ISSN 0026-2617, Microbiology, 2006, Vol. 75, No. 5, pp. 512–545. © Pleiades Publishing, Inc., 2006.
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REVIEW

Lithotrophic Microorganisms of the Oxidative Cycles of Sulfur and Iron

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Received May 25, 2006

Abstract—The review deals with sulfur bacteria (the first chemolithotrophs ever studied) and with the acidophilic bacteria of sulfur and iron cycles which were investigated as a result of Winogradsky's discovery. The diversity of these organisms and the factors and mechanism of its origin are emphasized; their metabolic functions and nutritional regulation are discussed.

DOI: 10.1134/S002626170605002X

Key words: colorless sulfur bacteria, acidithiobacilli, catabolism and anabolism, ecophysiology, variability, strain polymorphism, adaptation, acidophily, autotrophy, mixotrophy, heterotrophy.

A new field in life sciences was founded at the end of the 19th century; the role of S.N. Winogradsky's great discovery becomes more evident with the passage of time and with accumulation of data concerning chemoautotrophy.

Winogradsky's initial research on chemosynthesis was performed on sulfur bacteria, in particular, on a community of *Beggiatoa* with other microorganisms. Sulfur bacteria were demonstrated to oxidize hydrogen sulfide to sulfur; sulfur was accumulated in the cells and was later oxidized to sulfuric acid. This process is the only source of energy available to sulfur bacteria [1]. However, the capability of nonphotosynthetic bacteria to fix CO_2 and to grow autotrophically was confirmed only after Winogradsky isolated and investigated pure cultures of nitrifying bacteria.

The discovery of chemosynthesis in nitrifying bacteria confirmed the concept of a new type of bacterial physiology, termed autotrophy. The discovery of autotrophs heralded a new era in science.

S.V. Winogradsky was a pioneer in the study of microbial ecology and microbial geochemical activity. He established the importance of the study of microbial communities in their natural environment. He termed microbial activity in the environment "a collective self-organizing work" [2].

The authors of this review intended to use the example of research on colorless sulfur bacteria and acidophilic microorganisms in order to demonstrate the glorious fruits of Winogradky's ideas.

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COLORLESS SULFUR BACTERIA

Colorless sulfur bacteria (CSB) constitute a large group of morphologically conspicuous prokaryotes. Big cell size and intracellular deposition of elemental sulfur are the unique characteristics of this group. Under certain conditions, sulfur can be subsequently oxidized to sulfate, used as an electron acceptor in sustaining metabolism, and or reduced to sulfide. Some of the recently described CSB are among the largest known prokaryotes. The comparative morphological characteristics of some sulfur bacteria demonstrates that the cell volume of most of the marine and freshwater species is 3–8 orders of magnitude higher than that of common rod-shaped bacteria (Table 1). The large cells of sulfur bacteria have unique ecophysiological properties and can occupy ecological niches unavailable to other sulfur-oxidizing bacteria.

TAXONOMY OF COLORLESS SULFUR BACTERIA

CSB form a highly heterogeneous group from the taxonomic, phylogenetic, and physiological points of view. Most of the known morphotypes or phylotypes are uncultured organisms, and few of the representatives of this group have been studied in pure cultures. The application of modern molecular genetic methods to the analysis of material from environmental populations of sulfur bacteria over the last 20 years has resulted in marked progress in the study of communities of CSB, especially in marine environments. Thus, our understanding of the taxonomic composition, phylogeny, and evolutionary position of the representatives

Table 1. Taxonomy and morphology of colorless sulfur bacteria

Genera and species or groups of phylotypes	Cell shape	Maximal cell size,* $\frac{\mu m}{volume, \mu m^3}$	Cultures species or strains (st)	Phylo-types	Habitats		Type of metabo-lism **	Refer-ence
					Marine	Freshwater		
Filamentous								
Beggiatoa, group 1	Disk-shaped	$\frac{200 \times 60}{2000000}$	–	8	+	–	l, ?	[18]
Beggiatoa, group 2	Cylindrical		2 st	–	+	–	l, ?	[19]
Beggiatoa, group 3	Cylindrical	$\frac{4 \times 9}{110}$	5 st	–		+	l, o	[19–21]
Thioploca araucae	Disk-shaped	$\frac{43 \times 30^a}{40000}$	–	1	+	–	?	[22]
Thioploca sp.	Cylindrical	$\frac{18 \times 75}{10000}$	–	2	–	+	?	[24, 25]
Thiothrix spp.	Cylindrical	$\frac{2.5 \times 5}{25}$	11	–	–	+	4 l, 7 o	[23]
Leucothrix (3, species)	Cylindrical	$\frac{4 \times 6}{70}$	3	–	+	–	l	[14–16]
Sphaerotilus (*S. gallus*)	Cylindrical	$\frac{2.4 \times 10}{43}$	1		–	+	l, o	[17]
Unicellular								
Thiomargarita namibiensis	Spherical	$\frac{750}{200000000}$	–	1	+	–	l	[26]
Achromatium oxliferum	Ellipsoidal	$\frac{35 \times 95}{80000}$	–	3	+	+	l, o	[27]
Macromonas (*M. mobilis*)	Ellipsoidal and rod-shaped	$\frac{14 \times 30}{4500}$	–	1	-–	+		[28]
M. bipunctata	Ellipsoidal and rod-shaped	$\frac{4 \times 10}{80}$	1		–	+	o	[5]
Thiovulum (*T. majus*)	Spherical	$\frac{18}{3000}$	–	1	+	+	l	[29]
Spirillum	Spiral	$\frac{2.3 \times 50}{180}$	2		–	+	o	[11]
Titanospirillum	Spiral	$\frac{9 \times 30}{600}$		1	+	–		[9]
Aquaspirillum	Spiral	$\frac{2 \times 18}{55}$	1		–	+	o	[30]
Giesbergeria	Spiral	$\frac{2.1 \times 60}{210}$	2		–	+	o	[12]
Simplispira	Spiral	$\frac{1.3 \times 8.5}{110}$	2		–	+	o	[12]

Table 1. (Contd.)

Genera and species or groups of phylotypes	Cell shape	Maximal cell size,* $\frac{\mu m}{volume, \mu m^3}$	Cultures species or strains (st)	Phylo-types	Habitats		Type of metabo-lism**	Refer-ence
					Marine	Freshwate		
Levispirillum	Spiral	$\frac{1.4 \times 4}{6}$	1		–	+	o	[13]
Spirochaeta P[b]	Spiral	$\frac{0.4 \times 20–200}{4–40}$	1 (3 st)	–	1	–	o	[8]
Escherichia coli[c] (2 species)	Rod-shaped	$\frac{1 \times 2}{1.5}$	1	–		+	o	–

Note: [a], multicellular filaments, length up to several cm, containing thousands of cells; the size of individual cells is presented; [b], aerotolerant sulfur-oxidizing spirochetes from the "Thiodendron" sulfur mats; [c], *E. coli* is included for comparison.
"–", absent; ** l, lithotrophic; o, organotrophic; * from [3], supplemented.

of this group, as well as of a number of their physiological characteristics, has been improved [3, 4].

In the second edition of *Bergey's Manual of Systematic Bacteriology*, most of the known CSB were arbitrarily assigned to the new family *"Thiotrichaceae" of the order Thiotrichales* within the class *Gammaproteobacteria* [4]. This family includes the filamentous sulfur bacteria *Beggiatoa, Thiothrix, Thioploca*, and *Leucothrix*, and the unicellular sulfur bacteria *Achromatium, Thiomargarita, Thiobacterium*, and *Thiospira.* According to the results of 16S rRNA gene sequencing, the representatives of the known genus *Macromonas* were placed among betaproteobacteria, in the family *Comamonadaceae* [5, 6]. Other representatives of sulfur bacteria were classified with other taxa: the genus *Thiovulum* was placed in the family *Helicobacteriaceae* (class *Epsilonproteobacteria*) [7] and aerotolerant free-living spirochetes, the predominant component of the *"Thiodendron"* sulfur mats, in the new taxon in the family *Spirochaetaceae*, class *Spirochaetales* [18]. The phylogenetic position of the giant marine spirilla, which were described as a new genus *"Titanospirillum"* on the basis of morphological criteria [9], and of uncultured *Thiobacterium* species [10] is still not clear. The taxonomic characteristics of the species composition and the physiologo-morphological features of sulfur bacteria are presented in [4]. The phylogenetic position of the known CSB within the class *Gammaproteobacteria* is shown in Fig. 1.

A number of new genera of filamentous and unicellular sulfur bacteria have recently been described. Among these organisms are bacteria of the genus *Spirillum, S. winogradskii* (basonym *Thiospira winogradskii*), *S. kriegii* [11], and of the new genera *Giesbergeria* (*G. voronezhensis, G. kuznetsovii*) [12], *Simplicispira* (*S. metamorpha*) [12], and *Levispirillum* (*L. itersonii*) [13]. Three *Leucothrix* species, including the type species *L. mucor* [14–16] and the novel representative of the genus *Sphaerotilus, S. gallus* capable of lithotrophic growth by sulfide or thiosulfate oxidation [17] were classified as filamentous sulfur bacteria.

The data presented in Table 1 demonstrate that the majority of the known morphological types or phylotypes of sulfur bacteria are uncultured organisms. The possibilities for investigation of their metabolic diversity and of their function in natural environments are therefore limited.

Metabolically, CSB can be either lithotrophs or obligate organoheterotrophs. The representatives of the latter group perform oxidation of reduced sulfur compounds and accumulation of intracellular sulfur, not coupled with energy metabolism.

The metabolism and ecophysiology of these two groups will be described below.

LITHOTROPHIC COLORLESS SULFUR BACTERIA

Metabolism of Lithotrophic CSB

Utilization of inorganic sulfur compounds as electron donors for autotrophic, mixo-, or lithoheterotrophic growth has been strictly confirmed for a few of the numerous isolates of filamentous and unicellular sulfur bacteria (Table 2).

No correlation occurs between the inability of certain *Beggiatoa, Thiothrix*, and *Leucothrix* strains to grow autotrophically and the presence of the genes encoding the big subunit of RubisCO (ribulose 1,5-

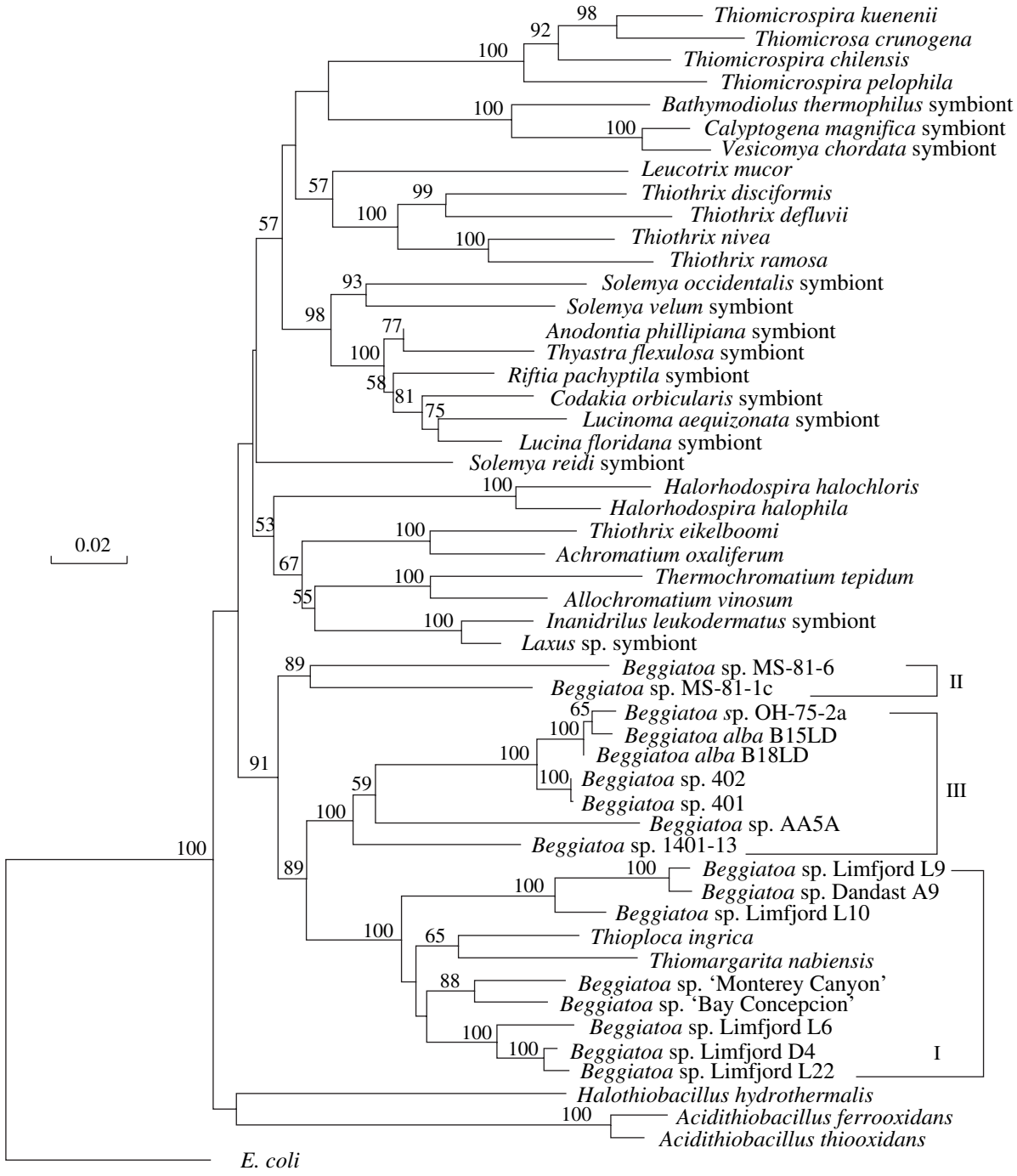

Fig. 1. Phylogenetic relations of freshwater and marine colorless sulfur bacteria of the class *Gammaproteobacteria* as revealed by 16S rRNA nucleotide sequence analysis [3, supplemented] I, *Thioploca, Thiomargarita*, big marine *Beggiatoa*; II, marine *Beggiatoa*; III, freshwater *Beggiatoa.* The scale corresponds to 2 nucleotide replacements per 100 nucleotides. Figures, the statistical reliability of the branching order determined by bootstrap analysis (the values above 95 are significant).

bisphosphate carboxylase), the key enzyme of the Calvin cycle. These strains utilize sulfur as an energy source for mixotrophic or lithoheterotrophic growth. Decreased expression of the RubisCO gene under unfavorable environmental conditions may be one of the causes of the absence of autotrophic growth [31]. This gene may have been lost in the course of evolution due to adaptation of the organism to the more oxidized conditions of freshwater environments [21]. Numerous attempts to obtain autotrophic or mixotrophic growth of marine and freshwater *Beggiatoa* strains in H_2S–O_2 gradients or in chemostat cultures have given no positive results [21, 32, 35, 36].

Table 2. Effect of environmental factors and some genomic characteristics on the metabolic type of lithotrophic sulfur bacteria

Species and strains	Presence of RubisCO		Type of metabolism					References
			lithoautotrophic		mixotrophic	lithoheterotrophic	organotrophic	
	Activity	Gene and form*	O_2, μM		Organic substrate			
			≥15	≤10	excess	limitation		
Beggiatoa alba B15 LD, ATCC 35556							+[a]	[33]
B. alba B 18 LD (neotype), ATCC 35555	+[b]	–				+	++	[35–37]
B. alba OH–75–2a	+[b]	+	–	–	–	–	++	[35, 36]
B. leptomitiformis D402 (neotype), DSM 74945	+	Gene *cbbL* (red type form I RubisCO)	–	+	+	++	+++[a]	[20, 21]
B. leptomitiformis D405	–	–	–	–	–	–	+++[a]	[20]
Beggiatoa spp. (13 strains from Pringsheim collection)	–	ND	–	–	–		+++[a]	[33]
Thiothrix nivea DSM 5205	+	+	+	ND	+	+	+++	[38]
T. ramosa T2	+	ND	+	ND	–	+[c]	++[a]	[39–41]
T. arctophila IN	–	Gene *cbbM* (form II RubisCO)	–	–	+	+	++	[42]
T. unzii Al, ATCC 49747	ND	ND	–	–	–	+	+	[44]
Thiothrix spp., strains No 4 and No 19	ND	ND	+	+	–	++	++	[47]
Sphaerotilus gallus 377	–	ND	–	–	–	+	+++	[17]
Beggiatoa MS-81 – 1 c	+	+	–	+	–	–	–	[45, 46]
Beggiatoa MS-81 – 6 c	+	+	–	+	–	+[b]	+	[45, 46]
Leucothrix mucor DSMZ 2157	–	-	–	–	–	+	+++	[16]
L. thiophila 2 WS, DSMZ 13 602	–	Gene *cbbL* (green type form I RubisCO)	–	–	+	++	+	[14, 43]
Leucothrix sp. strain 5 WS, DSM 13604	–	–	–	–	+	+	+	[14, 43]

Note: [a], lysis in the absence of thiosulfate; [b], RubisCO activity not at physiological level, several orders of magnitude lower than in marine strains; [c], diauxy; ND, not determined; "–" no growth or no RubisCO activity detected; * RubisCO forms and genes are given according to [34], bor *B. alba*, according to [37].

Within a species, even in the case of phenotypically and genetically very close strains, the capability to grow autotrophically can vary. For example, in spite of the 99.5% DNA homology between two *B. leptomitiformis* strains, D405 and D402, only the latter can grow autotrophically, lithoheterotrophically, and mixotrophically, as well as heterotrophically in the absence of reduced sulfur in the medium. Since the gene encoding RubisCO was not detected in the second strain, D405, its inability to utilize sulfur compounds in dissimilatory processes is probably genetically determined [34]. For other species and strains of filamentous sulfur bacteria, *Beggiatoa, Leucothrix*, and *Thiothrix*, a similar picture was observed (Table 2).

The application of molecular techniques of cloning and DNA analysis for RubisCO-encoding genes to environmental populations of sulfur bacteria from marine sulfur mats, together with direct measurement of the enzymatic activity in cell suspensions has improved our knowledge concerning the autotrophic

potential and metabolism of a number of uncultured sulfur bacteria, their taxonomical diversity, and phylogenetic relations. Apart from the presence of the RubisCO-encoding gene, the capability of autotrophic growth of marine populations of *Beggiatoa* and *Thioploca* was confirmed by the discovery of high activity of the Calvin cycle enzymes (RubisCO and phosphoribulokinase) and of sulfite oxidoreductase, the key enzyme of sulfur metabolism [64]. The presence of membrane-bound nitrate reductase in the cells of marine [48] and freshwater [49, 50] *Beggiatoa* and *Thioploca* populations indicates the possibility of anaerobic sulfur oxidation coupled with nitrate reduction or, more probably, with denitrification. The in situ determination of CO_2 fixation rates and radioautography with radioactive carbon isotopes were used to demonstrate autotrophic growth of filamentous (*Beggiatoa*, *Thiothrix, Thioploca* [51, 68]) and unicellular (*Achromatium* [53]) sulfur bacteria from the sulfur mats of marine hydrotherms. However, the results of radioautography of various populations of sulfur bacteria also demonstrated their capability to utilize, apart from CO_2, other carbon sources (acetate, certain amino acids, etc.), probably for mixotrophic or heterotrophic growth. The types of carbon metabolism in environmental populations of filamentous and unicellular sulfur bacteria are therefore probably heterogeneous. The rates of $^{14}CO_2$ fixation determined for some marine populations did not exceed the average level of heterotrophic CO_2assimilation [54].

Physiological studies of certain big marine and freshwater *Thioploca* [55] and, later, of *Beggiatoa* [48, 56] and *Thiomargarita* [26] from environmental samples led to the most important discovery of the last decade. Unique structures were found in the cytoplasm of these bacteria, huge vacuoles for nitrate accumulation; nitrate was used as an electron acceptor for anaerobic sulfide oxidation. Nitrate concentration in the vacuoles can be as high as 160–500 mM [48, 51, 55]. Vacuolated sulfur bacteria were capable of oxidizing sulfide to sulfur anaerobically; nitrate was reduced to NH_3. In some *Thioploca* populations, up to 15% of nitrate nitrogen was reduced to N_2 via denitrification [57]. The presence of nitrate reductase in the membrane fraction of giant *Beggiatoa* filaments confirms the relation between anaerobic sulfide oxidation and the functioning of an electron transport chain (ETC) [48]. It is still not clear whether this process participates in only maintenance metabolism, or whether bacteria are capable of prolonged activity with nitrate as an electron acceptor.

Thin filaments of marine and freshwater *Beggiatoa* deprived of vacuoles are not capable of such nitrate reduction. The nitrate reductase of the freshwater strain *B. alba* B18 LD [34] and in the thin filaments of several marine *Beggiatoa* strains belongs to the soluble cell fraction and probably does not participate in dissimilatory oxidation of sulfur compounds.

A number of freshwater and marine *Beggiatoa* strains with small cells (groups 2 and 3 in Table 1) were found to be capable of dinitrogen fixation [57].

Novel big marine sulfur bacteria with both intracellular sulfur inclusions and large vacuoles were described in 2004. These bacteria are attached to the substrate; although morphologically close to *Thiothrix*, they are quite distant from it phylogenetically and cluster together with vacuolated marine *Beggiatoa* and *Thioploca*. The vacuoles of these sulfur bacteria were shown not to be involved in respiratory nitrate accumulation [59]. The authors suggested that their function was to store dissolved oxygen and probably to increase the buoyancy of nonmotile filaments.

Unlike marine *Beggiatoa* strains, a number of freshwater ones can utilize intracellular or exogenous elemental sulfur as an electron acceptor for maintenance metabolism; under anaerobic conditions, H_2S production is coupled with the oxidation of cellular storage compounds [35, 46, 60]. These strains can therefore survive short-term anaerobiosis, which occurs under the unstable oxygen regime of the surface layers of bottom sediments.

Thus, the cultured species of lithotrophic sulfur bacteria have a broad range of metabolic capabilities. As can be seen from the data of Table 2, their realization depends on both genetic determination and environmental conditions. The main factors determining the realization of the metabolic potential are the presence and availability of alternative electron donors and acceptors, H_2S (or organic matter for mixotrophic growth) and O_2 (or NO_3^- under anaerobic conditions).

Regulation of the Energy and Constructive Metabolism in CSB

Variability of the types of energy and construction metabolism has been reported for a number of species and strains of lithotrophic sulfur bacteria (Table 2). Under environmental growth limitation by electron donors or acceptors, these organisms gain advantages of both obligate autotrophs and heterotrophs. The mechanisms of metabolic switching caused by changes in growth conditions remained unclear until recently. In the presence of reduced sulfur, oxygen concentration and the presence of organic compounds in the medium were demonstrated to be the main factors determining the direction of energy and constructive metabolism in the freshwater strain *Beggiatoa leptomitiformis* D402 (Table 2) [19, 20]. Autotrophic growth with thiosulfate oxidation was possible only in the absence of organic substrates and under strictly anaerobic conditions (oxygen concentrations not above 3–16 μM). Oxygen concentration in the medium above 20 μM resulted in arrested growth due to the inhibition of RubisCO activity, although the enzymes of oxidative sulfur metabolism retained high activity even under aerobic conditions. Depending on acetate concentration in the

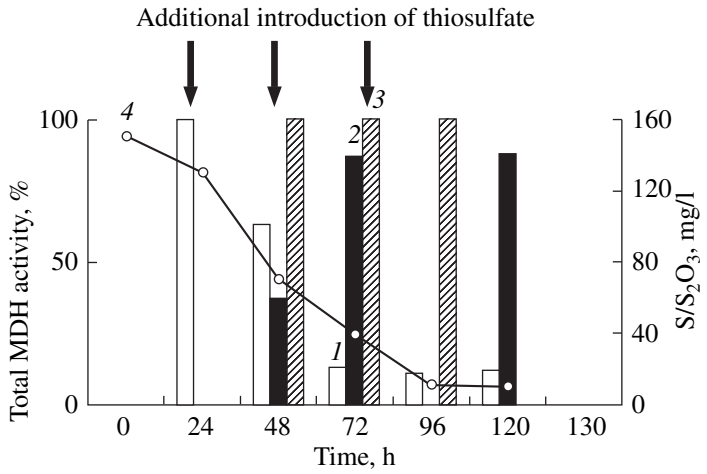

Fig. 2. Regulation of *Beggiatoa leptomitiformis* D402 constructive metabolism by the isoforms of malate dehydrogenase (MDH 2 and MDH 4) under limitation of mixotrophic growth by thiosulfate. Arrows indicate additional thiosulfate introduction in a series of parallel experiments. *1*, MDH 4; *2*, MDH 2; *3*, MDH 4 after additional thiosulfate introduction in a series of parallel experiments [62]; *4*, thiosulfate oxidation.

medium, bacteria grew mixotrophically, with thiosulfate and acetate as energy substrates, or lithoheterotrophically. In the last case, acetate was utilized only for assimilative purposes [20]. During transition of strain D402 from organotrophic to mixotrophic growth, carbon metabolism was modified and the enzymatic activity of the main metabolic pathways (TCA cycle and glyoxylate cycle) changed. In the presence of reduced sulfur compounds, the TCA cycle acted mostly as an anabolic pathway; the dehydrogenase portion was bridged by the glyoxylate cycle and produced the intermediates for biosynthetic purposes [61, 62]. Strain D402 reacted to the changes in nutrition type and aeration mode by changes of the respiratory chain. During organotrophic growth, an aa_3 type cytochrome oxidase was active in the terminal part of the ETC. Transition to mixotrophic, lithotrophic, or autotrophic growth resulted in the inhibition of aa_3 type oxidase biosynthesis and in enhanced biosynthesis of cbb_3 type oxidase, which is known to have high affinity to O_2 (K_M = 8–20 mM) [63].

In *B. leptomitiformis* D402, the regulation and switching of the metabolic pathways is achieved via restructuring of the malate dehydrogenase (MDH) system and production of the stable isomers of this enzyme, dimers, and trimers. MDH is the key enzyme of carbon metabolism. A tetrameric form of the enzyme is active under lithotrophic conditions; it is involved in the regulation of anabolic reactions of the glyoxylate cycle. The emergence of the dimeric form, which maintains the TCA cycle energetic metabolism, is linked to organotrophic growth [61].

The rate of restructuring of the metabolic pathways is important for bacterial adaptation to environmental conditions. This can be exemplified by the transition of *B. leptomitiformis* D402 from mixotrophic to organotrophic growth caused by the exhaustion of thiosulfate, and back to mixotrophic growth on thiosulfate addition (Fig. 2). The ratio of MDH isomers in the course of bacterial development reflects the relative contribution of organic and inorganic substrates in the energy metabolism [61].

The synthesis of an additional MDH isoform was detected in the heterotrophic strain *B. alba* DSM 1416 under oxygen stress. Compared to microaerobic growth, it resulted in the intensification of the glyoxylate cycle and in the activation of biosynthetic processes not related to growth (enhanced exopolysaccharide synthesis as protection from oxygen excess). Heterotrophic growth of all known sulfur bacteria is inhibited at the concentrations of organic substrates above 0.05–0.1% due to intracellular H_2O_2 accumulation.

Thus, in a number of filamentous sulfur bacteria, the adaptation to the changes in growth conditions is achieved by means of restructuring of the key enzyme of carbon metabolism; this leads, in turn, to changes in the activity of the major enzymatic pathways and redistribution of the flow of reduced equivalents in the cell. Mechanisms of metabolic regulation in response to changing growth conditions similar to those of *B. leptomitiformis* and *B. alba* were revealed in other filamentous sulfur bacteria *Leucothrix mucor* and *L. thiophila* [15, 16]; they are probably common to a number of organisms with variable metabolic types.

Ecophysiology of Lithotrophic CSB

Simultaneous presence of oxygen and sulfide is a necessary condition for growth of lithotrophic sulfur bacteria. The coexistence of these compounds in water is unstable due to both the high reactivity of sulfide and such factors as diurnal oxygen rhythms, tidal fluctuations of water level, and other hydrodynamic effects in

the zone where subterranean or hydrothermal sulfide-rich flows mix with oxygen-saturated water.

While the representatives of colorless sulfur bacteria have respiratory metabolism, they differ in their resistance to O_2 and the capability to utilize alternative electron acceptors (NO_3^-, S^0). Few *Thiothrix* species can grow in the flow of oxygen-saturated medium mixed with sulfide-containing water (in mineral springs, in underwater hydrothermal vents or low-temperature deep water, and in freshwater sediments with low rates of H_2S production). Most of the known sulfur bacteria, however, are obligatory or facultative microaerophiles or gradient microorganisms. The latter can thrive only in the narrow zone of H_2S–O_2 microgradient, at substrate concentrations not exceeding 2–10 μM.

Unique physiological features and ecophysiological adaptive mechanisms of sulfur bacteria living in contact with both O_2 and H_2S were revealed with the help of high-resolution selective microelectrodes (for characterization of the physicochemical properties of their habitats) together with molecular (fluorescent in situ hybridization), enzymological, and other modern techniques.

Due to the large size of their cells, sulfur bacteria gain some ecological and physiological advantages. High motion rates (an order of magnitude higher than those of small bacterial cells) and the presence of giant vacuoles (up to 80–98% of the cell volume) used for storage of alternative electron acceptors (nitrate and possibly oxygen under O_2 deficiency) are among these advantages. Various strategies and adaptive mechanisms are used in order to achieve the optimal growth conditions within the H_2S–O_2 gradient. Gradient sulfur bacteria *Beggiatoa* and *Thiovuhum* for example, exhibit pronounced chemotactic reactions and phobic responses to H_2S and O_2. The narrow *Beggiatoa* growth zone is located either on the surface of bottom sediments in the microgradient at H_2S and O_2 concentrations 2–10 μM or less, or as a narrow 50–100 μM band precisely in the zone of oxygen–sulfide contact. The filaments glide rapidly and purposefully along the border between H_2S and O_2 layers, and in the case of their increased diffusion flow, they maintain a stable gradient by active consumption of the substrates [64]. The chemotactic behavior of *Beggiatoa* and other filamentous sulfur bacteria (*Thioploca*) is believed to determine their capacity to form sulfur mats [65].

The unicellular gradient organism *Thiovulum majus* utilizes a different strategy [29, 52]. Single highly motile cells aggregate, forming mucous suspensions (veils), which can move into the zone of optimal conditions, usually in the water 0.5 mm above the sediment. The main function of this mucous structure is to create a physical barrier preventing contact of the cells with nonoptimal O_2 and H_2S concentrations.

In both cases, the purposeful (caused by chemotaxis) movement of *Beggiatoa* and *Thiovulum* counteracts the diffusion substrate limitation.

In marine [49, 55, 64] and freshwater [24, 25, 50] representatives of *Thioploca*, unique mechanisms were revealed for the adaptation to environmental conditions in the microniches occupied by sulfur bacteria. The giant filaments of marine *Thioploca* (trichomes 130–200 μm wide), which form massive sulfur mats in the upwelling zones along the Peruvian and Chilean coasts, perform sulfide oxidation coupled with nitrate reduction [66]. The *Thioploca* mats occur in highly productive marine coastal regions with high sedimentation rates; oxygen is absent from the bottom water, while nitrates are accumulated. It was already mentioned above that the *Thioploca* cells contain huge vacuoles (up to 98% of the cell volume) where nitrate is accumulated in concentrations of up to 500 mM. The cytoplasm forms a thin peripheral layer; S^0 inclusions are accumulated in the periplasm [49, 64]. The thickness of *Thioploca* mats can sometimes reach several meters [65].

Similar intracellular structures were revealed in the filaments of marine *Beggiatoa* from underwater hydrotherms [48, 64].

Big marine, nitrate-storing *Thioploca* and *Beggiatoa* occupy different microniches. While the *Thioploca* filaments migrate within their sheaths from the surface to the lower, sulfide-rich layers of the sediment, *Beggiatoa* niches are located at the border between anoxic, nitrate-containing bottom waters and the upper sediment layer with relatively high H_2S concentrations. The intracellular nitrate store is used as an electron acceptor for nitrate reduction in the course of sulfide oxidation to sulfur and then to sulfate [48, 64].

The structure of the sulfur mats formed by some *Thioploca* populations is unique. The sheaths and filaments are vertical; the latter can move shuttle-like within the sheaths, from the near-bottom anoxic, nitrate-containing water, to the lower layers of sulfide sediments. The sulfur mat can be 30 cm thick or more [55, 65].

These filamentous sulfur bacteria, therefore, utilize a sort of active shuttle-like transport system for nitrate storage with subsequent utilization in the lower mat horizons, which are in contact with sulfide-containing sediments. Thus, the supply of energy substrates becomes independent of diffusion flows. As was mentioned above, the detection of active membrane-bound nitrate reductase, the enzymes of sulfur metabolism, and the high activity of RubisCO in large *Beggiatoa* and *Thioploca* indicated the existence of such a mechanism; this mechanism can conceivably be used for chemoautotrophic growth under strictly anaerobic conditions. The data indicating the close phylogenetic relationship between large marine *Beggiatoa* and *Thioploca* correlate well with the similarity of their metabolism, i.e., their capacity to accumulate nitrates and to oxidize sulfur anaerobically. Thus, marine sulfur bacte-

ria *Beggiatoa* and *Thioploca* occupy ecological niches unavailable to other sulfur-oxidizing bacteria, where oxygen, nitrate, and sulfide are absent. Some of them can utilize a supplementary mechanism to receive the energy substrate due to their symbiotic relations with filamentous sulfate-reducing *Desulfonema. Desulfonema* filaments form a layer on the surface of *Thioploca* sheaths; they consume the organic compounds produced by sulfur bacteria and provide them with sulfide originating from sulfate reduction [68].

The biggest known prokaryotes, *Thiomargarita namibiensis*, use a similar mechanism to overcome substrate limitation by nitrate accumulation [26]. Unlike *Thioploca* and *Beggiatoa*, in this bacterium, nitrate accumulation in the vacuoles and sulfide oxidation are separated in time due to the seasonal and periodic supply of electron donors and acceptors.

Nonmotile filamentous sulfur bacteria *Thiothrix*, and probably the sulfur-oxidizing populations of *Leucothrix* apply several strategies for the optimization of their growth conditions. These bacteria overgrow solid substrates, aquatic plants, or form ectosymbioses with a number of marine animals. They develop in sulfide mineral springs where oxygen- and sulfide-saturated waters mix in a turbulent flow. They also grow on marine and freshwater shallows with intense H_2S input and an unstable oxygen regime. *Thiothrix* grows abundantly in such anthropogenic systems as activated sludge and the waters of purification plants. As with *Thiomargarita*, a number of adaptive mechanisms exist which compensate for the immobility of the filaments incapable of choosing the optimal zone. These mechanisms include the presence of motile gonidia migrating into the zone of optimal conditions, metabolic versatility [39, 41, 66, 67], and the formation of syntrophic associations. For example, up to 10^7 cells of sulfur reducers were detected in the mucous layer of *Thiothrix* overgrowth of the White Sea littoral. Ectosymbiosis of *Thiothrix*-related sulfur bacteria is known with various representatives of marine fauna, including the shrimps of hydrothermal vents [69–71]. Filamentous sulfur bacteria are believed to gain advantages for mixotrophic or possibly autotrophic growth in the vicinity of hydrothermal vents; shrimps deliver them to these environments and persist in the water column in the zone of hydrothermal sulfide flows.

Most of the cultured *Thiothrix* species and natural populations are relatively independent from high oxygen concentrations. This is to a great degree due to their thick polysaccharide sheaths (or mucous capsules in the case of *Leucothrix*), which regulate oxygen diffusion to nonmotile filaments. Exopolysaccharides (mucous sheaths, capsules, mucous dredge) play a similar role of protecting microaerophilic cells from toxic high concentrations of O_2 and H_2S.

HETEROTROPHIC COLORLESS SULFUR BACTERIA

Taxonomic Position and Phylogenetic Relations

The morphologically conspicuous microorganisms capable of intracellular sulfur accumulation but not able to utilize reduced sulfur compounds in energy metabolism form a group of heterotrophic colorless sulfur bacteria. This group is highly heterogeneous from the evolutionary and taxonomic viewpoints, including the representatives of phylogenetically remote taxa of alpha-, beta-, and gammaproteobacteria, aerotolerant *"Spirochaetaceae"* of the order *"Spirochaetales"*, and possibly the members of other taxa (with the taxonomic position not yet established according to the modern principles of classification). The numerous isolates from the known genera of lithotrophic sulfur bacteria (*Beggiatoa* and *Thiothrix*) with strictly heterotrophic metabolism and genetically determined absence of the enzymes of autotrophic CO_2 fixation and dissimilatory oxidation of sulfur compounds should also be assigned to heterotrophic sulfur bacteria. For example, only four of the eleven known *Thiothrix* species are lithotrophs [23]. Of the *Sphaerotilus* strains isolated from sulfide-rich environments (mineral sulfur springs, water treatment plants), only one strain of the new species *S. gallus* 373 was capable of lithotrophic growth by oxidation of sulfur compounds [17]. Heterotrophic sulfur bacteria belonging to the five known and novel genera of spiral-shaped cells constitute a large taxonomic group (Table 1). The ability to accumulate elemental sulfur in the course of sulfide oxidation was previously reported for *Aquaspirillum serpens*, the type species of this genus of heterotrophic spirilla [30]; other representatives of the known genera of heterotrophic spirilla possibly share this feature.

Functional Role of Inorganic Sulfur Compounds in Heterotrophic Sulfur Bacteria

Although massive accumulation of elemental sulfur on sulfide-containing media is similar in lithotrophic and heterotrophic sulfur bacteria, the metabolic role and the mechanism of oxidation of sulfur compounds in these groups are profoundly different. No organotrophic sulfur bacteria are known to have dissimilative enzymatic systems participating in the oxidation of sulfur compounds; this oxidation is therefore not coupled with ETC functioning and energy metabolism [60, 74].

The heterotrophic sulfur bacteria listed above differ from the lithotrophic species also in the mechanisms and terminal products of transformation of reduced sulfur compounds. They can oxidize sulfide only incompletely, to S^0; thiosulfate is qualitatively oxidized to tetrathionate. Experiments with environmental samples of an uncultured freshwater *Achromatium* revealed H_2S oxidation only to S^0 [27], unlike marine autotrophic populations, which accumulate sulfate as well as sulfur.

Most sulfur bacteria, both lithotrophic and heterotrophic, are microaerophilic. Their microaerophily, however, is the result of different physiological reasons. Many lithotrophs are microaerophilic because they require the simultaneous presence of both electron donor and acceptor (S^{2-} and O_2^-); bacteria became physiologically adapted to the specific environmental conditions where the supply of growth substrates is provided. Heterotrophic sulfur bacteria are microaerophiles because under these conditions they avoid the oxidative stress of aerated water in the absence of external detoxifying agents acting on reactive oxygen species (ROS).

Production of ROS, primarily H_2O_2 and O_2^-, in lytic concentrations is the main cause of the oxidative stress dangerous for many microaerophiles [35, 73, 75]. Production and accumulation of H_2O_2 in the cells of heterotrophic bacteria are known to be the result of several factors. The characteristics of ETC composition and function are the most important. Accumulation of H_2O_2 can occur at the initial dehydrogenase part of the ETC [75, 76] or (in the case of unbalanced ETC functioning) at the level of the soluble cytochrome *c* acting as the terminal oxidase. The latter can be due, among other factors, to the low activity of dehydrogenases that supply reductive equivalents to ETC [16, 77]. In heterotrophic strains of *Beggiatoa alba* and *B. leptomitiformis* D405, as well as in the facultatively autotrophic strain D402 grown heterotrophically, formation of noticeable amounts of H_2O_2 has been revealed [21, 63, 75]. The absence or low activity of the enzymatic system for ROS protection, primarily of cytoplasmic catalase and periplasmic cytochrome *c* peroxidase, promotes H_2O_2 accumulation in the cells. Most of the heterotrophic and lithotrophic sulfur bacteria lack catalase, or it is 1–2 orders of magnitude less active than in aerobic heterotrophs. Thus, microaerophily and production of lytic ROS concentrations can be caused by multiple factors.

Prevention of the toxic effect of H_2O_2 and other ROS on aerobically grown sulfur bacteria is the physiological rationale for the processes of oxidation of sulfur compounds. For *Spirillum winogradskii* D427, up to 80% of the cell yield in the exponential growth phase was shown to be lysed by H_2O_2 in the course of aerobic cultivation. In *S kriegii* D430, another species of sulfur spirilla, microaerophily is more pronounced, and the lytic effect of H_2O_2 is much stronger.

The net positive effect of thiosulfate is to raise the so-called antioxidative state of the cells of various sulfur bacteria to several times higher than of other aerobic, oxygen-resistant heterotrophs [73]. This integral parameter reflects the nonspecific increase in the cell antioxidant functions caused by the activation and maintenance of a high pool of SH-containing cell components (proteins, amino acids, and enzymes) [74].

The degree of the positive role of thiosulfate can be understood by assessing its effect on such growth characteristics as the maximum yield and the efficiency of utilization of the main substrate. The former is the result of prevention of direct toxic damage of cell structures by H_2O_2 and oxygen radicals, as well as of stabilization of certain enzymatic reactions and metabolic processes. The latter is caused by a decrease in expenses for the assimilatory processes not related to growth. Polysaccharide synthesis for protection against excessive oxygen concentrations, which requires high energy consumption, is an example of such processes.

The Role of Reduced Sulfur Compounds in the Metabolism of Aerotolerant Sulfur-Oxidizing Spirochetes

Aerotolerant sulfur-oxidizing spirochetes are the sole or the dominant mat-forming component of *"Thiodendron"* bacterial communities [8]. Their mass development occurs in highly productive regions of marine coasts, in marine and oceanic environments with extensive supply of biogenic, volcanic, or hydrothermal sulfide, or in saline sulfur springs. Unlike the other sulfuret types formed by photo- or chemoautotrophic sulfur-oxidizing bacteria, *"Thiodendron"* sulfur mats are formed by spirochetes; obligate heterotrophs with fermentative metabolism [79, 80]. In nature, such mats are formed on or above the sediment surface, often in the presence of *Fucus* algae, under an unstable oxygen regime with O_2 concentrations varying from 0.01 to 5–7 mg/l. Laboratory experiments revealed that oxygen concentrations 8–25 O_2 were optimal for spirochete growth when reduced sulfur compounds or thiosulfate were present.

Spirochetes grown under microaerobic conditions were found to profit from involving oxygen in the catabolic processes not related to ATP accumulation; an alternative pathway of glucose metabolism was used. The catabolic reactions of pyruvate (the product of glucose glycolysis) transformation along a shortened pathway resulted in the formation of more oxidized products, mainly acetate and CO_2, unlike strictly anaerobic glucose decomposition to ethanol, formate, H_2, and pyruvate. The aerobic pathway of glucose utilization is more profitable for spirochetes; it results in a twofold increase in cell yield and substrate utilization efficiency. Enzymatic formation of H_2O_2 from O_2 by NADH peroxidase is among the undesired effects of aerobic metabolism. Nonenzymatic H_2O_2 removal occurs by its interaction with sulfide or thiosulfate; it results in the deposition of large amounts of elemental sulfur in the periplasmic space of the spirochetes. Due to this process of H_2O_2 removal, the presence of reduced sulfur compounds is obligatory for the aerobic growth of aerotolerant spirochetes. In the absence of O_2, intracellular sulfur is used as an external electron acceptor to drain the excess of reductive equivalents, i.e., to remove H_2 produced by fermentation [81]. Aerotolerant spirochetes, like other sulfur bacteria, use accumulation of exopolysaccharides in the sulfur mat

zones and in culture media in order to decrease excessive O_2 concentrations.

The efficiency of aerobic glucose utilization is twice as high as that under anaerobic conditions, even with the energy and nutrient expense in gluconeogenesis for polysaccharide synthesis taken into account. A more stable oxygen regime for spirochete development in sulfur mats, as well as an additional supply of sulfide are achieved due to the dense interactions with the sulfur-reducing bacteria of the genera *Desulfobacter* and *Dethiosulfovibrio* [8, 82]. This dense symbiotic association was originally described by B.V. Perfil'ev on the basis of microscopic investigations of sulfur mats as a *Thiodendron latens* bacterium with a complex growth cycle [78]. The symbiosis is profitable for both components; the sulfur reducers utilize acetate and pyruvate, the products of glucose metabolism, while the spirochetes gain an additional sulfide supply.

It was mentioned above that such close associations are known for some marine sulfur bacteria (marine communities of *Thioploca–Desulfonema* and *Thiothrix*–sulfidogenic bacteria). The trophic relations within such communities of sulfur mats are, however, practically not studied.

Aerotolerant spirochetes profit from the simultaneous utilization of O_2in the enzymatic reactions not related to energy metabolism, and of H_2S for H_2O_2 removal in order to maintain aerobic growth.

Research with radiolabeled $Na_2^{35}S$ was performed in the Crater Bay in the Sea of Japan and in a mineral spring of the Staraya Russa resort in order to assess the rate of sulfide oxidation by spirochetes in the *"Thiodendron"* sulfur mats. The values obtained (up to 200 mg S^{2-}/m^2 day) [79] were comparable to or exceeded the rates of the oxidation processes in bacterial communities of the sulfur mats formed in marine environments by lithotrophic bacteria [54, 69, 71]. Such studies, however, are not numerous, and it is therefore impossible to qualitatively assess the environmental role of lithotrophic and heterotrophic colorless sulfur bacteria in the oxidative reactions of the sulfur cycle and in the processes of destruction and production in the carbon cycle.

Research on communities of sulfur bacteria has provided new information in microbial biology concerning the structure of prokaryotic cells and their taxonomic, physiological, and phylogenetic diversity. Further research on colorless sulfur bacteria is impeded by the difficulty of their isolation and of maintaining them on laboratory media under stable conditions. The development of new cultivation techniques considering the characteristics and adaptive capabilities of colorless sulfur bacteria is essential for further investigation of the cooperative trophic and functional relations with other microbial groups in natural environments and for the understanding of the functioning of natural ecosystems.

ACIDOPHILIC CHEMOLITHOTROPHIC MICROORGANISMS

Phenotypic Diversity

The most remarkable characteristic of acidophilic chemolithotrophic microorganisms is their striking phylogenetic heterogeneity. Although they belong to remote taxa at the phylum, class, order, and family level (Tables 3–5), they share some physiological characteristics, i.e., acidophily, utilization of inorganic substrates as energy sources, and high resistance to metal ions.

Acidophilic chemolithotrophs can be subdivided into three groups. The first includes the microorganisms with a broad range of energy substrates (Fe^{2+}, S^{2-}/S^0, and sulfide minerals). They belong to the phyla *Proteobacteria, Firmicutes*, and *Crenarchaeota* (Table 3).

Acidithiobacillus ferrooxidans are strictly aerobic, obligately chemolithoautotrophic gram-negative motile rods, 0.3–0.5 × 1.0–1.7 μm. Some strains oxidize H_2. Oxidation of S^0 can occur under anaerobic conditions, with Fe^{3+} as the terminal electron acceptor [83].

The genus *Sulfobacillus* comprises gram-positive nonmotile rods, 0.6–0.8 × 1.0–4.0 μm, with round or slightly oval endospores located terminally or subterminally. They are strictly aerobic, mixotrophic organisms, able to survive only several transfers under heterotrophic conditions. Some species grow on S^0 anaerobically, with Fe^{3+} as the terminal electron acceptor [84–86].

Acidianus brierleyi are nonmotile archaea of spherical shape, 1.0–1.5 μm. Gram reaction is negative. The organism is a facultative autotroph capable of organotrophic growth on such complex organic substrates as yeast extract [87, 88].

Metallosphaera sedula and *M. prunae* are gram-negative, motile aerobic coccoid archaea, 0.8–1.2 μm. Gram reaction is negative. These organisms are facultative chemolithoautotrophs, capable of aerobic H_2 oxidation. Organotrophic growth occurs on complex organic compounds; S^0 is not reduced under anaerobic conditions in the presence of H_2 [89, 90].

Sulfurococcus yellowstonensis are gram-negative aerobic coccoid archaea, 0.8–1.2 μm. Gram reaction is negative. They are facultative lithotrophs and grow heterotrophically on complex organic compounds [91].

Sulfolobus metallicus are gram-negative aerobic coccoid archaea, 0.8–2.0 μm. Gram reaction is negative. They grow organotrophically on complex organic substrates and carbohydrates [92].

Acidophilic microorganisms of the second group oxidize practically exclusively Fe^{2+} and consist of acidophilic representatives of the phyla *Nitrospirae, Actinobacteria*, and *Euryarchaeota* (Table 4). Representatives of the three phyla discussed above have not yet been found in this group,

Leptospirillum ferrooxidans are strictly aerobic, obligately chemolithoautotrophic, gram-negative,

Table 3. Acidophilic chemolithotrophic microorganisms oxidizing Fe^{2+}, S^{2-}/S^0, and sulfide minerals

Domain, phylum	Class	Order	Family	Genus	Species	G+C, mol %	Temperature (range), optimum	pH (range), optimum
Bacteria, Proteobacteria	*Gammaproteobacteria*	*Acidithiobacillales*	*Acidithiobacillaceae*	*Acidithiobacillus*	*ferrooxidans*	55–57	(2–37) 28–30	(0.8–6.0) 1.8–2.0
Bacteria, "Firmicutes"	*Bacilli*	*Bacillales*	*"Alicyclobacillaceae"*	*Sulfobacillus*	*thermosulfidooxidans*	47.2	(20–60) 50–55	(1.1–2.4) 1.7–1.8
					sibiricus	48.2	(17–60) 55	(1.1–2.6) 2.0
					thermotolerans	48.1	(20–60) 40–42	(1.2–2.4) 2.0
					acidophilus	55–57	45–50	2.0
Archaea, Crenarchaeota	*Thermoprotei*	*Sulfolobales*	*Sulfolobaceae*	*Sulfolobus*	*metallicus*	38	(50–75) 70	(1.0–4.5)
				Acidianus	*brierleyi*	~31	(45–75) 70	(1.0–6.0)
				Metallosphaera	*sedula*	~45	(50–80) 75	(1.0–4.5)
					prunae	~46	(55–80) 75	(1.0–4.5)
				Sulfurococcus	*yellowstonensis*	44.6	60–65	(1.0–5.5) 2.0–2.4

Table 4. Acidophilic chemolithotrophic microorganisms oxidizing only Fe^{2+}

Domain, phylum	Class	Order	Family	Genus	Species	G+C, mol %	Temperature (range), optimum	pH (range), optimum
Bacteria, Nitrospirae	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	*Leptospirillum*	*ferrooxidans*	51–56	(2–37) 28–30	(1.1–2.5) 2.0
					"ferriphilum"	55–58	41	1.0–1.5
					thermoferrooxidans	56	(30–55) 45–50	(min 1.3) 1.65–1.90
Actinobacteria	*Actinobacteria*	*Acidimicrobiales*	*Acidimicrobiaceae*	*Acidimicrobium*	*ferrooxidans*	67–69	~45–50	(1.5–5.0) 2.0
Archaea, Euryarchaeota	*Thermoplasma*	*Thermoplasmatales*	*Ferroplasmaceae*	*Ferroplasma*	*acidiphilum**	36.5	(15–47) 35–36	(1.3–2.2) 1.7
					acidarmanus	ND	(32–51) 42	(0.2–2.5) 1.2

* Some strains grow at the optimal temperature 40–42°C. ND, no data.

motile vibrios, spirals, or pseudococci, 0.2–0.5 × 0.9–2.0 μm [93, 94].

Leptospirillum thermoferrooxidans are phenotypically close to *L. ferrooxidans*, but have different temperature requirements. The organism can grow mixotrophically in the presence of 0.02% yeast extract [95].

Acidimicrobium ferrooxidans are acidophilic, thermotolerant or moderately thermophilic, gram-positive rods, 0.35–0.4 × 1.0–1.5 μm. They grow autotrophically with Fe^{2+} and heterotrophically on yeast extract. Heterotrophically grown cells are motile. Aerobes [96].

Ferroplasma acidiphilum are strictly aerobic, acidophilic, gram-negative coccoid archaea. Cell shape can vary from spherical to threadlike, 0.3–1.0 × 1.0–3.0 μm. Gram reaction is negative. The cell wall is absent. Strict chemolithotrophs, incapable of growth on organic compounds; require yeast extract as a growth factor [97, 98].

Ferroplasma acidarmanus is close to *F. acidiphilum*. The organism, however, is chemoorganotrophic and grows at higher temperature and lower pH values (Table 4) [99].

Table 5. Acidophilic chemolithotrophic microorganisms oxidizing only S^{2-}/S^0

Domain, phylum	Class	Order	Family	Genus	Species	G+C, mol %	Temperature (range), optimum	pH (range), optimum
Bacteria, Proteobacteria	*Gammaproteobacteria*	*Acidithiobacillales*	*Acidithiobacillaceae*	*Acidithiobacillus*	*thiooxidans*	55–57	(2–37) 28–30	(0.5–5.5) 2.0–3.0
					caldus	63.1–63.9	(32–52) 45	(1.0–3.5) 2.0–2.5
					albertensis	61.5	25–30	(2.0–4.5) 3.5–4.0
Archaea, Crenarchaeota	*Thermoprotei*	*Sulfolobales*	*Sulfolobaceae*	*Sulfolobus*	*acidocaldarius*	~37	(55–85) 70–75	(1.0–6.0) 2.0–3.0
					solfataricus	~35	(50–87) 75–87	(2.0–5.5) ~4.5
					shibatae	35	(50–86) 81	(1.0–4.0) 3.0
					hakonensis	38.4	(50–80) 70	(1.0–4.0) 3.0
				Acidianus	*infernus*	~31	(65–96) 90	(1.0–5.5) ~2.0
					ambivalens	~32.7	(60–87) 80	(1.0–3.5) ~2.5
				Stydiolobus	*azoricus*	38	(57–89) 80	(1.0–5.0) 2.5
				Sulfurococcus	*mirabilis*	43–46	(50–85) 70–75	(1.0–5.8) 2.0–2.6

The third group of acidophilic chemolithotrophs comprises the microorganisms capable of oxidizing only elemental sulfur and reduced sulfur compounds. They belong to the phyla *Proteobacteria* and *Crenarchaeota* (Table 5). Representatives of *Euryarchaeota* and *Firmicutes* were not found in this group.

Acidithiobacillus thiooxidans are mesophilic, aerobic, gram-negative motile rods, 0.5 × 1.0–2.0 μm, obligate autotrophs [100, 101].

Acidithiobacillus caldus are moderately thermophilic, aerobic, gram-negative motile rods, 0.7–0.8 × 1.2–1.8 μm. Mixotrophs; growth on $S_4O_6^{2-}$ occurs with glucose or yeast extract [102].

Acidithiobacillus albertensis are mesophilic, strictly aerobic, gram-negative motile rods, 0.45 × 1.2–1.5 μm, obligate autotrophs [103].

Sulfolobus acidocaldarius are thermophilic, aerobic, nonmotile coccoid cells, 0.7–2.0 μm. Gram reaction is negative. Grow on complex organic substrates, are facultative chemolithoautotrophs, weakly oxidize H_2 [104, 105].

Sulfolobus solfataricus are aerobic, coccoid archaea, 0.8–2.0 μm. Gram reaction is negative. Grow on complex organic substrates; weak growth on H_2. Facultative chemolithoautotrophs [104, 106].

Sulfolobus shibatae are thermophilic, aerobic, weakly motile coccoid archaea 0.7–1.5 μm. Gram reaction is negative. Grow on organic substrates. Facultative chemolithoautotrophic growth occurs by sulfur oxidation [107, 108].

Sulfolobus hakonensis are aerobic, thermophilic, nonmotile coccoid archaea, 0.9–1.1 μm. Gram reaction is negative. Facultative chemolithoautotrophs; weak growth occurs on organic media with 1.0% maltose, glutamic acid, or tryptophan as sole carbon and energy source [109].

Acidianus infernus are facultatively anaerobic, thermophilic, coccoid archaea, 0.5–2.0 μm. Gram reaction is negative. Grow autotrophically or mixotrophically. Some strains grow heterotrophically on complex organic substrates. Grow aerobically by S^0 or H_2 oxidation. Under anaerobic conditions, reduce sulfur and oxidize H_2 [110, 111].

Stydiolobus azoricus are thermophilic, motile coccoid archaea, 0.5–1.8 μm. Gram reaction is negative. Obligate aerobes, chemolithoautotrophs capable of S^0 reduction with H_2 oxidation. Yeast extract at 0.005–0.02% (wt/vol) is stimulatory [112].

Sulfurococcus mirabilis are thermophilic, budding, motile coccoid archaea, 0.8–1.2 μm. Gram reaction is negative. Aerobes, facultative autotrophs. Yeast extract at 0.005–0.02% stimulates growth and sulfur oxidation. Grow on organic substrates at concentrations not exceeding 0.1–0.25% [113].

Acidianus ambivalens are nonmotile coccoid archaea, 0.5–2.0 μm. Gram reaction is negative. Obligate chemolithoautotrophs, oxidize or reduce sulfur [114, 115].

The evolution of various acidophilic chemolithotrophic microorganisms in specific ecological

niches evidently took two main directions: development of a specific type of energy metabolism based on mineral energy sources (electron donors) and temperature (emergence of mesophilic, moderately thermophilic, and thermophilic chemolithotrophs).

Sulfur-oxidizing communities were formed to a great degree in sulfur-containing therms in regions of active volcanism (Table 5). The conditions in ore deposits and in sulfide-containing hydrotherms favored the evolution of microorganisms oxidizing Fe^{2+}, sulfide minerals, and sulfur (Tables 3, 4).

The diversity in various taxa of adaptive mutation processes aimed at providing a supply of electron donors can not be assessed at present; it will be considered below with acidophilic chemolithotrophs as an example.

The evolution of constructive metabolism in chemolithotrophs (autotrophic, mixotrophic, or heterotrophic) was probably affected by another factor, viz., the gaseous environment, specifically O_2 and CO_2, since their solubility at elevated temperatures decreases drastically, especially in acidic media enriched with metal ions and deprived of carbonates.

This is possibly the reason why, unlike mesophilic chemolithotrophs, practically all acidophilic thermophilic and moderately thermophilic bacteria and archaea, no matter what their taxonomic position, possess wider metabolic capabilities. Apart from CO_2, they metabolize organic compounds. They are either mixotrophs or facultative autotrophs.

Variability in Acidophilic Chemolithotrophic Microorganisms

Variability is a way for organisms to adapt to new environmental conditions, leading to the emergence of organisms with new features. Survival of a species depends on its ability to vary. The more flexible its genome, the quicker an organism adapts to changing environmental conditions. Strain polymorphism within a species is a manifestation of this capability.

Strain polymorphism in acidophilic chemolithotrophs. The diversity of the phenotypic characteristics between strains of acidophilic chemolithotrophs is known. Among them, growth rate, the rate of oxidation of an energy source, pH and temperature values optimal for growth and energetic processes, and resistance to metal ions are the most important [98, 116–118]. The strains of *A. ferrooxidans* differ also in the rate and efficiency of their adaptation to new energy substrates [119], to pH below optimal values [120], and to elevated concentrations of heavy and toxic elements [118].

The phenotypic strain diversity of *A. ferrooxidans* is the result of genotypic strain polymorphism. The strains of *A. ferrooxidans* differ in genome size (from 2.2×10^9 to 2.8×10^9 Da) and in DNA G+C content (from 56.1 to 58.1 mol %) [121]. DNA–DNA hybridization analysis of total genomes of 23 strains, with 17 used as reference ones, revealed the high degree of genetic heterogeneity. Four groups (genomovars) were revealed, which were characterized by high degrees of genomic similarity; six strains did not belong to any of these groups. The genomic similarity of these strains with each other and with the other genomovar strains was from 13 to 43%; this finding implies the existence of at least ten genomovars of this chemolithotrophic species. Genetic heterogeneity of *A. ferrooxidans* can be observed at the level of comparative analysis of 16S rRNA gene sequences, which are considerably more conservative than the nucleotide sequences of the total bacterial genome. Several phylogenetic groups of *A. ferrooxidans* were revealed within the phylogenetic cluster.

All the strains of *A. ferrooxidans*, as well as of *Sulfobacillus* and archaeal *Ferroplasma*, possess a unique structure of chromosome DNA, as indicated by the different profiles of DNA fragments produced by a single restriction endonuclease and analyzed by pulse electrophoresis [122]. The polymorphism of the chromosome DNA structure is the result of transpositions of big genomic segments; some authors [123] believe the latter to be caused by homologous recombinations between the transposable elements distributed in the genome. These are the main source of genome plasticity. The polymorphism of the chromosome DNA structure is a stable strain characteristics; it can be used for strain identification and monitoring in the environment, in technological processes, and in experiments.

Strain polymorphism is a stage of formation of new microbial species in the course of accumulation of differences in the DNA nucleotide sequences encoding vitally important characteristics.

Information on strain polymorphism in the structure of individual genes in chemolithotrophs is being accumulated. For example, in *A. ferrooxidans* ATCC 19859, a set of RubisCO genes, 1422 and 333 bp, was found, encoding a big and small unit, respectively [124]. Two sets of genes encoding this enzyme were revealed in *A. ferrooxidans* Fe1, *rbcL*1-*rbcS*1, and *rbcL*2-*rbcS*2 [125]. In *A. ferrooxidans* TFI-35, multiple gene copies were revealed by Southern blot hybridization of cloven genomic DNA with ^{32}P-labeled *Pst*I/*Eco*RI, the fragment of the pLS401 plasmid containing the RubisCO big subunit gene [126]. The number of copies of this gene is not surprising, considering that in *A. ferrooxidans*, RubisCO constitutes from 2 to 5% of the total protein. Strain polymorphism in the number of RubisCO gene sets and their copies can be expected in this bacterial species.

Strain polymorphism in *A. ferrooxidans* was demonstrated in the structure of *iro* gene encoding the first key enzyme in the chain of electron transfer from Fe^{2+}–Fe(II) oxygenase [127].

One to seven plasmids (from 2 to 70 kbp) were revealed in the cells of 75% of *A. ferrooxidans* strains

[128]. Among the 20 strains from different geographic locations, isolated from the substrates of different composition and ratio of sulfide minerals, 16 strains contained from one to six plasmids of various size and in varying number of copies [129, 130].

In the *A. ferrooxidans* genome, IS elements belonging to the families IS*Afe1* (IS*T1*) and IS*T2*, with the size of 1300 and 1400 bp respectively, are present [131]. Among the 22 analyzed strains isolated from different continents (America, Europe, Asia), the majority (12 strains) contained IS elements of both families in their genomes. One strain contained only the IS*T1* element and three strains only IS*T2*; in six strains, IS elements were not revealed. In the strains containing IS elements, the number of IS*T2* copies varied from 15 to 25 and the number of IS*T1* copies from 1 to 10. A new IS element, IS*T3091*, was found in the pTFI91 plasmid of *A. ferrooxidans* TFI-91 [132]. Its length was 1063 bp. In a number of strains, from 10 to 20 copies per genome of another IS element, IS*T3091* were present [133]. Its size was 1200 bp. While IS*T2* elements were revealed in all of the five *A. ferrooxidans* strains isolated from ore concentrates of different composition, IS*Afe1* elements were present in four of them [134]. The partial sequencing of IS*Afe1* 5' and 3' terminal nucleotide sequences in two strains, TFBk and TFL-2, and complete sequencing of IS*Afe1* from strain TFBk revealed nucleotide substitutions in comparison with the prototype, IS*Afe1* element of *A. ferrooxidans* ATCC 19859. The partial sequencing of IS*T2* 5' and 3' terminal nucleotide sequences in strains TFO, TFBk, and TFL-2 revealed numerous nucleotide substitutions in comparison with the IS*T2* element of the prototype strain. Complete sequencing of IS*T2* from strain TFBk revealed 21.2% divergence between IS*T2* of strain TFBK and the prototype. Three *A. ferrooxidans* strains, TFV-1, TFN-d, and TFO, contained an IS element 600 bp long, named IS*Afe600*. The new IS element differed from the known ones in its high guanine and cytosine content. The strains differed in the number of copies of IS elements and in their localization on the chromosome DNA fragments. Strain TFBk contained the highest number of copies of IS elements.

Compared to *A. ferrooxidans*, other representatives of acidophilic chemolithotrophic microorganisms are poorly studied. However, in some of them (*S. sibiricus* and *F. acidiphilum*), strain polymorphism was reported in DNA G+C content and in the level of DNA homology [119, 135].

The structure of the chromosome DNA analyzed by pulse electrophoresis is the most characteristic manifestation of strain polymorphism in acidophilic chemolithotrophs. Every strain of *A. ferrooxidans*, *A. thiooxidans*, *S. thermosulfidooxidans*, *S. sibiricus*, and *F. acidiphilum* has a unique structure of the chromosome DNA [135–137].

These examples of strain polymorphism in acidophilic chemolithotrophic microorganisms demonstrate the divergence of their characteristics within a species, caused by natural selection of the clones best adapted to environmental conditions within genetically heterogeneous natural populations. A successful clone becomes the predominant one in the population and the progenitor of a new strain. Strain polymorphism is a stage in the formation of new microbial species in the course of accumulation of differences in important DNA nucleotide sequences encoding vitally important characteristics.

Environmental factors and strain polymorphism. The energy substrate, pH value, temperature, and concentrations of metal ions are the environmental factors most important for acidophilic chemolithotrophs. The effect of these factors on strains, primarily of *A. ferrooxidans*, was investigated in relation to their chromosome DNA structure as revealed by pulse electrophoresis, Southern hybridization of phosphorus-labeled plasmids with chromosome DNA fragments, and analysis of the number of copies and localization of IS elements on the fragments of chromosomal DNA.

Adaptation of *A. ferrooxidans* strains to pH values below optimum revealed the lowest pH (0.8) for growth and oxidation of the energy source (Fe^{2+}) [120]. No changes in the structure of chromosomal DNA were found in adapted strains compared to the original ones. The pH values reached in the course of adaptation were possibly within the reaction norm of each of the strains.

The effect of increased resistance to the ions of copper, arsenic, nickel, mercury, zinc, and ferric iron on the structure of chromosomal DNA was investigated. Each strain had a specific threshold concentration of metal ions, which enabled growth and oxidation of ferrous iron. Increase of resistance to nickel (0.18 g/l) and copper (20.0 g/l) did not result in changes of the chromosomal DNA structure. Increase of resistance to trivalent arsenic (from 1.5 g/l in the original strain VKM B-458 to 4.0 g/l in strain TFAs2) or to zinc ions (from 40 g/l in the original strain to 70 g/l in strain TFZ) resulted in amplification of individual DNA fragments: 28 kbp in the arsenic-resistant strain and 98 kbp in the zinc-resistant one [122]. Amplification of the 28 kbp DNA fragment also occurred in strain 458 after prolonged cultivation on media with arsenic-containing concentrates from the ores of several deposits [138]. These changes were reversible and disappeared after transfers on arsenic-free medium.

After prolonged cultivation of *A. ferrooxidans* TFI on the medium with gradually increasing concentrations of ferric iron, irreversible changes in the structure of chromosomal DNA were observed [118]. The strain TFI-Fe, resistant to 50 g/l of Fe^{3+} was obtained; the structure of its chromosomal DNA was altered compared to the parent strain. These structural changes did not disappear after multiple transfers of strain TFI-Fe in the medium with 9 g/l iron. Thus, a new strain with the chromosomal DNA structure changed compared to the parent strain and with increased resistance to Fe^{3+} ions

was obtained under experimental conditions of increasing concentrations of ferric iron. Formation of new *A. ferrooxidans* strains caused by changed concentrations of metal ions is evidently possible in nature.

The energy substrate is the environmental factor most important for acidophilic chemolithotrophs. Changes in the structure of chromosomal DNA were observed in the course of adaptation of five *A. ferrooxidans* strains (collection strain VKM B-458 and TFBk, TFN-d, TFO, and TFV-1 isolated from dense ore pulps) to new oxidized substrates (iron, elemental sulfur, pyrites from the ores of different deposits, arsenopyrite concentrate, and gold-arsenic pyrite-arsenopyrite concentrates from the ores of different deposits, including those containing pyrrhotite and copper ore). For example, changes in the chromosomal DNA structure were revealed in strain TFBk in the course of adaptation to elemental sulfur, pyrite from Akchatau deposits, and ore concentrates of the Olimpiadinskoe and Nezhdaninskoe deposits [138, 139]. Adaptation to the ore concentrate of the Nezhdaninskoe deposits resulted in changes to the chromosomal DNA structure in strains TFN-d and TFV-1. These changes were usually reversible and disappeared after transfer to the medium with ferrous iron. For two other strains, 458 and TFO, mutants with irreversible changes in the structure of chromosomal DNA were obtained as a result of energy substrate changes [138, 140]. Strain 458M was isolated after numerous transfers of strain 458 onto media with pyrite-arsenopyrite concentrates from ores of various deposits. In the mutant, the size of one fragment decreased by 10 kbp and the numbers of two other fragments changed. Unlike strain TFO, isolated from the surface ore of the Olimpiadinskoe deposit, strain FTO-2 was isolated from the ore concentrate of deeper levels of this deposit, with a lower degree of oxidation. Strains TFO and TFO-2 differed in the structure of chromosomal DNA. Thus, one strain was obtained experimentally, while the other was isolated due to the changes in the characteristics of the natural substrate.

In *S. thermosulfidooxidans* 41 strain 41 [136] and in an archaeon of the genus *Ferroplasma* [141], changes in the structure of chromosomal DNA in response to changes of the substrates were revealed.

The influence of the physical, chemical, and physicochemical characteristics of two pyrites with different types of conductivity (electron and vacancy-based) on the genotypic characteristics of two *A. ferrooxidans* strains, TFBk and TFV-1, isolated from different energy substrates was investigated [142]. In both strains adapted to pyrites (TFV-1 adapted to pyrite with electron-type conductivity and TFBk adapted to both pyrite types), changes in the chromosomal DNA structure were revealed. Changes in plasmid size and numbers were detected in strain TFV-1. In strain TFBk, the plasmid composition was the same on the medium with ferrous iron and after adaptation to pyrites.

Thus, the energy substrate was demonstrated to be one of the main environmental factors causing both reversible and irreversible changes in the structure of chromosomal DNA. It is therefore the factor which causes the microevolutionary processes leading to the formation of new strains.

The mechanisms causing strain polymorphism. Changes in the structure of chromosomal DNA can be the result of genome changes caused by mutation, recombination, and insertion (of IS elements, transposons, and plasmids). Plasmids are present in most *A. ferrooxidans* strains. They are believed to be cryptic. We revealed plasmids in all strains isolated from gold-containing pyrite-arsenopyrite ores and concentrates of complex composition, in 9 out of the 11 strains isolated from ores and concentrates of more simple composition containing nonferrous metals, and only in a half of the strains isolated from such substrates as mine water, pyritized coals, and activated sludge [129]. The simpler the substrate composition, the higher is the ratio of strains containing no plasmids. Plasmids, therefore, possibly participate in the regulation of the processes of oxidation of energy substrates.

Plasmid profiles were obtained for the strains grown on media with ferrous iron and then adapted to different substrates (elemental sulfur, pyrite, sulfide concentrate) [143–145]. For some strains (TFL-2, TFO, TFBk), the number of plasmid copies was shown to depend on the oxidizing substrate. The number of plasmids was changed as a result of adaptation of strain TFN-d to the concentrate and of strain TFV-1 to sulfur, pyrite, and concentrate.

Although the plasmids of this species are believed to be cryptic, our results indicate their possible contribution to the adaptation to changing environmental conditions, as well as to the information exchange between plasmid and chromosomal DNA and to the regulation of the activity of chromosomal genes. Localization of expressed genes of resistance to mercury on chromosomal DNA and of certain genes of a deficient mercury operon on *A. ferrooxidans* pTF-FC2 plasmid are examples of such exchange [146].

Laboratory simulation of the conditions inducing the genotypic variability revealed the possible role of plasmids and IS elements in the microevolution processes and in the emergence of strain polymorphism. Plasmids of strain TFBk were used as probes for Southern hybridization with the blots of macrorestriction profiles of the chromosomal DNA from several strains adapted to different energy substrates [145]. Weak hybridization signals were detected on many fragments of the chromosomal DNA of all the strains investigated. Localization of the bands with weak hybridization signals changed in some cases of the adaptation to new oxidized substrates. These results demonstrated that multiple nucleotide sequences complementary to the sequences of the strain TFBk plasmid were present in the chromosomal DNA of all these strains. These

sequences belong to IS elements, as is indicated by their small size (evidenced by the low intensity of hybridization signals), their multiple number, and localization on chromosomal and plasmid DNA. Changes in the localization of IS elements in the course of adaptation to new oxidation substrates suggest their participation in the regulation of the processes of oxidation of new energy sources. This hypothesis was initially proposed by Holmes and Haq [146]; they suggested the role of mobile genomic elements in the mechanism of adaptation to changing environmental conditions, in the evolution of gene structure and function, in the regulation of expression of the biochemical pathways, and in exchange of genetic information between strains.

In some cases, for example, in strains TFO and TFN-d adapted to pyrite, apart from multiple weak hybridization signals, stronger DNA hybridization with pTFK2 plasmid was revealed. The cells of these strains contain plasmids with extensive nucleotide sequences homologous to pTFK2 plasmid of strain TFBk. Strong hybridization signals were possibly the result of incorporation of the plasmid DNA into the chromosome in the course of metabolic switching to the oxidation of a new energy substrate.

Thus, the possibility was demonstrated for interaction between the plasmid and chromosomal DNA in *A. ferrooxidans* strains in the course of their adaptation to new energy substrates. This mechanism may be involved in the intraspecific variability underlining the microevolution processes, which result in genotypic and phenotypic strain polymorphism.

The role of IS elements in the adaptation to new energy substrates was demonstrated in experiments on localization and the number of copies of IS elements in chromosomal DNA fragments of *A. ferrooxidans* grown on media with ferrous iron or adapted to elemental sulfur [134]. The labeled DNA of IS*T2* element, ^{32}P-labeled DNA of the complete IS*Afe1* element, and the ^{32}P-labeled internal part of the IS*Afe1* element were used as a probe for Southern hybridization; the latter probe is unique and has no homologies with the known sequenced prokaryotic nucleotide sequences. The labeled probes were hybridized with *Eco*RI restricts of five *A. ferrooxidans* strains. Changes in the number and intensity of hybridization bands were revealed in the course of adaptation of *A. ferrooxidans* strains to elemental sulfur. The reaction of IS elements to the adaptation to a new energy substrate varied from strain to strain. Strains TFBk and TFO exhibited the most pronounced changes; in the latter case, adaptation to sulfur resulted in numerous hybridization bands in *Eco*RI restricts which were not present in the strain grown on ferrous iron. Strain TFO was originally isolated from the pyrrhotite-containing concentrate of a pyrite-arsenopyrite ore; its oxidation results in the formation of large amounts of elemental sulfur. This strain was more active in oxidizing elemental sulfur and quicker to adapt to the new substrate than strain TFBk [119]. In the course of adaptation to elemental sulfur, transposition of IS*T2* elements to new loci was revealed in both strains TFO and TFBk and transposition of IS*Afe1* elements was revealed in strain TFO. When the complete IS*Afe1* element was used as a probe for Southern hybridization, changes in the localization of IS elements were detected in all the *A. ferrooxidans* strains studied. These results indicate the role of IS elements in the regulation of metabolic switching from ferrous iron to sulfur oxidation. In some strains (TFO), IS*Afe1* and in others (TFBk), IS*T2* elements probably play the major part in the adaptation to elemental sulfur.

Transpositions of IS elements in *A. ferrooxidans* chromosomal DNA caused by changes in the cultivation conditions have been described previously. For example, genomic localization of repeating IS*T1* DNA elements (IS*Afe1*) was analyzed before and after several months' transfers of *A. ferrooxidans* ATCC 19859 on copper-containing silicon waste [147]. Changes in the position of one of the copies of the IS*T1* element were revealed. In the cells adapted to the Cu^{2+} concentrations toxic for the culture, other positional changes were observed. The authors concluded that the changes in the localization of IS elements were related to the process of adaptation and that the genetic instability of bacteria containing IS elements could provide for the genotypic and phenotypic flexibility in response to changing environmental conditions. Other authors also reported the phenotypic changes of *A. ferrooxidans* strains caused by changes in the localization of IS elements [147–150].

The ability to adapt to changes in the environment is the main characteristic of every living organism. The genotypic and phenotypic heterogeneity of every natural microbial population is the result of mutations, chromosomal recombinations, the presence of labile genomic elements (plasmids, transposons, and IS elements), and of their participation in genetic variability.

The wide distribution of acidophilic chemolithotrophic microorganisms in nature and their high variability are the result of the diversity of conditions in their habitats, primarily with respect to the broad spectrum of such oxidized energy substrates as sulfide minerals; their combination and quantitative proportion in sulfide ores is variable. For example, several strains of the same species *S. sibiricus* differing in the structure of chromosomal DNA were recovered from a small parcel (4 m^2) in the zone of spontaneous heating of a pyrite-arsenopyrite ore [135]. The strains differed in the rates of oxidation of ferrous iron, elemental sulfur, and individual sulfide minerals; their optimal temperature and pH values were different (unpublished results). Evidently, an active process of strain microevolution occurs, which helps the species to maintain viability under changing environmental conditions.

These processes occur under technological conditions. For example, a change of the *A. ferrooxidans*

strain dominant in the oxidation of the zinc-containing industrial product occurred after the pH of the medium was experimentally changed and maintained for at least a month at the new level [118]. Decrease in the degree of ore oxidation and increase in its antimony content also resulted in the substitution of the microbial strains predominant in the process of biohydrometallurgy [140].

It was stated above that changes in the energy substrate and increase of the concentration of metal ions in experiments with pure cultures of acidophilic chemolithotrophic microorganisms often result in changes in the structure of chromosomal DNA (including localization of IS elements on chromosomal DNA), of the number of plasmids and their copies. This may be the result of either the genetic heterogeneity of the population under study, or of adaptive mutagenesis. Although microbial strains for every experiment were obtained from individual colonies, the population became genetically heterogeneous after one cell division. Thus, the changes in chromosomal DNA structure which were revealed in a strain which had been adapted, for example, to a new energy substrate for several transfers, could have been explained by the substitution of another clone within the population, more adapted to the oxidation of the new energy source. In such a case, reversal to the original energy substrate would have also required several transfers for all the changes in the chromosomal DNA structure to disappear. If the changes in the structure of chromosomal DNA disappeared in the first transfer to the original energy substrate, they were possibly the result of adaptive mutagenesis caused by other mechanisms (reversible intrachromosomal or plasmid–chromosomal recombinations, changes in numbers or localization of IS elements). Evidently, the same mechanisms of adaptation of microbial populations to environmental changes operate under natural conditions as well.

Pure microbial cultures are unknown to nature. Microbial associations participating in decomposition of specific substrates and exchanging products of their metabolism, as well as microbial communities of a certain ecological niche are constantly changing systems, which respond to environmental changes and thus enable the survival of the community as a whole and of most of its component species.

Acidophily of Chemolithotrophs

Hot solutions, including acidic ones, are the normal environment for many chemolithotrophic bacteria and archaea, which oxidize sulfur or sulfide minerals. This is their main physiological characteristic. Sulfuric acid is the end product of microbial oxidation. However, the nature of acidophily is not completely understood. Since the surface structures contact with the medium at pH 0 and higher, while the intracellular pH is about 5.5–6.8 [151–153], the role of these structures is certainly important.

Morphological heterogeneity of the cellular surface structures. Acidophilic chemolithotrophs exhibit a great diversity of cellular surface structures, even under similar acidophilic conditions with elevated concentrations of metal ions, in a broad temperature range.

The cell wall of gram-negative acidophilic chemolithotrophs is typical for this bacterial group. It includes an external triple membrane, a murein layer, and periplasmic space. Depending on environmental conditions, mucous microcapsules can be present. Surface membrane structures were revealed in *A. thiooxidans* grown on elemental sulfur [154, 155].

The cell wall of gram-positive acidophilic sulfobacilli is typical for this bacterial group. It includes a peptidoglycan layer with an adjacent S layer. The S layer consists of regularly organized protein or glycoprotein subunits. These are ordered to such a high degree that the structure can be termed paracrystalline [156–158]. The specific porous structure of S layers is the result of their paracrystalline nature.

In acidophilic archaea, the S layer is the only component of the cell wall, attached directly to the cytoplasmic membrane [159, 160]. Acidophilic archaea of the genus *Ferroplasma* lack the cell wall completely [97].

The periplasmic space is the most important compartmented structure of the cell wall [160], especially the periplasm, i.e., the functionally active compounds of the periplasmic space. The fact that the pH there is close to external values (~2.0 and lower) and the proteins (enzymes) are acid-resistant is important. Thus, the cytoplasmic membrane, rather than the surface structures that have other important functions, is the main chemical barrier between the cell and its environment.

Many authors have demonstrated that the periplasmic space is one of the most active and important metabolic centers of a prokaryotic cell [159, 160]. Beveridge [160] maintained that all bacteria, with rare exceptions, possess a periplasm. The periplasm, i.e., the functionally active compounds of the cellular surface structures, must be present, although not necessarily as a compartmentalized structure. The periplasm of archaea and gram-positive bacteria may be integrated with their S layers.

It is still difficult to explain how archaea without cell walls, such as *Ferroplasma* and *Thermoplasma*, function under acidic conditions. The cytoplasmic membrane evidently performs all the functions of the surface structures; alternatively, some yet unknown lipid structures may exist on the membrane surface.

Analysis of the chemical composition of the cell walls revealed that in gram-negative bacteria *A. ferrooxidans*, lipopolysaccharides are among its major components, with phosphatidyl serine as the major component of the phospholipid part of the lipopolysaccharide complex, which was found both in the cells and in the medium. The amino acid composition of S layers of some archaea and eubacteria was investigated. It was

characterized by the prevalence of acidic amino acids over basic ones. For example, acidic amino acids constituted 21.5% of the *S. thermosulfidooxidans* S layer, and basic ones, 8.2% [156].

Interestingly, the S layer proteins of gram-positive bacteria, including chemolithotrophic sulfobacilli are similar to those of sulfur-oxidizing archaea [156]. The S layers, being proteins with paracrystalline structure can possibly be understood, at least in certain functional respects, as specific surface biological membranes. The functional unity is evident in all the morphological diversity of the surface structures of chemolithotrophic eubacteria and archaea.

Heterogeneity of lipid composition. The surface structures contain proteins which are stable at low pH values. The physiologically active compounds contained therein function at low pH. In the cytoplasm, under near-neutral pH, the conditions are entirely different.

The cytoplasmic membrane, specifically its lipids that maintain its stability and the proton gradient, acts as a biochemical border between the cell surface and the cytoplasm.

The lipid composition of acidophilic archaea is known to be unique [161, 162]. Unlike eubacteria, the lipids of thermophilic archaea *Sulfolobus* do not contain glycerol esters of fatty acids. They mostly contain isoprenoid and hydroisoprenoid hydrocarbons and isoprenyl glycerol esters. The lipids of thermophilic archaea *Acidianus* consist of isoprenoid esters, those of *Thermoplasma*, of dibiphytanilic (C_{40}) diglycerol tetraesters. The main lipid components of mesophilic archaea *Ferroplasma* are β-D-glycopyranosyl caldarchaetidyglycerol (~55%) and trihexosyl caldarchaetidyglycerol (26%) [163]. Depending on the growth conditions, up to 60% of ω-cyclohexylundecanic and up to 10% of ω-cyclohexyltridecanic acid are found in gram-positive moderately thermophilic bacteria *Sulfobacillus.* Up to 3% of ω-cyclohexyl-α-oxyundecanic acid was also detected. These acids are present in heterotrophic *Alicyclobacillus*. Branched fatty acids, mostly antheiso acids constitute the main part of the fatty acids of sulfobacilli [164].

Gram-negative acidophilic *Acidithiobacillus* are rich in lipopolysaccharides and phospholipids.

The nature of acidophily. The major differences in cell structure and biochemistry between acidophilic chemolithotrophic microorganisms did not enable us to discern specific information concerning the nature of their acidophilic behavior. The membrane lipids are known to play an important role in maintaining the constant cellular pH and the membrane proton gradient; they also provide for the functioning of the membrane-bound ATP synthase. Concerning acidophilic archaea with proton-impermeable tetraester membranes, Maklady and coworkers [165] suggested that the lipid monolayer of the membrane played the key role in survival in highly acidic conditions. This concept can possibly be applied to other acidophilic microorganisms, with other types of membrane lipids performing similar functions.

Kinetic and Metabolic Characteristics of Acidophilic Chemolithotrophs

Chemolithotrophic growth. Acidophilic chemolithotrophs can be subdivided into two groups, obligate chemolithoautotrophs and mixotrophs (or, in some cases, facultative autotrophs). Only mesophilic bacteria of the genera *Acidithiobacillus* and *Leptospirillum* and archaea of the genus *Ferroplasma* (*F. acidiphilum*) are obligate acidophilic chemolithoautotrophs (Tables 3, 4). The moderately thermophilic *Sulfobacillus* and probably practically all the thermophilic and moderately thermophilic archaea are mixotrophs (Tables 3, 5). It was demonstrated for many strains of sulfobacilli that stable growth occurred only under mixotrophic conditions; autotrophic or heterotrophic growth resulted in lysis after between two and ten transfers, depending on the strain. There is less information concerning archaeal behavior in this respect. In some hydrotherms where CO_2 is available via abyssal supply, autotrophic metabolism of certain archaea can probably be expected in the presence of sulfur or H_2.

Acidophilic chemolithotrophs, whatever their taxonomic position, have relatively low growth rates on mineral substrates (Table 6). In obligate mesophilic autotrophs, e.g., in archaeal *Ferroplasma*, it is lower. The rate of CO_2 fixation by *F. acidiphilum* is ca. 12.5 times lower than by *A. ferrooxidans* under optimal conditions (Table 7) [98]. For the moderately thermophilic bacteria *Sulfobacillus*, the highest growth rate was observed under mixotrophic conditions, with Fe^{2+} and S^0 as electron donors. Growth under autotrophic or heterotrophic conditions is weak and occurs only for several transfers. With the exception of *A. infernus*, the archaea oxidizing only S^0 have lower growth rates (Table 5).

Unfortunately, in most of these studies, growth kinetics was analyzed for a limited number of strains of one and the same species; no adaptation for the substrates or other growth conditions was performed. Fig. 3 demonstrates the different rates of S^0 oxidation by various aboriginal *A. ferrooxidans* strains; all of them were less active than *A. thiooxidans* T. 3S. These differences are the result of their previous history in the environment, primarily related to their sulfur sources (the type of sulfide minerals and the presence of sulfur). Adaptation to a substrate can considerably enhance growth and oxidation of Fe^{2+}, S^0, and sulfide minerals by the strains (Fig. 4–6).

The ecology of the strains, their pheno- and genotypic polymorphism and adaptive variability are usually not considered in such works. Together with the application of artificial growth media, this constitutes a

Table 6. Growth rates (μ_{max}, h^{-1}) and generation times (t_d, h) for some acidophilic chemolithotrophic strains under different growth conditions

Microorganisms (substrate)	μ_{max}/t_d			Microorganisms (substrate)	μ_{max}/t_d		
	Autotrophic	Mixotrophic with yeast extract	Heterotrophic		Autotrophic	Mixotrophic with yeast extract	Heterotrophic
Moderately thermophilic mixotrophs				*S. hakonensis* (S^0)	–	0.075 / 9.2	–
S. thermosulfidooxidans subsp. *asporogenes*, 41 (Fe^{2+})	0.035 / 19.8	0.33 / 2.1	–	*A. infernus* (S^0)	–	0.028 / 2.5	–
" (S^0)	–	0.21 / 3.3	–	*A. ambivalens* (S^0)	–	0.17 / 4.0	–
Gold-arsenic sulfide concentrate	–	0.14 / 5	–	*F. acidiphilum* (Fe^{2+})	0.016 / 43.3	–	–
S. thermosulfidooxidans, 1269 (Fe^{2+})	0.09 / 7.7	0.29 / 2.39	0.09 / 7.7	Mesophilic autotrophs			
S. sibiricus, N 1 (Fe^{2+})	0.06 / 11.55	0.53 / 1.3	0.063 / 11	*A. ferrooxidans* (Fe^{2+})	0.19 / 3.65	–	–
S. thermotolerans, Kr1 (S^0)	–	0.38 / 1.82	–	*A. ferrooxidans* ($S_2O_3^{2-}$)	0.05 / 14	–	–
" (Fe^{2+})	–	0.35 / 1.98	–	" (S^0)	0.069 / 10	–	–
S. acidophilus, N, ALV, NAL, 2B	–	–	0.12 / 5.78	" ($CuFeS_2$)	0.05 / 14	–	–
", BC1, 3C, LM1	–	–	0.087 / 7.97	" (CuS, Cu_2S, Cu_2FeS_4)	0.04 / 18	–	–
Archaea				*A. thiooxidans* (S^0)	0.046 / 15	–	–
S. yellowstonensis (Fe^{2+})	–	0.023 / 30	0.013 / 53.3 (on fructose)	*L. ferrooxidans* (Fe^{2+})	0.069 / 10	–	–
" (S^0)	–	0.033 / 21		Acidophilic heterotrophs			
" (FeS_2)	–	0.004 / 173.3	–	*Alicyclobacillus*	–	–	0.46 / 1.5
M. sedula (ore)	–	0.13 / 5.3	–				

serous shortcoming. As A.J. Kluyver stated, many of the pure cultures were physiological artifacts [quote 2].

In natural or stable technogenic environments, chemolithotrophic communities participate in the transformations of complex sulfide concentrates. They either compete for the substrate, as Winogradsky described, or interact (e.g., chemolithoautotrophs provide organic compounds for the mixotrophic sulfobacilli and archaea). Laboratory results on growth kinetics can therefore reflect only growth under specific conditions.

Carbon metabolism in chemolithotrophs. Even a century after its discovery, autotrophy, including obligate autotrophy still requires a satisfactory explanation [166].

Table 7. $^{14}CO_2$ fixation by strains Y^T and Y-2 in comparison with *A. ferrooxidans*

Microorganism	Cell radioactivity, pulse/(min mg protein)	CO_2 fixation, nmol ^{14}C/(min mg protein)
A. ferrooxidans	190592	0.125
F. acidiphilum Y-2	28258	0.01
F. acidiphilum Y^T	30783	0.01

The concept of obligate autotrophs emerged after the discovery of chemosynthesis by Winogradsky.

A number of organic compounds are toxic to chemolithoautotrophs for various reasons, such as penetration into the cell and affecting the intracellular pH or disruption of the regulatory metabolic mechanisms. However, formic acid was demonstrated to act as the sole energy source for *A. ferrooxidans* [167].

Analyzing obligate methanotrophy and obligate autotrophy in chemo- and photolithotrophic bacteria, Wood et al. [166] hypothesized that the absence of active α-ketoglutarate dehydrogenase could be the reason for autotrophy. This implies an incomplete TCA cycle broken at the level of 2-oxoglutarate; such a cycle can perform only biosynthetic functions. However, mixotrophic *Sulfobacillus* exhibit the same feature. They possess all the enzymes of the TCA cycle, except for 2-oxoketoglutarate dehydrogenase [168]. Sulfobacilli are known to be capable of autotrophic or heterotrophic growth for several transfers, although they lyse afterwards. The specificity of autotrophic genomes is, most probably, the essence of autotrophy. All the autotrophs have genomes of a small size; this size, however, should be sufficient for obligate autotrophy [169]. The average genome size measured for 23 *A. ferrooxidans* strains was 3.8 Mbp [121]. The most common genome size was 3.6–3.8 Mbp. Other authors determined the chromosome size of autotrophic *A. ferrooxidans* (eight strains), *A. thiooxidans*, and *L. ferrooxidans* as 2.29–3.33, 3.14, and 1.90 Mbp, respectively [170, 171]. Genome size of mixotrophic acidophilic *S. thermosulfidooxidans* 1269, *S. sibiricus* N1, and *S. thermosulfidooxidans* subsp. *asporogenes* 41 was determined as 5.85, 5.53, and 4.7 Mbp, respectively [84, 85, 172]. These values are close to those of such heterotrophs as *E. coli* (4.64 Mbp), *B. subtilis* (4.21 Mbp), and *Streptomyces* (8.66 Mbp). Microorganisms with greater genome size probably have greater metabolic possibilities due to the expression of a greater number of active enzymes.

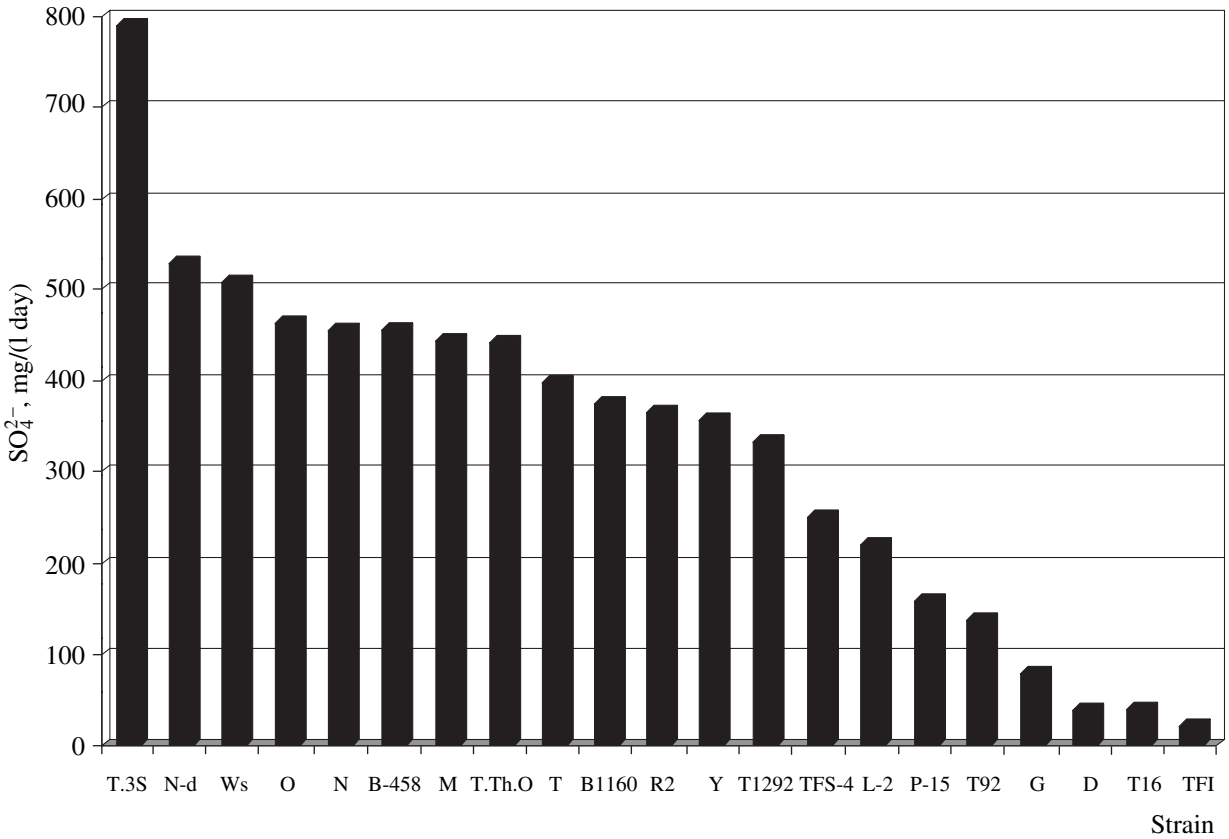

Fig. 3. Rates of growth and S^0 oxidation by *A. ferrooxidans* strains.

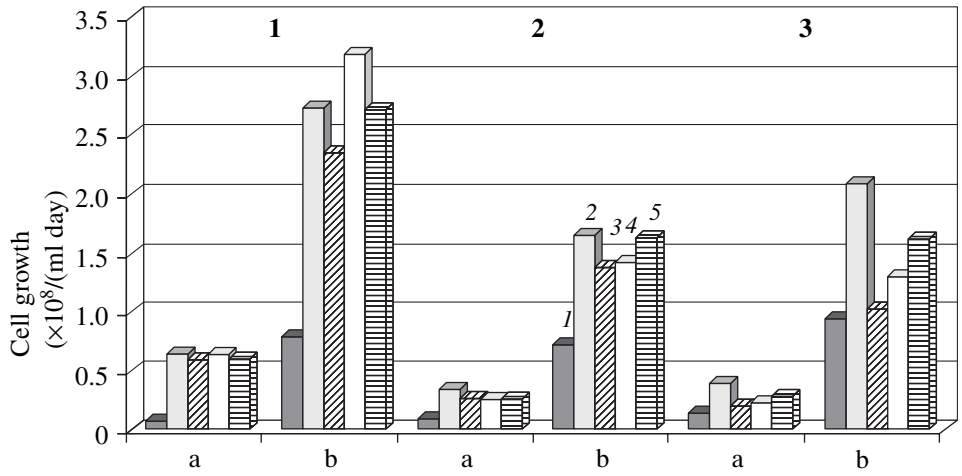

Fig. 4 Growth of original (a) and adapted (b) *A. ferrooxidans* strains on the medium with S^0 (**1**), FeS_2 (**2**), or concentrate (**3**). Strains: *1*, TFV-1; *2*, TFN-d; *3*, TFBk; *4*, TFO; *5*, TFL-2.

Chemolithoautotrophs are slow-growing, highly specialized microorganisms with limited metabolic capabilities.

Biochemistry of CO_2 assimilation. The enzymatic conversion of CO_2 to the carboxyl of 3-phosphoglycerate, reduction of this product to triosephosphate and regeneration of the CO_2 acceptor occur in the well-known Calvin–Benson reductive pentose phosphate cycle. RubisCO, the key enzyme of this cycle, was found in the extracts of practically all the known mesophilic chemolithotrophic aerobes and facultative anaerobes. It catalyzes the initial stage of carbon assimilation via the Calvin cycle. Dissolved nonhydrated CO_2, rather than bicarbonate ion, is the real substrate of this enzyme. Multiple copies of two sets of RubisCO genes were revealed in the *A. ferrooxidans* genome [124–126]. Two forms of RubisCO differing in molecular weight (460 and 640 kDa), in their affinity to the substrates, and in the relative role of carboxylase and oxygenase functions were revealed in *A. thiooxidans* [173]. We detected RubisCO in all the studied strains of sulfobacilli [174–176]. These organisms are mixotrophic and survive only a few transfers under autotrophic or heterotrophic conditions. The maximal RubisCO activity under autotrophic, mixotrophic, and heterotrophic conditions was 44.6, 18.0, and 1.5 nmol CO_2 /min mg protein respectively for *S. thermosulfidooxidans* [174] and 12.8, 7.0, and 4.8 nmol CO_2/(min mg protein) respectively for *S. sibiricus* N1 [175]. Yeast extract (0.02%) was added for mixotrophic growth. However, high activity of this carboxylase in the cells of strain 41 (31.0 nmol CO_2/(min mg protein)) persisted after the introduction of 0.02–0.05% glucose as a carbon source. The inability of sulfobacilli to grow autotrophically is the result of some factor other than the activity of these carboxylases.

Phosphoenolpyruvate carboxylase (FEP carboxylase) and pyruvate carboxylase activities were revealed in sulfobacillar strains 41 and N1. The highest FEP carboxylase activity in *S. thermosulfidooxidans* 41 was achieved under mixotrophic conditions with yeast extract and Fe^{2+} (20.7 nmol/(min mg protein)); under autotrophic and heterotrophic conditions, it decreased to 8.7 and 0.2 nmol/(min mg protein) respectively [174]. The level of this enzyme activity in strain N1 was lower under autotrophic (1.8 nmol/(min mg protein)) and mixotrophic (0.6 nmol/(min mg protein)) conditions than during heterotrophic growth (2.2 nmol/(min mg protein)) [175]. Both strains exhibited low pyruvate carboxylase activity under various growth conditions. *S. sibiricus* N1 grown mixotrophically had the highest activity (9.8 nmol/(min mg protein)).

Reactions of pyruvate and phosphoenolpyruvate carboxylation leading to oxalacetate regeneration are probably one of the mechanisms which supply the TCA cycle with amino acids precursors.

Carbohydrate metabolism. Carbohydrate metabolism is best studied for some sulfobacilli [175–177]. The key enzymes of the pentosephosphate pathway and

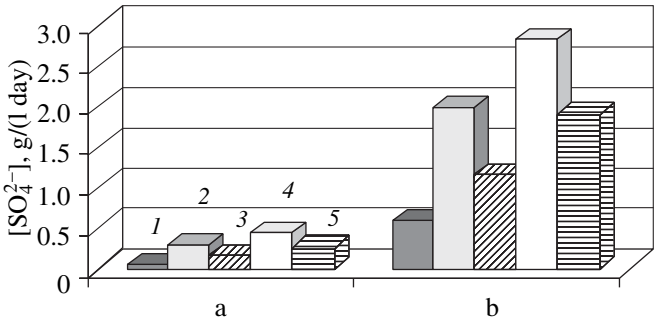

Fig. 5. Oxidation of S^0 by original (a) and adapted (b) *A. ferrooxidans* strains. Strain designations are the same as on Fig. 4.

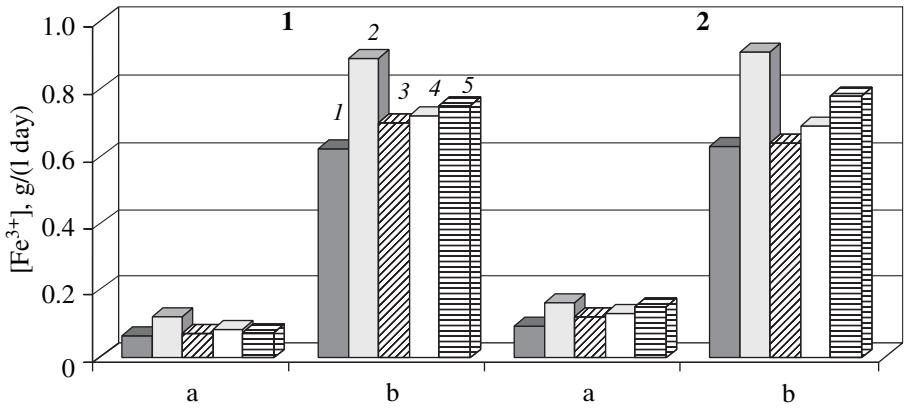

Fig. 6. Oxidation of FeS_2 (**1**) and concentrate (**2**) by original (a) and adapted (b) *A. ferrooxidans* strains. Strain designations are the same as on Fig. 4.

of the Embden–Meyerhof–Parnas and Entner–Doudoroff pathways were detected in the cell-free extracts of *S. thermosulfidooxidans* 1269 and 41; only the first two pathways were active in *S. sibiricus* N1, while the Entner–Doudoroff pathway was not involved in sugar metabolism. In *S. acidophilus* ALV, the activity of the key enzymes of the Entner–Doudoroff pathway was also not detected [178]. However, both the activity of the enzymes of carbohydrate metabolism and the amount of sugar utilized indicate that these two strains, N1 and AVL, are more tolerant to heterotrophic conditions than strains 1269 and 41.

Functions of the TCA cycle. Like other microorganisms, chemolithotrophs perform the oxidation of organic substrates via TCA cycle reactions. Chemolithotrophs lack the complete TCA cycle, as was mentioned above; it is opened due to the absence of 2-oxoglutarate dehydrogenase. Its individual reactions, however, function under all growth conditions. They perform biosynthetic functions. In none of the studied thiobacilli, was the activity of isocitrate lyase, one of the key enzymes of the glyoxylate pathway, revealed. The glyoxylate pathway in these organisms is therefore inactive [174, 175].

Since under mixotrophic conditions, protein accumulation by sulfobacilli is maximal and the enzymes of carbohydrate metabolism are more active, sulfobacilli can probably use glucose oxidation as a supplementary energy source for mixotrophic growth. Growth under these conditions is stable.

Carbon metabolism in chemolithotrophic archaea is scarcely studied. CO_2 assimilation in the autotrophically growing thermophilic archaeon *S. brierleyi* (*A. brierleyi*) was reported to occur via the reductive TCA cycle [179].

Bioenergetics of acidophilic chemolithotrophs. Oxidation of inorganic substrates and energy generation by acidophilic chemolithotrophic microorganisms occurs in accordance to P. Mitchel's chemiosmotic theory. The process is best studied in the mesophilic *A. ferrooxidans* grown on Fe^{2+} [151, 152, 180]. This ion penetrates into the periplasmic space (probably in a complex with lipopolysaccharides); the electrons ($Fe^{2+} \longrightarrow Fe^{3+} + \bar{e}$) are then transported to the terminal acceptor, O_2, via the electron-transport chain. This system includes the porins of the external membrane; Fe^{2+} oxidase bound to cytochrome c_{552}; at least one c_4 type of cytochrome c_{552}; rusticyanin, a small, copper-containing protein; and the terminal cytochrome *c* oxidase. Electron transport along the ETC generates the transmembrane electrochemical gradient of hydrogen ions ($\Delta\mu_{H^+}$), a combination of the electrochemical ($\Delta\psi$) and the chemical (gradient of H^+ concentration ΔpH; pH ~2.0 in the medium and in the surface structures and pH 6.5–6.8 in the cytosol) components. Proton influx into the cells is performed by H^+ translocating ATPase, and the membrane potential of 256 mV for ATP synthesis is generated. The protons are consumed via an oxidase reaction. The mechanism of intracellular pH regulation is also coupled with the second half of the Fe^{2+} oxidation reaction ($2\bar{e} + 2H^+ + 1/2O_2 \longrightarrow H_2O$). Thus, microbial anodic oxidation of mineral substrates is a typical electrochemical reaction catalyzed by enzymes. A high-molecular-weight cytochrome *c* (22 kDa) was recently isolated from the cell wall membrane [181]. It probably acts as the first electron transporter in the course of Fe^{2+} oxidation in the electron transport chain.

The sulfur-oxidizing system of mesophilic chemolithotrophs is known to a lesser degree. In *A. ferrooxidans*, the enzymes sulfur(sulfide)-Fe^{3+} oxidoreductase ($S^0 + 4Fe^{3+} + 3H_2O \longrightarrow H_2SO_3 + 4Fe^{2+} + 4H^+$), sulfite-$Fe^{2+}$ oxidoreductase ($H_2SO_3 + 2Fe^{3+} + 4H_2O \longrightarrow H_2SO_4 + 2Fe^{2+} + 2H^+$), and sulfite-cytochrome *c* oxidoreductase were revealed [182]. *A. thiooxidans* contains membrane-bound sulfite oxidase with a high molecular mass (400 kDa) resembling the sulfite-Fe^{3+}oxidoreductase of Fe^{2+}-grown *A. ferrooxidans* (650 kDa). Mansch and Sand [183] believe

that cytochromes *b* and aa_3 constitute a part of the *A. ferrooxidans* sulfur-oxidizing system. Since sulfur oxidation by this organism was strongly inhibited by CN^- and N_3^-, terminal oxidases were probably involved in this process. The cells of *A. ferrooxidans* grown on sulfur contained S^0 dioxygenase, thiosulfate dehydrogenase, rhodanese, APS reductase, and sulfite oxygenase. Mixotrophically grown *Sulfobacillus* strains oxidizing $S_4O_6^{2-}$ and S^0 exhibited activity of thiosulfate oxidase, tetrathionate hydrolase, rhodanese, and S^0 oxygenase [176, 183]. This enzymatic system is similar to that of acidithiobacilli.

The enzymatic electron transporting systems of *L. ferrooxidans* are scarcely studied. Sugio and coworkers [184] detected sulfur(sulfide)-Fe^{3+}-OH oxidoreductase, although its activity was low. Rusticyanin, a copper-containing protein, was not found [185]. The cells grown with Fe^{2+} contained large amounts of a red, acid-resistant, acid-soluble cytochrome *c* with an adsorption peak at 579 nm. This is the key protein in the respiratory chain of these bacteria [186].

A number of experimental articles dealt with the organization of the respiratory chain of acidophilic archaea. Many of these works were summarized by Schafer and coworkers [187].

A. ambivalens was found to contain the simplest aerobic respiratory chain. It consisted of reduced NADH dehydrogenase of type II, succinate: quinone oxidoreductase [188], caldariellaquinone, and a simple aa_3-type terminal quinone oxidase [189–192].

The respiratory chain of *S. acidocaldarius* is much more complex. It includes two different terminal oxidase complexes, the SoxABCD complex and SoxM supercomplex. SoxM includes FeS/a^{587}, sulfocyanin (a blue copper-containing protein), and cytochrome ba_3. SoxABCD includes cytochromes a^{587}/ and aa_3 [193–195]. The major NADH dehydrogenase of *S. metallicus* is a type II enzyme with unusual properties. Its flavin group is covalently bound and contains flavin mononucleotide instead of flavin dinucleotide [196]. The spectroscopic analysis of the respiratory chain revealed, however, a new type of iron–sulfur clusters and certain respiratory enzymes [187, 197]. NADH dehydrogenase (complex I), an important component of the bacterial respiratory chain, was not detected in *Sulfolobales* [187, 198]. The representatives of *Sulfolobales* contain a unique type of quinones. Apart from caldariellaquinone (CQ) [199, 200], benzo-[β]-thiophene-4,7 quinone, termed "sulfolobus quinone" (SQ) was found in large amounts in *A. infernus* and *A. ambivalens* and in trace amounts in *S. acidocaldarius* [201]. These components of the respiratory chain vary with growth conditions. Aerobically grown *A. ambivalens* cells contained only 1/3 of the SQ amount present in anaerobically grown cells. In *A. infernus*, SQ was not present under aerobic conditions and CQ was detected under anaerobic conditions. Strain *Sulfolobus* MT4 contained a tricyclic benzo[1,2-β; 4,5-β] dithiophene-4,8 quinone [202]. This was not found in *M. sedula* [89]; in *S. solfataricus*, the ratio between SQ and CQ varied significantly depending on the growth temperature [203].

Activity of CoA-acylated 2-oxoid:ferredoxin oxidoreductase was detected in cell-free extracts of *S. acidocaldarius* [204]. Two $[4Fe\text{-}4S]^{2+(2+,\,1+)}$ clusters per ferredoxin molecule are probably present. A [3Fe-4s] cluster was found in *Sulfolobales*. Ferredoxin is a small monomeric protein with one $[3Fe\text{-}4S]^{+/0}$ center and one $[4Fe\text{-}4S]^{2+/+}$] center [205, 206]. The subsequent elements of the *S. acidocaldarius* respiratory chain consist of *a*- and *b*-type cytochromes [207, 208]. The genes encoding Rieske proteins and cytochromes *b* were detected in genomes of various *Sulfolobus* species [198, 209]. Type *c* cytochromes are not present in Sulfolobales.

The electron-transport chain of *Ferroplasma* archaea (type II, organotrophic growth) was reconstructed using the sequence library created by environmental genome shotgun. A presumable terminal oxidase containing copper and iron and cytochrome *b* associated with an in iron-sulfur Rieske protein and a blue copper-containing protein were revealed [210]. These proteins are supposed to form a terminal oxidase supercomplex, similar to the *S. acidocaldarius* SoxN supercomplex. The unique respiratory supercomplex had been previously demonstrated to combine the features of quinol and cytochrome *c* oxidase [211].

The sequencing of the blue copper-containing protein of some *Ferroplasma* strains revealed homology with *A. ferrooxidans* rusticyanin and *Sulfolobus* sulfocyanin (SoxE). This protein was probably a component of the electron transport chain used for Fe^{2+} oxidation and heterotrophic growth [210]. Analysis of the level of protein expression in several strains closely related to the chemoorganotrophic species *F. acidarmanus* revealed that Fe^{2+} oxidation can occur via a copper-containing heme protein sulfocyanin; it transports electrons to the terminal electron acceptor, cbb_3 [212]. Sulfocyanin (596 nm) was present in the membrane during mixotrophic growth, while during chemoorganotrophic growth its content decreased drastically.

The electron transport chain is probably basically similar in various chemolithotrophic acidophilic archaea, although its deeper study may reveal certain differences. It is, however, different from the ETC of bacteria *Acidithiobacillus*, *Sulfobacillus, Leptospirillum*, and some others.

The mechanism of ATP formation in acidophilic chemolithotrophic archaea is possibly also related to the existence of two systems of proton translocation, by the ETC and by H^+-ATPase. As with chemolithotrophic bacteria, the activity of these systems results in the formation of the transmembrane electrochemical proton potential and in ATP synthesis. Membrane-bound ATPases were isolated from a number of representatives of *Sulfolobus*.

Apart from ATP, reduced pyridine nucleotide ($HADH_2$) is required for CO_2 fixation. It has a low potential of ca. –320 mV. The substrates oxidized by chemolithotrophs (electron donors: Fe^{2+}, S^0, etc.) have higher potentials (+420 mV for Fe^{2+}). They are incorporated into the respiration chain at the cytochrome level and therefore obviously cannot reduce NAD^+. A system of reverse electron transport exists at least in acidithiobacilli (*A. ferrooxidans* and *A. thiooxidans*) and in some other chemolithotrophic bacteria; this system consumes energy as ATP [213].

The mechanisms of oxidation of inorganic substrates (Fe^{2+}, S^{2-}/S^0, and sulfide minerals) by acidophilic chemolithotrophic archaea are insufficiently studied [214]. The pathway of sulfur oxidation in *A. ambivalens* was described. Soluble sulfur oxidoreductase (SOR) catalyses initial stages of sulfur oxidation by sulfur disproportionation with sulfite, thiosulfate, and sulfide, the products of these reactions. Thiosulfate is oxidized to $S_4O_6^{2-}$ by a new type of thiosulfate:quinone oxidoreductase [215].

Sulfite, another SOR product, is oxidized by two different pathways [216]. Membrane-bound sulfite:acceptor oxidoreductase activity was revealed. Soluble enzymes, including adenylyl sulfate adenylyl transferase and adenosine phosphate reductase, also oxidize sulfite with energy conservation via substrate phosphorylation.

CONCLUSIONS

Winogradsky's concept of chemolithotrophy and chemolithoautotrophy has developed successfully and has brilliant prospects. It is an important branch of modern natural sciences, in accord with the development of biosphere science and of geological microbiology. Chemolithotrophic microorganisms with similar functions exhibit a striking taxonomic diversity. Acidophilic chemolithotrophs are the best studied in this respect. It is still not clear, however, how the evolution of the same function, i.e., of the ability to gain energy from the oxidation of in organic substrates, occurred in phylogenetically remote microbial groups. The energy substrate, the carbon source, and the temperature in a given environment were likely to be the main factors affecting the evolutionary processes.

Acidophilic chemolithotrophs with relatively small genomes form a highly specialized microbial group inhabiting low-temperature niches (up to 35°C). Both bacteria and archaea (some ferroplasms) are present in this group. More complicated survival mechanisms were required at higher temperatures, when CO_2 solubility under acidic conditions decreases drastically. Thermophiles and moderate thermophiles developed more complicated genomes and consequently, a more complex enzymatic apparatus for the anabolic and catabolic processes. Mixotrophs predominate in this group.

The phylogenetic diversity of the organisms discussed in this review could be the result of the horizontal transfer of genetic information between the organisms of different taxa, which existed simultaneously in the same ecological niche. Their subsequent evolution resulted in divergence, which caused a striking diversity of morphotypes, energetic, and constructive metabolic pathways. They, however, retained their main characteristics, i.e., the ability to generate the transmembrane electrochemical proton gradient, which is the basis for ATP synthesis and maintenance of intracellular homeostasis.

Physiological and biochemical inventarization of chemolithotrophs in the biosphere should be a high-priority task for future progress.

This review does not deal with ecologo-geographical studies of chemolithotrophs. This research is, however, highly important for the understanding of potential and actual capabilities of chemolithotrophs in environmental communities. The production of bacterial chemosynthesis in the regions of hydrothermal and volcanic activity in the oceans is known to be comparable with the production in the photic zones of these regions. Chemolithoautotrophs act as primary producers in the hydrothermal ecosystems of ocean depths; they gain energy by metabolizing the reduced gases from the interior of the earth. A new type of symbiosis was revealed and explained—the ectosymbiosis of aquatic fauna with filamentous sulfur bacteria [217].

Acidophilic chemolithotrophic microorganisms produce sulfuric acid as a result of oxidation of reduced sulfur compounds. The scale of these processes is enormous. They result in the formation of oxidation zones in ore deposits, in carrying out of heavy metal ions, etc.

Large-scale research of the biosphere promoted the solution of a number of practical problems. The scientific basis was developed for the estimation of the role of chemolithotrophs in the turnovers of sulfur, iron, and nitrogen, especially for the conditions of increasing anthropogenic load. Biogeotechnology, a new branch of applied science was developed in order to deal with the problems of extraction and recovery of valuable minerals. The biohydrometallurgic technology for recovery of gold-arsenic ores is already functioning in the Russian Federation (Siberia) and in other countries. Realization of other biohydrometallurgic industrial technologies and development of their new generations is a prospective task.

Modern science requires an integrated approach to various problems. Classical microbiological techniques are relevant for this field, as well as molecular biological techniques, which provide knowledge on microbial activity in natural and technogenic environments, and physicochemical techniques, which provide knowledge concerning the environment and the properties of the oxidized substrates.

Chemolithoautotrophy exemplifies the close relation, if not the unity, of life sciences and earth sciences. The concept of Winogradsky is in this sense planetary.

The work was supported by the Russian Foundation for Basic Research, project nos.04-04-48602, 03-04-049294; the Presidium of the Russian Academy of Sciences Program for Basic Research "Molecular and Cellular Biology"; the Ministry of Science and Industry of the Russian Federation ("Research and Development in Priority Fields of Science and Technology, 2002–2006"); State contract no. 43.073.1.1.2515; grant of the President of the Russian Federation for support of the leading scientific schools no. NSh-1742.2003.04, and the program of the Ministry of Education and Science of the Russian Federation "Development of the System of Leading Scientific Schools as the Medium for Knowledge Generation and Training of Highly Skilled Personnel.Research in Priority Directions and Programs", no. RI-112/001/324.

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