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EXPERIMENTAL ARTICLES

Reduction of Synthetic Ferrihydrite by a Binary Anaerobic Culture of *Anaerobacillus alkalilacustris* and *Geoalkalibacter ferrihydriticus* Grown on Mannitol at pH 9.5

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Abstract—In the course of an investigation of alkaliphilic iron reduction, metabiotic interactions in a binary culture reducing synthetic ferrihydrite (SF) have been studied. The binary culture contained two anaerobic bacteria: the alkaliphilic organotrophic bacillus *Anaerobacillus alkalilacustris*, which ferments sugars and sugar alcohols and is incapable of iron reduction, and the dissimilatory iron-reducing bacterium *Geoalkalibacter ferrihydriticus*, which is able to grow on acetate at the expense of anaerobic respiration. The experiments were performed under conditions of SF excess and deficiency. It was expected that *G. ferrihydriticus* would oxidize the acetate formed in the course of mannitol fermentation by *A. alkalilacustris*. The results were different from the expected ones: in the binary culture, fermentation products other than acetate were used for iron reduction; these were primarily formate and ethanol, which led to acetate accumulation rather than consumption. The reduction of SF to magnetite and/or siderite followed the earlier established regularities. The preferential order of donor utilization by *G. ferrihydriticus* did not conform to the energy yields of the corresponding reactions. Thus, it has been shown that there may be interactions in microbial communities that cannot be predicted from the characteristics of pure cultures. The degradation pathways of organic matter in communities may differ considerably from those observed in pure cultures, even in pure cultures of highly specialized organisms.

Keywords: combined cultures, *Anaerobacillus alkalilacustris, Geoalkalibacter ferrihydriticus*, iron reduction, siderite, magnetite, soda lakes, acetate.

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Decomposition of organic matter under anaerobic conditions is performed by the microbial community, in which bacteria are linked by trophic interactions. The trophic structure of the microbial community has been thoroughly studied by microbiologists over the past decades. Both microbial communities of natural ecosystems [1–4] and communities developing in anthropogenic habitats, such as waste treatment plants [5, 6], have been studied. The main metabolic pathways underlying trophic chains are by and large universal and inherent to the microbial communities of various ecosystems, whereas the species affiliation of the microorganisms is determined by the physicochemical parameters of the habitat [7]. The microbial communities of soda lakes are trophically autonomous systems in which complete degradation of allochthonous or autochthonous organic matter occurs due to the functioning of specialized microorganisms [7]. The key compound that is accumulated in the course of anaerobic decomposition of organic matter is acetate, whose degradation either requires compounds of variable-valency elements as exogenous electron acceptors or occurs syntrophically due to the interaction of two microorganisms [8, 9]. In the latter case, one microorganism oxidizes acetate with the production of hydrogen, and the other prevents hydrogen accumulation by consuming it; this makes the reaction of acetate oxidation thermodynamically favorable [8]. Hydrogen oxidation requires electron acceptor; therefore, at the last stages of organic matter decomposition by anaerobic communities, the key role is played by secondary anaerobes, represented by methanogens, sulfate reducers, or iron reducers. Iron reducers that perform reduction of ferrihydrite are widespread in the hypergenesis zone [10–12]. Representatives of this group have recently been shown to also be present in the microbial communities of soda lakes [13]. From an enrichment culture obtained from bottom sediment of Lake Khadyn (Tuva) on sulfate-free medium with acetate and synthetic ferrihydrite

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Table 1. Variants of experiments and controls

Parameters	Experiments		Controls				
	E1	E2	C1	C2	C3	C4	C5
A. alkalilacustris	+	+	+	+	–	–	–
G. ferrihydriticus	+	+	–	–	+	–	–
mannitol, 1 g/l	+	+	+	+	–	+	–
acetate, 1 g/l	–	–	–	–	+	–	+
SF, 10 mM Fe(III)	+	–	–	+/–	+/–	+/–	+/–
SF, 100 mM Fe(III)	–	+	–	–/+	–/+	–/+	–/+

Designations: (+) presence; (–) absence; (+/–) variants of SF content.

(SF), new alkaliphilic bacteria were isolated: the alkaliphilic dissimilatory iron reducer *Geoalkalibacter ferrihydriticus*, utilizing some nonfermentable fermentation products, including acetate [13]; the peptolytic bacterium *Natroniella ferrireducens*, carrying out facilitated fermentation in the presence of Fe(III) [14]; and the anaerobic bacillus *Anaerobacillus alkalilacustris* [15], fermenting sugars and sugar alcohols but incapable of iron reduction.

When metabiotic interactions in the microbial communities are studied, it is assumed that the participants of the trophic chain utilize the metabolic products of the preceding participants [1, 6]. Such theoretical constructions are based on the results of studies of pure cultures. The operation scheme of the microbial community outlined based on the studies of pure cultures does not necessarily reflect the actual interactions in natural habitats, where numerous additional factors may significantly alter the interactions deduced from laboratory experiments with pure cultures. Study of combined cultures makes it possible to advance toward the understanding of the trophic interactions under natural conditions.

This work presents the results of our study of SF reduction by a binary culture of the alkaliphilic anaerobic bacteria *A. alkalilacustris* and *G. ferrihydriticus*. As the substrate, we chose the sugar alcohol mannitol (CH_2OH-$(CHOH)_4$-CH_2OH), which, unlike e.g. glucose, does not reduce iron abiogenically and is not used by *G. ferrihydriticus* as an electron donor. Mannitol is fermented by a pure culture of *A. alkalilacustris* to formate, lactate, ethanol, acetate, and succinate. It was expected that acetate would be used by the iron reducer as the electron donor; however, the interactions in the binary cultures proved to be quite different.

MATERIALS AND METHODS

Bacterial strains used. This work used the type strains *Anaerobacillus alkalilacustris* Z-0521^T (= VKM B-2403^T = DSM 18345^T) and *Geoalkalibacter ferrihydriticus* Z-0531^T (= VKM B-2401^T = DSM 17813^T), isolated from anoxic bottom sediments of soda lake Khadyn (Tuva) with pH 9.5 and mineralization of 17 g/l [13, 15].

Cultivation conditions. The experiments were performed using anaerobic medium optimal for both microorganisms (g/l): KH_2PO_4, 0.2; $MgCl_2$, 0.1; NH_4Cl, 0.5; KCl, 0.2; NaCl, 4.0; Na_2CO_3, 3.0; $NaHCO_3$, 10.0; yeast extract, 0.1; anthraquinone disulfonate, 0.1; cysteine, 0.3; trace element solution [16], 1 ml/l. Mannitol (1 g/l) was added as the substrate; as the electron acceptor, we employed SF, prepared by titration of a $FeCl_3$ solution with 10% NaOH as described earlier [13]. The content of Fe(III) suspension in the medium was 10 or 100 mM. The pH after sterilization was 9.5. The medium was dispensed anaerobically under an N_2 flow into 120-ml glass flasks in 50-ml portions. The flasks were inoculated with an *A. alkalilacustris* culture (2 vol %) and a culture of *G. ferrihydriticus* (5 vol %). The incubation was in the dark at 35°C.

Experimental variants. Experiments with the binary culture were performed at two donor/acceptor ratios, 1 : 2 (E1 series) and 1 : 20 (E2 series). In each series, there were six replicates and controls in two replicates. Details of the experimental and control variants are presented in Table 1. In additional experiments with a pure culture of *G. ferrihydriticus*, the electron donors formate (2 mM), acetate (2 mM), lactate (2 mM), ethanol (1 mM), and succinate (2 mM) were added to the medium together or separately. In all of the experiments, the electron acceptor was SF, the content of Fe(III) in the medium being 10 or 100 mM.

Analytical methods. The growth of the cultures was monitored by direct count of the morphologically distinct cells under a Zetopan phase-contrast microscope (Austria).

Consumption of mannitol and formation of its fermentation products was monitored by HPLC on a Stayer chromatograph (Aquilon, Russia) equipped with an Aminex HPX-87H column (Biorad, United States) and a Smartline 2300 refractometric detector (Knauer, Germany); the elution was with 5 mM H_2SO_4. Samples for chromatography were obtained by culture centrifugation at 15 000 rpm for 3 min followed

by acidification of the clear supernatant with 5 M H_2SO_4 to pH 2.0.

The content of Fe(II) formed as a result of SF reduction was determined in a colorimetric reaction with ferrozine [17].

The precipitate samples intended for solid phase analysis were obtained by culture centrifugation at 2000 rpm for 5 min with further drying in a desiccator at 35°C in a 100% N_2 atmosphere over silica gel.

The morphology of the solid phase was studied under a Hitachi S-405A scanning electron microscope. To provide for electric conductivity, the specimens were sputter-coated with gold on an Eiko IB-3 ion-sputtering device under an argon atmosphere for 5 min at the ion current of 6 mA and a pressure of 0.1 mmHg.

The composition of the newly formed mineral phases was determined by Mössbauer spectroscopy as follows. The spectra of ^{57}Fe nuclei were taken in a temperature interval of 4.5–295°K on an MS-1104Em spectrometer (Solar, Russia) operated in a constant acceleration mode. Spectrum measurements at low temperatures were performed in the SHI-850-5 (Janis Research Co, United States) and MKKMI (TsMII VNIIFTRI, Russia) helium and nitrogen cryostats. The spectrometer was calibrated at room temperature using standard α-Fe absorbent and a ^{57}Co source in an Rh matrix. For processing and analysis of the Mössbauer spectra, we used model deciphering methods (SpectrRelax) [18] and methods for reconstruction of the distribution of superfine parameters of partial spectra (DISTRI) [19], implemented in the MSTools software package.

RESULTS

SF reduction by a binary culture of *A. alkalilacustris* and *G. ferrihydriticus* grown on mannitol. In the experimental series E1 and E2, visually noticeable iron reduction began on the third day after inoculation. Averaged data on the cell count in supernatant and on the contents of fermentation products and reduced iron are presented in Table 2 and Fig. 1. Over the first two days, only *A. alkalilacustris* cells were observed in the supernatant; their number was independent of the initial amount of SF, reaching 10^7 cells/ml on the first day and then declining as a result of lysis. Lysis was faster in the experimental variant with an excess of SF: in this case, no *A. alkalilacustris* cells could be observed in the medium as soon as on the third day. Single cells of *G. ferrihydriticus* were observed on the second day; in both experimental series, growth of the iron reducer occurred on days 2 to 4, its cell number reaching $4–6 \times 10^6$ cells/ml in the series with SF deficiency (E1) and $3–4 \times 10^7$ cells/ml in the series with SF excess (E2). Then, the growth of *G. ferrihydriticus* entered stationary phase and its cell number did not change significantly.

Average values of changes in the concentrations of mannitol and fermentation products formed by *A. alkalilacustris* in the experimental series E1 and E2 and controls are presented in Table 2 and Figs. 1b and 1c. These results were in agreement with the data on changes in the bacterial cell numbers. On the first day, rapid decrease in the mannitol concentration occurred, accompanied by accumulation of the fermentation products: formate, ethanol, acetate, and minor amounts of lactate and succinate. As soon as on the second day, mannitol was virtually exhausted, which corresponded to the beginning of lysis of *A. alkalilacustris* cells. Beginning with the third day, gradual decrease in the formate and ethanol concentrations occurred, accompanied by further accumulation of acetate. In the experimental series E1 (SF deficiency), formate and ethanol concentrations decreased on average from 4.0 to 1.5 mM and from 2.7 to 2.5 mM, respectively, by the end of the experiment, and the acetate concentration increased from 1.0 mM to 2.1 mM. In the case of SF excess (E2 series), mannitol oxidation was also completed on day two, but the dynamics of formate, ethanol, and acetate concentrations were different from those under SF deficiency (E1 series). In E2 series, formate was completely utilized by *G. ferrihydriticus* as soon as on the fourth day, and the decrease in the concentration of ethanol was greater: from 1.86 mM to 0.49 mM by the end of the experiment. In some experiments of the E2 series, the consumption of lactate also occurred, although, on average, its concentration did not change significantly: under both SF excess and SF deficiency, it remained at a level of 0.5-0.6 mM. The acetate concentration increased considerably, from 2.1 to 4.75 mM by the end of the experiment. Figures 1b and 1c, which demonstrate the dynamics of the concentrations of mannitol and products of its fermentation by *A. alkalilacustris* in the coculture with *G. ferrihydriticus*, do not show changes in the lactate and succinate concentrations. In the control with a pure culture of *A. alkalilacustris*, excess of SF influenced the ratio of the mannitol fermentation products, whereas at SF deficiency, the ratios of the fermentation products were virtually the same as in the absence of SF (see Table 2). In the control with excess of SF, the final concentration of acetate was higher and that of ethanol was lower than in the controls without SF or with SF deficiency.

The results of determinations of Fe^{2+} concentrations in the experiments and controls are presented in Table 2. The reduction of iron began on the second day of incubation, correlating with the beginning of *G. ferrihydriticus* growth. In the E1 experimental series, the precipitate formed did not possess magnetic properties, whereas in the E2 series, the precipitate was attracted by a magnet beginning with the third day. Therefore, we undertook a special study of the solid phase composition.

Table 2. SF reduction by the binary culture on mannitol (averaged data of experimental and control series)

E1, 10 mM Fe(III)	Days					
	0	1	2	3	4	7
Mannitol, mM	4.0	1.3	0.04	0.03	0.03	0.04
Formate, mM	0	3.3	4.0	3.3	2.5	1.5
Ethanol, mM	0	1.7	2.6	2.6	2.7	2.5
Acetate, mM	0	1.0	1.7	1.8	1.9	2.1
Lactate, mM	0	0.3	0.4	0.4	0.4	0.4
Succinate, mM	0	0.1	0.4	0.4	0.5	0.4
Fe(II), mM	0	0.3	2.3	4.8	5.4	6.6
A. alkalilacustris, cells/ml × 10^6	0	6.5	2.6	0.2	0	0
G. ferrihydriticus, cells/ml × 10^6	0	0	0.3	1.3	5	4.8
C2, 10 mM Fe(III)						
Mannitol, mM	5.2	1.66	0.32	0.03	0.03	–
Formate, mM	0.23	2.77	6.3	6.25	6.15	–
Ethanol, mM	0	1.1	2.96	3.1	3.08	–
Acetate, mM	0	0.93	1.08	1.15	1.12	–
Lactate, mM	0	Traces				–
Succinate, mM	0	0.18	0.28	0.3	0.26	–
Fe(II), mM	0	0	0	0	0	–
A. alkalilacustris, cells/ml × 10^6	0	8	3.5	0.4	0	–
C3, 10 mM Fe(III)						
Acetate, mM	5.69	5	4.84	4.81	4.12	3.54
Fe(II), mM	0	1.68	2.24	3.58	5.69	6.95
G. ferrihydriticus, cells/ml × 10^6						
C4, 10 mM Fe(III)						
Mannitol, mM	3.42	–	3.58	–	3.65	3.5
Fe(II), mM	0	–	0	–	0	0
C5, 10 mM Fe(III)						
Acetate, mM	8.16	–	8.08	–	8.39	8.3
Fe(II), mM	0.5	–	0	–	0	0
E2, 100 mM Fe(III)						
Mannitol, mM	2.44	1.09	0.01	0.01	0.01	0.02
Formate, mM	0	1.64	1.19	0.15	0	0
Ethanol, mM	0	1.47	1.86	1.24	0.89	0.49
Acetate, mM	0.03	0.72	2.08	3.26	3.73	4.75
Lactate, mM	0	0.14	0.16	0.21	0.19	0.22
Succinate, mM	0	0.07	0.38	0.36	0.38	0.38
Fe(II), mM	0.7	6.4	34.48	35.52	36.9	46.42

Table 2. (Contd.)

E2, 100 mM Fe(III)	Days					
	0	1	2	3	4	7
A. alkalilacustris, cells/ml × 10^6	0	6	1.5	0	0	0
G. ferrihydriticus, cells/ml × 10^6	0	0	0.4	8.2	28	29
C2, 100 mM Fe(III)						
Mannitol, mM	2.38	0.6	0	0	0	–
Formate, mM	0.33	2.74	3.35	4.56	4.79	–
Ethanol, mM	0.41	0.69	1.13	2.28	2.13	–
Acetate, mM	0.13	0.85	0.94	1.7	1.67	–
Lactate, mM			Traces			–
Succinate, mM	0.04	0.14	0.24	0.4	0.36	–
Fe(II), mM	0	0	0	0	0	–
A. alkalilacustris, cells/ml × 10^6	0.5	10	2.6	0	0	
C3, 100 mM Fe(III)						
Acetate, mM	11.07	9.36	8.72	8.28	7.7	7.8
Fe(II), mM	0.5	2.5	11.36	15.89	23	23.6
G. ferrihydriticus, cells/ml × 10^6	0.5	1.3	2.8	5.6	7.8	7.5
C4, 100 mM Fe(III)						
Mannitol, mM	4.99	–	4.73	–	5.05	4.8
Fe(II), mM	0	–	0	–	0	0
C5, 100 mM Fe(III)						
Acetate, mM	5.84	–	5.7	–	5.75	5.8
Fe(II), mM	0	–	0	–	0	0
C1						
Mannitol, mM	4.44	1.56	0.87	0.04	0.05	–
Formate, mM	0	4.5	4.9	5.7	5.57	–
Ethanol, mM	0	0.81	2.13	3.5	3.46	–
Acetate, mM	0	0.79	0.8	0.85	0.89	–
Lactate, mM			Traces			–
Succinate, mM	0	0.05	0.12	0.23	0.2–0.7	–
A. alkalilacustris, cells/ml × 10^6	0	6.3	2.8	0	0	–

Notes: The designations of experimental and control series are deciphered in Table 1. "–" stands for "not determined."

Study of the solid phase. The Mössbauer study showed that in the E1 experimental series the formation of magnetically ordered phase did not take place. The spectrum taken at room temperature (Fig. 2b) was a superposition of three quadrupole doublets: one of them corresponded to Fe(III) atoms, and two, to the atoms of Fe(II). The parameters of the quadrupole doublets containing Fe(II) atoms corresponded to siderite ($FeCO_3$) and to an unidentified phase. The partial spectrum corresponding to Fe(III) atoms differed from the SF spectrum; its parameters corresponded to lepidocrocite (γ-FeOOH). In the E2 experimental series (SF excess) (Fig. 2a), magnetically ordered phase was formed. The spectrum taken at 77°K was a superposition of a quadrupole doublet corresponding to Fe(II) atoms and a sextet corresponding to iron atoms in a magnetically ordered phase. Determinations at low temperatures showed that the magnetically ordered phase was represented by superparamagnetic particles of nonstoichiometric magnetite (Fe_3O_4) or a mixture of magnetite and maghemite (γ-Fe_2O_3).

The Mössbauer study of the precipitates in the controls (Fig. 2) showed that the growth of *A. alkalilacus-*

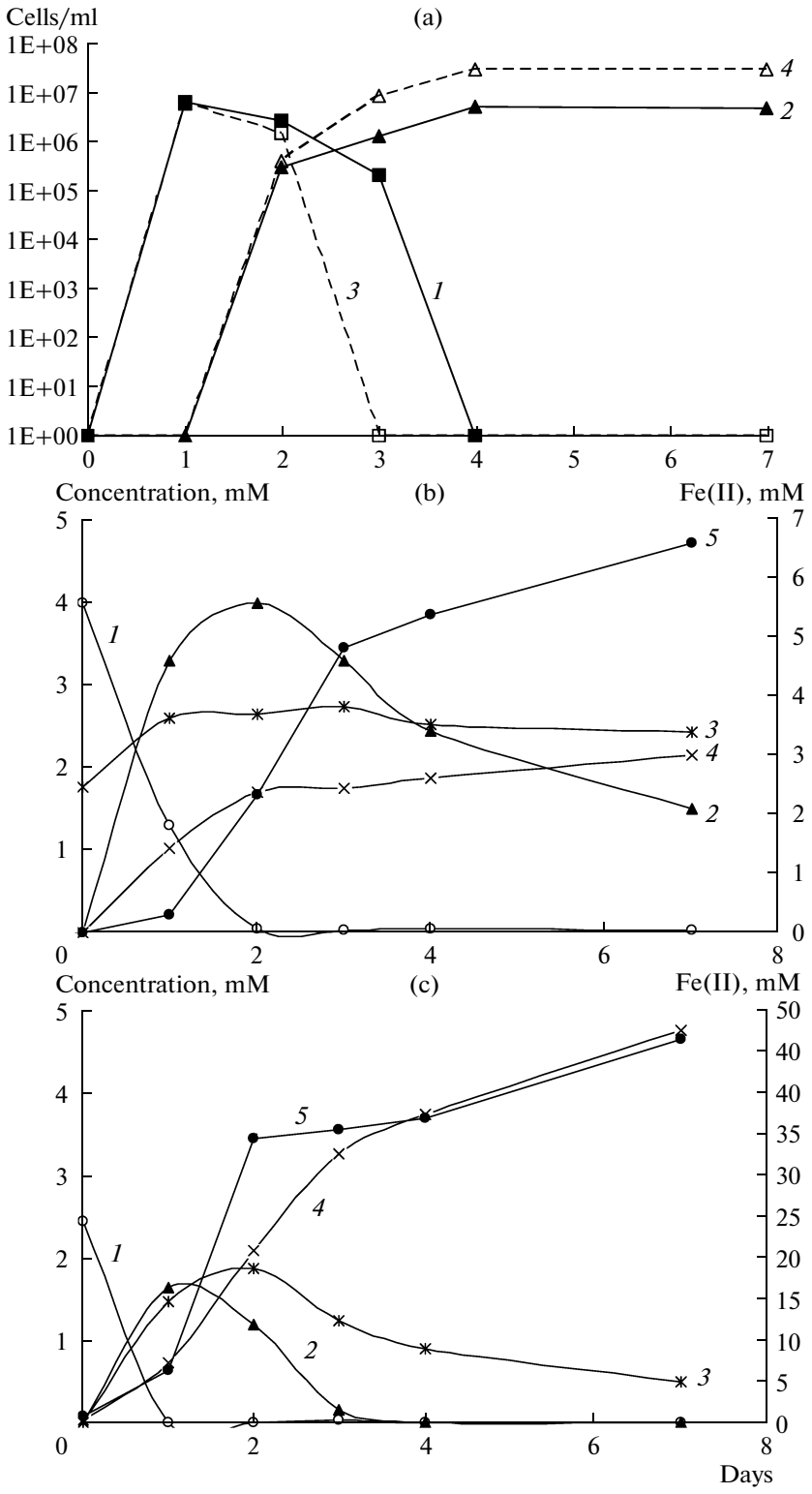

Fig. 1. (a) Curves of growth of (*1, 3*) *A. alkalilacustris* and (*2, 4*) *G. ferrihydriticus* in the binary culture in experiments with (*1, 2*) deficiency of SF (E1) or (*3, 4*) excess of SF (E2). (b, c) Reduction of SF and formation of mannitol utilization products in the *A. alkalilacustris–G. ferrihydriticus* binary culture on mannitol under (b) SF deficiency (E1) or (c) SF excess (E2): *1*, mannitol; *2*, formate; *3*, ethanol; *4*, acetate; *5*, Fe(II).

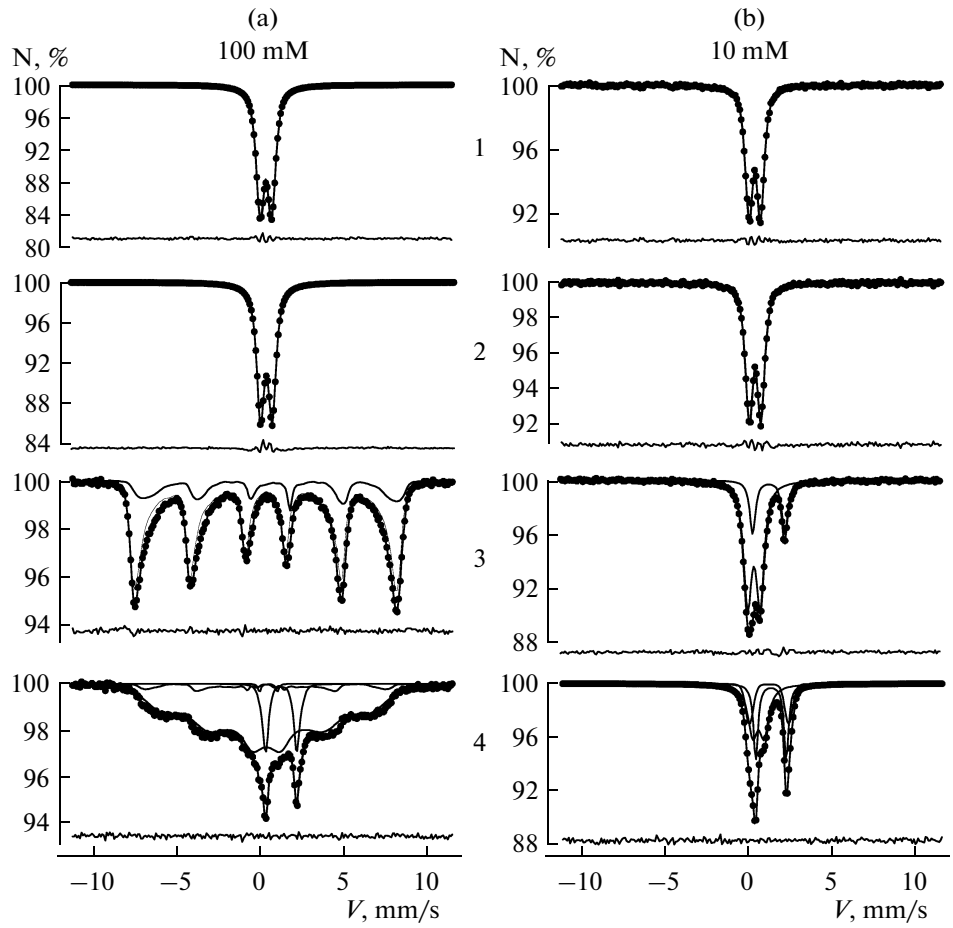

Fig. 2. Mössbauer spectra (taken at room temperature) of the solid phases obtained at (a) SF excess or (b) SF deficiency. 1, C5; 2, C2; 3, C3; 4, experiments.

tris on mannitol was not accompanied by SF reduction either under SF excess or SF deficiency: the parameters of the precipitate spectrum after growth were the same as those of the initial SF, although transformation of a part of SF to lepidocrocite had occurred. In the control variant C3 (*G. ferrihydriticus* grown on acetate as electron donor) under SF deficiency, no magnetically ordered phase was formed, and formation of siderite occurred. Under SF excess, relative content of siderite decreased, and the major phase formed during growth of *G. ferrihydriticus* was nonstoichiometric magnetite. In abiogenic controls with mannitol or acetate under SF deficiency, a part of SF was transformed to lepidocrocite, whereas under SF excess the spectrum parameters did not differ from those of the initial SF. Relative contents of iron in the initial and newly formed phases in experimental and control series are shown in Table 3.

The data from the Mössbauer studies were in good agreement with the examinations of the morphology of the initial and newly formed phases. Thus, in the E1 experimental series, the newly formed phase was represented by siderite spherulites, highly homogeneous in their size (3 to 5 μm) (Fig. 3a). Conversely, the precipitate formed in the E2 experimental series consisted of extremely heterogeneous particles that formed aggregates in which individual crystals were difficult to discern. Figure 3c presents a micrograph of the initial SF; it was typical of those of the controls in which no SF reduction occurred. Upon drying, such precipitate turned into a dense crust consisting of very small particles typical of amorphous or poorly crystalline compounds.

SF reduction by *G. ferrihydriticus* grown with a combination of electron donors. As mentioned above, accumulation of acetate and decrease in formate concentration were observed in both experimental series (E1 and E2) during the binary culture growth. The oxidation of formate or ethanol by a pure culture of *G. ferrihydriticus* had not been reported earlier [13], and this possibility was to be tested in additional experiments, the results of which are presented in Table 4 and Figs. 4a and 4b. We simultaneously added, in equal concentrations, all of the products formed during mannitol fermentation by *A. alkalilacustris* as electron donors for *G. ferrihydriticus*; SF was provided

Table 3. Relative content of iron atoms (%) in the mineral phases of solid phase samples from experimental and control series* (averaged data)

Mineral phases, %	10 mM Fe(III)					100 mM Fe(III)				
	E1	C2	C3	C4	C5	E2	C2	C3	C4	C5
SF	35	65	74	78	33	0	70	0	71	87
Lepidocrocite	0	35	0	22	67	0	30	0	29	13
Siderite	41	0	26	0	0	12	0	10	0	0
Magnetically ordered phase	0	0	0	0	0	88	0	90	0	0
Unidentified phase (Fe^{2+})	24	0	0	0	0	0	0	0	0	0

* Designations of experimental and control series are deciphered in Table 1.

Table 4. Growth of *G. ferrihydriticus* on medium with a combination of electron donors and on media with individual donors under conditions of deficiency (10 mM) or excess (100 mM) of iron (data averaged over 3 replicates)

Electron donors and Fe(II)	10 mM Fe(III)						100 mM Fe(III)					
	days						days					
	0	2	3	4	7	22	0	2	3	4	7	22
Formate, mM	1.8	0.6	0.2	0.2	0.0	0.0	1.8	0.1	0.0	0.0	0.0	0.0
Ethanol, mM	0.8	0.6	0.4	0.2	0.0	0.0	0.9	0.4	0.0	0.0	0.0	0.0
Acetate, mM	1.7	2.0	2.2	2.3	3.0	3.7	1.9	2.2	2.3	2.3	2.3	2.4
Lactate, mM	1.4	1.3	1.3	1.3	0.9	0.6	1.4	1.0	0.5	0.2	0.0	0.0
Succinate, mM	1.8	1.7	1.8	1.8	1.7	1.8	1.8	1.8	1.8	1.8	1.7	1.8
Fe(II), mM	0.2	0.0	0.0	0.8	3.9	5.8	0.2	7.0	21.6	29.7	32.0	31.9
Formate, mM	2	1.4	0.55	0	0	0	1.84	1.35	0.21	0.02	0	0
Fe(II), mM	0	1.82	3.8	4.5	4.28	4.1	0	1.68	4.51	5.85	5.13	5.25
Ethanol, mM	0.7	0.3	0	0	0	0	0.8	0.3	0	0	0	0
Acetate, mM (product)	0	0.63	1.03	1.11	0.8	0.65	0	0.25	0	0	0	0
Fe(II), mM	0	0	0	2.6	5.8	5.1	0.07	2.74	6.8	7.6	10.1	7.25
Lactate, mM	1.33	1.15	0.62	0.67	0	0	1.37	0.74	0.22	0	0	0
Acetate, mM (product)	0	0	0.38	0.49	0.99	0.83	0	0	0	0	0	0
Fe(II), mM	0	0	0	0	3.4	11.1	0.07	2.99	7.3	6.6	12.5	11.1
Acetate, mM	1.84	1.35	0.85	0.93	0.87	0.92	1.78	1.53	0.62	0.1	0.02	0
Fe(II), mM	0	2.43	3.56	4.81	8.9	8.32	0	2.15	10.1	20.8	18.5	22.5
Succinate, mM	1.77	1.86	1.86	1.53	1.42	1.51	1.78	1.75	1.28	0.96	0.84	0.7
Fe(II), mM	0	0	0	0	3.4	11.1	0.03	1.1	4	4.8	10.15	8.75

either in excess or deficient amount. The growth of *G. ferrihydriticus* on this medium resulted in SF reduction, which was due to successive utilization by the bacterium of the available donors, which occurred in the following order: formate–ethanol–lactate. In case of SF deficiency, only half of lactate was oxidized. The concentration of succinate remained constant, and that of acetate increased, like in the E1 and E2 experimental series; however, as distinct from the processes in E1 and E2, acetate accumulation was more intense under SF deficiency and not under SF excess.

SF reduction by *G. ferrihydriticus* grown with individual electron donors. Since succinate was not utilized in any of the experiments discussed above, it was necessary to check the feasibility of its utilization by *G. ferrihydriticus*. For this, we set up one more experiment, in which all of the products formed during mannitol fermentation by *A. alkalilacustris* were added to the medium as electron donors separately. In addition, we set up an experiment on the utilization by *G. ferrihydriticus* of molecular hydrogen, which proved to be utilized (data not shown). The results obtained with other donors are presented in Table 4 and Figs. 5a and

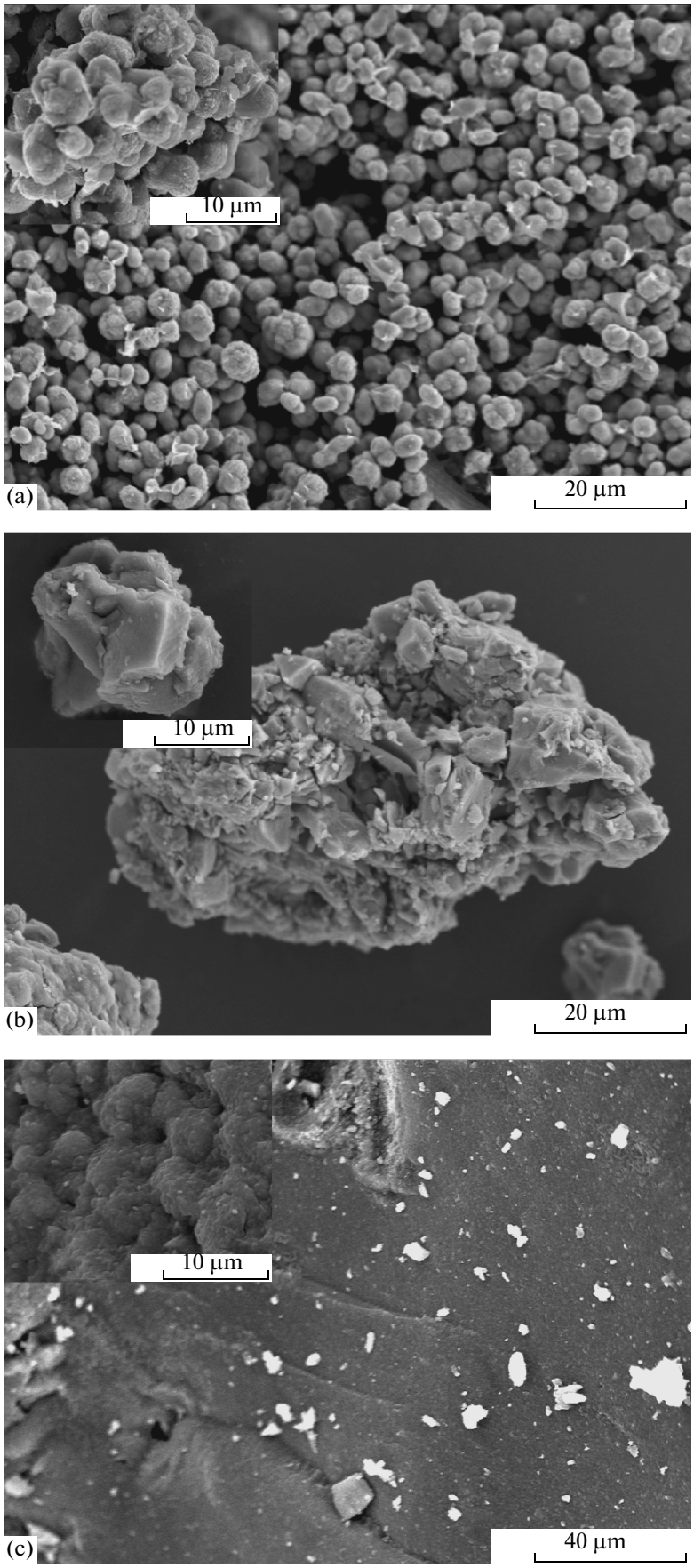

Fig. 3. Morphology of precipitates obtained in (a) E1, (b) E2, and (c) C5. Bars correspond to (a, b) 20 μm and 10 μm in the inserts and to (c) 40 μm and 10 μm in the insert.

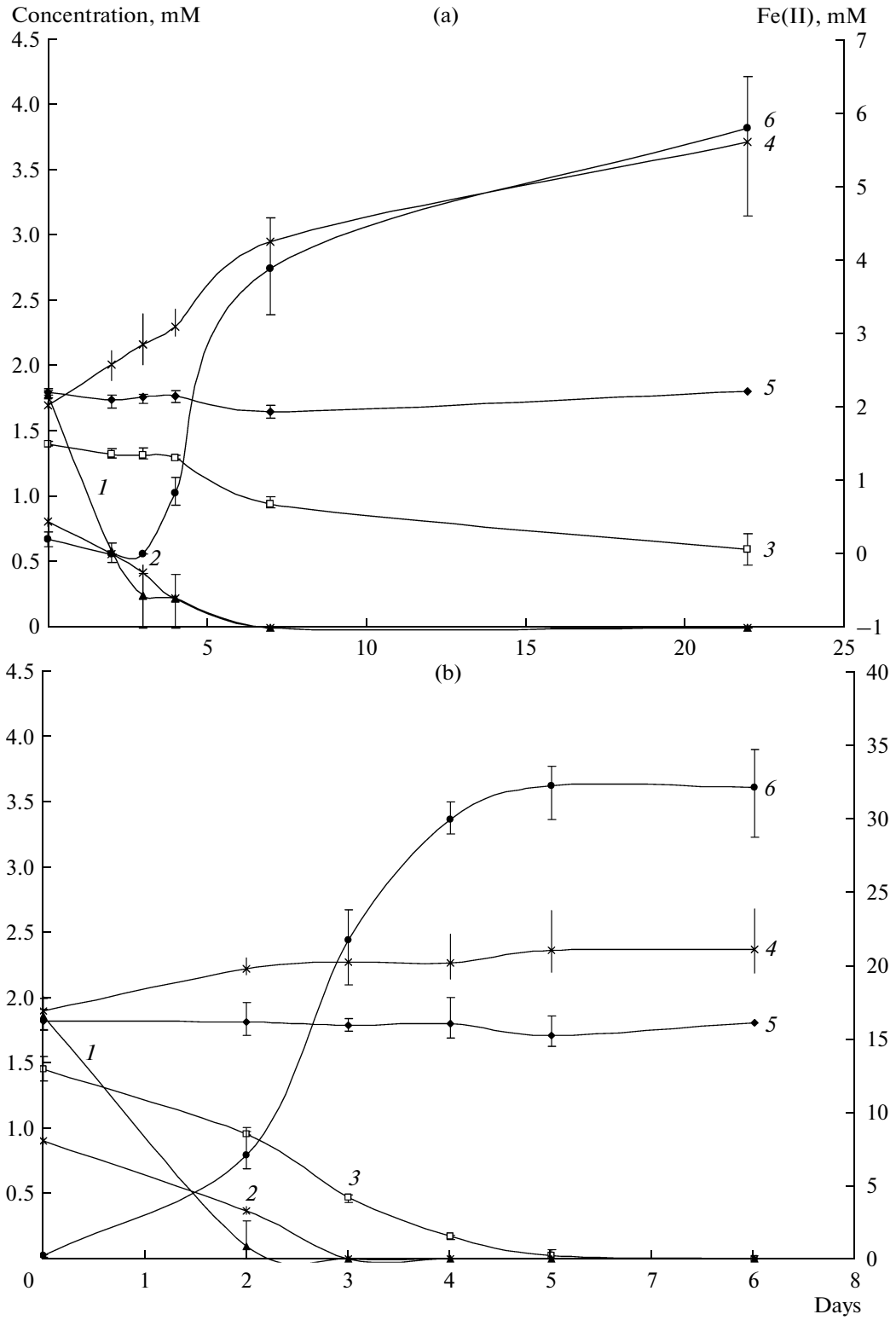

Fig. 4. Utilization of a substrate combination by *G. ferrihydriticus* at (a) SF deficiency and (b) SF excess. *1*, formate; *2*, ethanol; *3*, lactate; *4*, acetate; *5*, succinate; *6*, Fe(II).

Table 5. Experimentally determined (K_C, K_E) and theoretical (K_T) stoichiometric coefficients for mannitol oxidation by the pure culture of *A. alkalilacustris* (K_C) and the binary culture of *A. alkalilacustris* and *G. ferrihydriticus* (K_E) at the early growth phases

Products	K_C			K_E		K_T
	C1	C2, 10 mM Fe(III)	C2, 100 mM Fe(III)	E1, 10 mM Fe(III)	E2, 100 mM Fe(III)	
Formate	1.25	1.18	2.01	0.98	1.07	1.2
Ethanol	0.78	0.59	0.89	0.55	0.99	1.12
Acetate	0.20	0.22	0.70	0.32	0.42	0.6
Lactate	<0.01	0.01	0.03	0.09	0.10	0.4
Succinate	0.06	0.05	0.15	0.03	0.04	0.04
Available mannitol*, mM (% utilized)	4.44 (100%)	5.20 (100%)	2.38 (100%)	3.20 (100%)	1.66 (100%)	

Designations: K_C, K_{OE}, K_T = mM product/mM substrate.

* The mannitol added to the medium (1 g/l) was sorbed by SF, due to which the actual available concentration of mannitol was different in different experiments.

5b. All of the compounds, including succinate and hydrogen, were used by *G. ferrihydriticus* as electron donors.

DISCUSSION

In combined cultures, trophic interactions between the partners are usually various forms of symbiosis. This is particularly true for pairs that include a fermenter and a secondary anaerobe with respiratory metabolism (e.g., syntrophic associations based on interspecies H_2 transfer [8]). In the pair of the anaerobic alkaliphiles *A. alkalilacustris* and *G. ferrihydriticus*, the interactions were purely metabiotic (utilization of the metabolic products of the preceding component of the trophic chain).

The dynamics of the development of the binary culture on mannitol and SF exhibited two clearly pronounced stages. During the first stage, which lasted three days, the fermentation of mannitol by *A. alkalilacustris* was not significantly influenced by the iron-reducing partner and by SF and proceeded according to the equation

$$12.5CH_2OH{-}(CHOH)_4{-}CH_2OH + H_2O =$$
$$= 15HCOO^- + 14C_2H_5OH + 7.5CH_3COO^-$$
$$+ 5CH_3CHOHCOO^- + 0.5(CH_2COO)_2^{2-} + 28.5H^+.$$

The comparison of the experimentally found stoichiometric coefficients with the theoretical ones is provided in Table 5. Although *A. alkalilacustris* does not require electron acceptors for mannitol fermentation, the addition of excess SF to a pure culture of *A. alkalilacustris* resulted in a shift in the composition of fermentation products toward formate, acetate, and succinate. However, for the early growth stage of the *A. alkalilacustris–G. ferrihydriticus* binary culture, this shift was not strongly pronounced. It was also found that the concentration of mannitol measured in the medium immediately after inoculation was lower than the calculated one, the difference being twofold in case of SF excess and lesser in the E1 experimental series. Since sugar alcohols are known to be potent complexing agents, it can be assumed that mannitol was abiogenically sorbed by SF; it is possible that the sorbed mannitol was not available for fermentation. It can be seen from Table 5 that experimentally determined stoichiometric coefficients were closest to the theoretical ones in the E2 experimental series at SF excess. This supports the assumption that *A. alkalilacustris* could utilize only dissolved mannitol.

The second stage corresponded to cessation of fermentation and peak accumulation of its products and to the beginning of *G. ferrihydriticus* development and iron reduction. The overlap of the two stages was very short in time, and it was probably the shortness of this period that restricted the mutual influence. However, in the binary culture, *G. ferrihydriticus* oxidized only formate and ethanol (Table 2, Fig. 1), whereas in the pure culture in the presence of Fe(III), it could oxidize other products of mannitol fermentation, including acetate and succinate, which was demonstrated in a

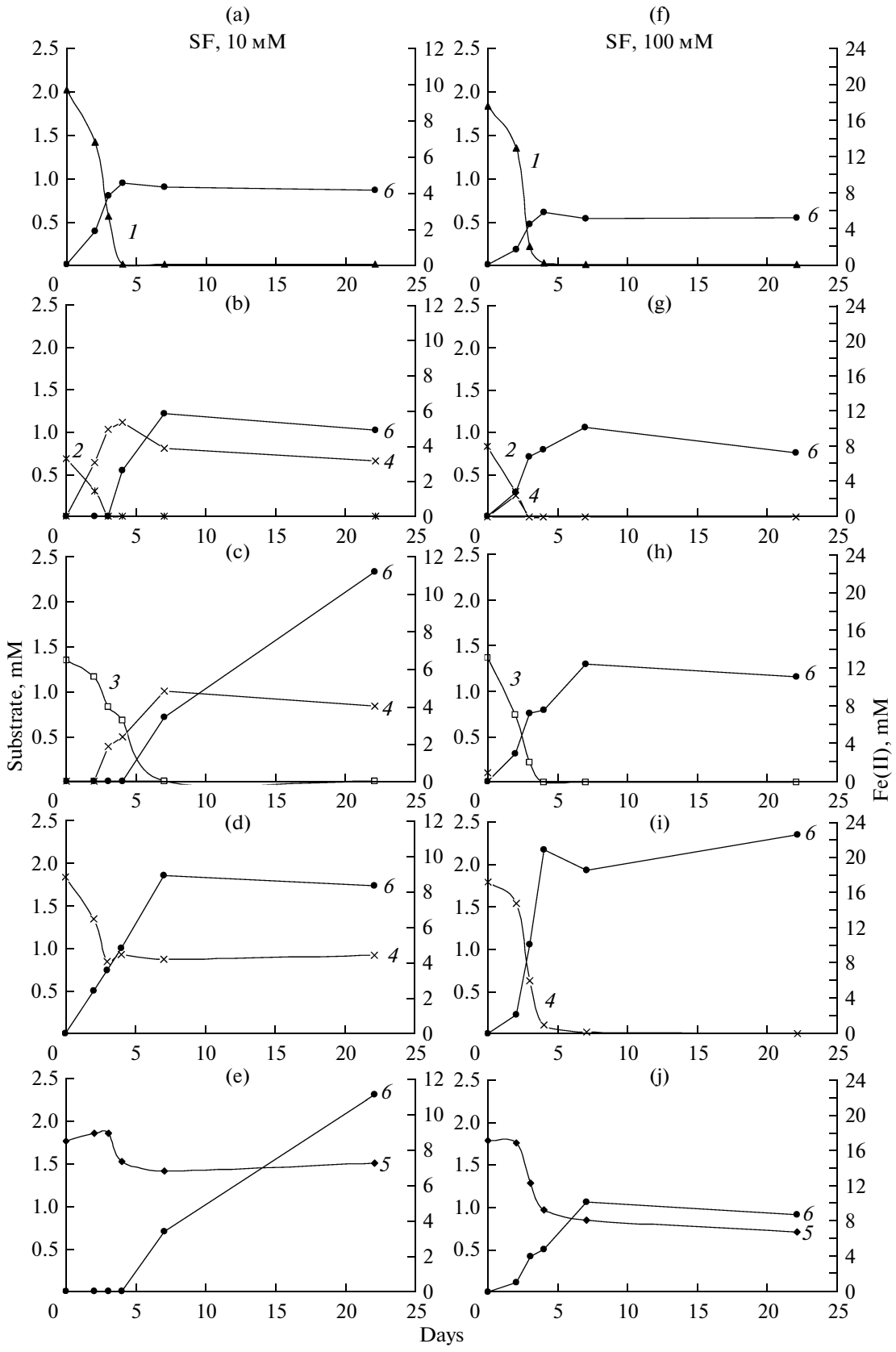

Fig. 5. Utilization of individual substrates by *G. ferrihydriticus* at (a–e) SF deficiency and (f–j) SF excess. *1*, formate; *2*, ethanol; *3*, lactate; *4*, acetate; *5*, succinate; *6*, Fe(II).

series of additional experiments (Table 4 and Figs. 4–5). The succession of substrate utilization (first formate and then ethanol) was independent of SF content and contradicted the energy yields of these reactions. This succession of utilization of mannitol fermentation products was a regularity that was reproduced in the additional experiment with a combination of donors (Fig. 4), in which a pure culture of *G. ferrihydriticus* used them in the following succession: formate–ethanol–lactate. No succinate consumption occurred. If we consider these reactions in terms of their energy yields, they will rank in the reverse order: succinate–lactate–ethanol–formate. The changes in the Gibbs free energy presented below (kJ/mol at pH 9.5) were calculated for two experimental variants, E1 (10 mM ferrihydrite introduced) and E2 (100 mM ferrihydrite introduced). In the E1 experimental variant, ferrihydrite was reduced to siderite ($FeCO_3$). Although both of these iron compounds are practically insoluble, their solubility products are known [20, 21], which allows the concentration of iron ions in the medium to be calculated. The changes in the Gibbs free energy were in this case calculated with taking into account the small concentrations of Fe(II) and Fe(III). In the E2 experimental variant, ferrihydrite was reduced to magnetite (Fe_3O_4), for which no data on solubility at normal conditions are available. Therefore, for the E2 variant, the changes in Gibbs free energy were calculated without taking into account Fe(II) and Fe(III). However, this did not affect the ranking of the reactions by their energy yield.

	O1, ΔG_f	O2, ΔG_f
$HCOO^- + 2Fe^{3+} + H_2O = HCO_3^- + 2Fe^{2+} + 2H^+$	−207.61	−106.89
$CH_3COO^- + 8Fe^{3+} + 4H_2O = 2HCO_3^- + 8Fe^{2+} + 9H^+$	−745.61	−342.74
$C_2H_5OH + 12Fe^{3+} + 5H_2O = 2HCO_3^- + 12Fe^{2+} + 14H^+$	−1168.17	−563.87
	O1, ΔG_f	O2, ΔG_f
$CH_3CHOHCOO^- + 12Fe^{3+} + 6H_2O = 3HCO_3^- + 12Fe^{2+} + 14H^+$	−1181.78	−577.48
$(CH_2COO)_2^{2-} + 14Fe^{3+} + 8H_2O = 4HCO_3^- + 14Fe^{2+} + 16\,H^+$	−1330.83	−625.81

In general, microbial processes obey the law of thermodynamic advantageousness of reactions, and reactions with $\Delta G_f < -31.8$ kJ/mol are driven by microorganisms only under special conditions, e.g., in case of syntrophic interactions [8, 9]. However, our experiments clearly demonstrate that the energy yields of the reactions do not always determine the choice of the substrate from the available set, and preference may be determined not by thermodynamic factors but by biochemical features of the cell enzymatic machinery. Lack of succinate or acetate consumption by *G. ferrihydriticus* from a substrate mixture may be determined by the inhibitory effect of other products of mannitol fermentation by *A. alkalilacustris*; this issue requires a separate study.

Of the above five reactions of organic donor oxidation with Fe(III), only two (those involving ethanol and lactate) have alternatives in which acetate is an oxidation product:

	O1, ΔG_f	O2, ΔG_f
$C_2H_5OH + 4Fe^{3+} + H_2O = CH_3COO^- + 4Fe^{2+} + 5H^+$	−422.563	−221.13
$CH_3CHOHCOO^- + 4Fe^{3+} + 2H_2O = CH_3COO^- + HCO_3^- + 4Fe^{2+} + 5H^+$	−881.923	−234.74

It is just these reactions that are likely to be responsible for acetate accumulation in the binary culture. This assumption is confirmed by data obtained in the additional experiments in which only ethanol or only acetate was added as the electron donor (Table 4, Fig. 5). However, even with these reactions taken into

account, the acetate formed in the experimental series E1 and E2 could not be balanced with the substrate consumed. This necessitates search for additional pathways of acetate formation; however, this search requires further experiments, and currently we can only hypothesize about these pathways. A possible assumption is that *G. ferrihydriticus* is capable of homoacetogenesis on ethanol, which is indirectly supported by the pattern of iron reduction in the additional experiments with individual electron donors (see Fig. 5). During growth on lactate or ethanol at SF deficiency, iron reduction occurred in parallel with acetate accumulation. Over the first three days, oxidation of lactate and ethanol was not accompanied by reduction of iron. The consumption of iron was insufficient to account for lactate and ethanol oxidation, which also indicated that iron reduction was most probably not the only reaction allowing energy conservation.

The Mössbauer studies of newly formed mineral phases, carried out in the E1 and E2 experimental series, confirmed the regularities of the formation of reduced mineral phases and their dependence on the initial SF amount (excess/deficiency), established earlier for pure cultures of thermophilic and alkaliphilic iron reducers [22, 23]. The formation of siderite or magnetite is independent of the rate of SF reduction and is determined by the area of the surface of the initial mineral phase impacted by bacteria. Studies of the solid phase in control series confirmed the inability of *A. alkalilacustris* to reduce SF. The emergence of lepidocrocite in the composition of the solid phase in some control series indicates a slow process of SF recrystallization.

Our results allow reappraisal of the acetate oxidation processes occurring in the anaerobic microbial community. As mentioned in the introduction, in this community, acetate is the major metabolite accumulated during organic matter decomposition, and it is considered to be consumed mainly by secondary anaerobes, a group comprising chemoorganotrophic and chemolithotrophic microorganisms that perform oxidation/reduction reactions in which organic acids, alcohols, or molecular hydrogen are electron donors, and the role of acceptors is played by inorganic compounds of S, Fe, N, C, As, Se, U and some other variable-valency elements whose compounds are stable under natural conditions. Secondary anaerobes have been isolated from virtually all ecological niches. *G. ferrihydriticus*, a typical representative of this group, was isolated from an enrichment grown on acetate as a selective substrate (electron donor). However, the results reported in the present paper clearly demonstrate that the ability of a pure culture to oxidize acetate may remain unrealized in the community, where there is always a choice of electron donor. Acetate oxidation in the community is likely to be carried out by highly specialized microorganisms, syntrophic ones first of all. The effectiveness of syntrophs in acetate oxidation has been demonstrated for alkaliphilic sulfidogenesis on cellulose as an example [24]: it was shown that the oxidation of acetate which accumulated in the course of cellulose degradation occurred only with the involvement of the obligately syntrophic bacterium *"Candidatus* Contubernalis alkalaceticum."

The results obtained in the present work allow the following conclusions to be made:

(1) Oxidation of mannitol in the binary culture resulted in SF reduction. The metabiotic nature of the interactions in the *A. alkalilacustris–G. ferrihydriticus* pair has been demonstrated. The organisms were mutually indifferent with the exception that the latter one used the metabolic products of the former, without affecting its metabolic pattern.

(2) The earlier unknown metabolic capacities of *G. ferrihydriticus* were revealed: it can use formate, ethanol, lactate, and molecular hydrogen as electron donors during SF reduction. This necessitates amendment of its taxonomic description.

(3) In the binary culture on a mixture of substrates, *G. ferrihydriticus* exhibited a paradoxical metabolic pattern, driving acetogenesis and not acetate oxidation. One of the reasons for acetate accumulation was the oxidation of ethanol to acetate by *G. ferrihydriticus* according to the reaction $C_2H_5OH + 4Fe^{3+} + H_2O = CH_3COO^- + 4Fe^{2+} + 5H^+$.

(4) For *G. ferrihydriticus* and most probably for bacteria in general, the energy yields of the reactions are not a decisive factor in the choice of a particular electron donor from the set of the donors available.

(5) Under conditions of SF excess, the major reduced phase formed by a coculture of *A. alkalilacustris* and *G. ferrihydriticus* grown on mannitol is represented by nonstoichiometric magnetite, and under SF deficiency, by siderite. This regularity, revealed earlier for pure cultures of thermophilic and alkaliphilic iron reducers, is likely to be universal.

(6) The capacity of a bacterium to oxidize acetate on selective media does not necessarily imply readiness to utilize it in the microbial community, where there is a choice of electron donors.

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