiosulfate, or sulfur), product formation (especially sulfuric acid, since this causes pH changes), and $^{14}CO_2$ fixation. Other, more specific techniques include the measurement of substrate-dependent respiration and immunofluorescent microscopy.

Cell Counts

With some of the more conspicuous bacteria (e.g., *Beggiatoa*, *Thiovulum*), it is possible to obtain a rough estimate of numbers based on direct cell counts. However, most of the colorless sulfur bacteria require cultivation before they can be counted. The choice of media and substrates for most-probable-number (MPN) estimates or direct plate counts is especially difficult for the colorless sulfur bacteria. The most obvious problem is that outside the chemostat there is no way of selectively growing facultative autotrophs or chemolithoheterotrophs. They must first be isolated on autotrophic or heterotrophic media, respectively, and then screened for sulfur oxidizing capacity. In addition, low recovery efficiency can be a problem with both plate counts and dilution series. Two other problems are associated with the obligate autotrophs: 1) thiosulfate is frequently used as an energy source in solid media, but this is not always the most suitable energy source. For some bacteria, agar plates containing colloidal sulfur (see Chapter 138) may be more appropriate, while other bacteria may require sulfide. The use of solid sulfide media can present technical problems with regard to toxicity and instability unless one of the less-soluble nontoxic sulfides (e.g., calcium sulfide) is used: 2) some autotrophic species do not give distinct colonies on agar, and moreover, the acidophiles may be inhibited by organic compounds resulting from chemical acid hydrolysis of the agar itself at their required growth pH values. To overcome these agar-associated problems, other techniques (such as the use of silica gel plates or floating filters [de Bruyn et al., 1990] may be more appropriate (G. C. Stefess, R. de Schrijver, and J. C. de Bruyn, unpublished observations). Some of the sulfur-oxidizers may have a requirement for an unidentified growth factor such as a vitamin or mineral.

Activity Measurements

Data on the rates of sulfide oxidation in natural systems are scattered and somewhat variable, possibly because of the difficulty of accurate sampling as well as the reactivity of the compounds involved.

Substrate Uptake and/or Transformation—Chemical and Radioassays. Once cell numbers have been estimated with a degree of confidence, they can only be used to provide an idea of the potential activity of colorless sulfur-oxidizing bacteria within that particular ecosystem. The measurement of substrate transformations (i.e, utilization or accumulation), preferably in situ, can be used as a measure of

actual activity. A major problem associated with the use and measurement of many reduced sulfur compounds, especially sulfide and sulfite, is that they are chemically very reactive and are readily oxidized spontaneously by oxygen. Appropriate controls can, to some extent, overcome this problem, but it must be remembered that in nature biological and chemical reactions compete, and equilibrium reactions causing the exchange of radiolabel in reduced sulfur compounds mean that extra caution must be used in the interpretation of results. Moreover, chemical oxidation rates are influenced by many of the environmental parameters that also affect biological activity (e.g., pH, temperature, chemical constitution of the solutions involved). In a few cases, where dominant populations of known colorless sulfur bacteria occur (e.g., *Sulfolobus* in solfataras, *Beggiatoa* mats), rough estimates have been made of the activity of these organisms. Mosser et al. (1973) found rates for sulfur oxidation to sulfate of 67 and 190 g m^{-2}·day^{-1} for mats of *Sulfolobus acidocaldarius* growing in two hot pools (Moose Pool and Sulfur Cauldron, Yellowstone National Park, respectively). In the Black Sea, a maximum rate of 710 nmol l^{-1}·day^{-1} was observed by Sorokin (1970). For an extended discussion of sulfur oxidation rates in nature, the reader is referred to Kuenen (1975) and to Jørgensen (1988).

Another problem is that the sulfur-oxidizing heterotrophs may also contribute to the turnover of reduced sulfur compounds at natural sites. In some cases, $^{14}CO_2$ fixation can be used to eliminate this but in many locations where mixotrophs or chemolithoheterotrophs are involved, CO_2 may not be the primary source of carbon. This type of experiment could, therefore, sometimes result in underestimates if it is not used in tandem with other measurements. An associated problem is that the specific activity of a given species can vary. For example, Beudeker et al. (1980) found that, when grown under carbon dioxide limitation, the ribulose bisphosphate carboxylase (Rubisco) activity in *T. neapolitanus* was 240 nmol min^{-1}·mg $protein^{-1}$. If, however, thiosulfate was the limiting factor, the enzyme level fell to 72 nmol min^{-1}·mg $protein^{-1}$. Other substrate conversion rates can also vary, especially among species. Thus, it has been found that *T. denitrificans* and *Thiomicrospira denitrificans* oxidize thiosulfate at rates of 0.86 and 2.9 mM thiosulfate g C^{-1}·h^{-1} respectively (Timmer ten Hoor, 1977).

A combination of CO_2 fixation and oxygen and hydrogen sulfide analysis was used to measure microbial activity in Saelenvaan Lake, in Norway. As can be seen from Fig. 11, a peak of CO_2 fixation was found to coincide with the very narrow zone where oxygen and sulfide coexisted.

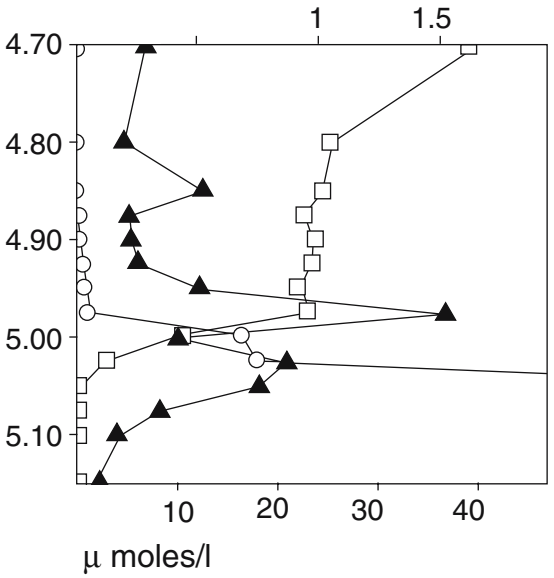

Fig. 11. Profiles of CO_2 fixation, dissolved oxygen, and dissolved hydrogen sulfide concentrations in Saelenvan Lake (Norway) sampled at 5 a.m. on 15 August 1978. The high resolution was due to the use of a special sampling device connected to a pump. CO_2 fixation rates (top horizontal axis) were obtained using $^{14}CO_2$ injected into dark bottles, which were incubated in situ. The left vertical axis is depth in m. The bottom horizontal axis is the concentration of dissolved gas in μmol $liter^{-2}$. Triangles, μmol CO_2 $liter^{-1}$·h^{-1}; squares μmol oxygen $liter^{-1}$; circles, μmol hydrogen sulfide $liter^{-1}$. (From Kelly and Kuenen, 1984.)

It should be noted that the sampling technique was critical for the success of these experiments. A special sampling device with an inlet that removes water from a horizontal area of the column at 1–2 cm intervals (Jørgensen et al., 1979) was necessary—if a less accurate device was used, the very narrow CO_2 fixation zone could not be seen because of dilution by the surrounding water.

Those working on the ecosystems around the hydrothermal vents have, of course, severe difficulties to overcome in making in situ measurements, especially since a new variable, pressure, must be considered (Jannasch, 1985). In order to measure the activity of autotrophic bacteria at these sites, $^{14}CO_2$ fixation was measured in syringes incubated on the sea bed (approximately 250 atmospheres, 3°C) and on board ship (1 atmosphere) at 3 and 23°C. Little or no difference was found between the two samples incubated at 3°C, and the bacteria responsible for the $^{14}CO_2$ fixation were thus obviously barotolerant rather than barophilic. Moreover, $^{14}CO_2$ fixation sharply increased if thiosulfate was added, or when the samples were incubated at 23°C, indicating that mesophilic colorless sulfur bacteria

were responsible (Tuttle et al., 1983; Wirsen et al., 1986).

MICROELECTRODES A technique that has been used with some success in the study of in situ bacterial biofilms and immobilization for biotechnology employs the use of microelectrodes that can be progressively moved through a biolayer, gradually registering the gradients present. The slope of the gradient, combined with data on the diffusion coefficient for the substrate measured, can provide direct information on the flux and turnover of substrates, and thus can give accurate information on in situ activities. These microelectrode systems are frequently linked to a computer that not only controls the rate of passage of the electrode tip through the biolayer, but also records and calculates the results (e.g., Revsbech et al., 1986). Among others, oxygen, pH, sulfide, carbon dioxide, and N_2O microelectrodes are available, but the use of some electrodes (e.g., sulfide, CO_2) is limited by their low sensitivity at commonly used pH values. However, the oxygen electrode has been extensively used, especially in systems where photosynthesis is involved and oxygen supply can easily be controlled by modifying the availability of light (e.g., Jensen and Revsbech, 1989; Revsbech and Ward, 1984). The construction of these electrodes, and their use in various ecosystems, was extensively reviewed by Revsbech and Jørgensen (1986). Their use, in conjunction with some of the other methods mentioned above, may at least provide a means of measuring actual activities in gradients, rather than potential activities in in vitro cultures.

Summary and Conclusion

The carbon metabolism of the colorless sulfur bacteria is the best-known facet of their physiology and biochemistry. New insights into their pathways of sulfur metabolism have done away with the old unifying concept of sulfur metabolism, as it is now clear that there are diverse pathways in the organisms investigated thus far.

With the use of new techniques for cultivating the more fastidious colorless sulfur bacteria (e.g., *Beggiatoa*) in gradient cultures, and with pure cultures of other strains (e.g., *Macromonas*) now available, the way is now open for further research into their (eco)physiology and biochemistry.

It is hoped that microelectrodes, in combination with improved isotope techniques, will also provide more detailed information about the activities of these bacteria in nature.

However, one important question remains—should the colorless sulfur bacteria still be considered a taxonomic group? As discussed throughout this paper, the use as a taxonomic criterion of the ability to gain energy from the oxidation of inorganic reduced sulfur compounds has resulted in the definition of a very heterogeneous group, collectively known as the colorless sulfur bacteria. It is possible that the possession of the relevant pathways for growth on reduced sulfur compounds is of no greater taxonomic relevance than the ability to use the Calvin cycle or to grow on hydrogen. Moreover, it seems likely, in view of the results obtained with 5S and 16S RNA analysis, that we are seeing the result of evolutionary convergence towards the (eco)physiological properties encountered in many of the colorless sulfur bacteria. The extreme heterogeneity of the group is further emphasized as other long-known bacteria are found to also possess the properties of colorless sulfur bacteria. Indeed, the common lack of a test for thiosulfate or sulfide oxidation in routine taxonomic screening has meant that the sulfur-oxidizing potential of species of genera such as *Paracoccus*, *Pseudomonas*, and *Alcaligenes* are only now being recognized.

Despite their morphological and phylogenetic diversity, the colorless sulfur bacteria present a coherent picture in physiological terms. As it is generally the physiological specifications of an organism that define its ecological significance, the reclassification of the colorless sulfur bacteria may present something of a microbiological dilemma because the relationships suggested by the rRNA analysis (Table 8) bear little relation to the ecophysiological activities of the organisms. Thus, in spite of the reallocation of species among different genera, research can only profit from the retaining of physiological, rather than taxonomic, groupings—such as the sulfate reducers, nitrogen fixers, denitrifiers, and colorless sulfur bacteria.

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