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Fe(III) and Fe(II) ions different effects on *Enterococcus hirae* cell growth and membrane-associated ATPase activity

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ABSTRACT

Enterococcus hirae is able to grow under anaerobic conditions during glucose fermentation (pH 8.0) which is accompanied by acidification of the medium and drop in its oxidation–reduction potential (E_h) from positive values to negative ones (down to ~−200 mV). In this study, iron (III) ions (Fe^{3+}) have been shown to affect bacterial growth in a concentration-dependent manner (within the range of 0.05–2 mM) by decreasing lag phase duration and increasing specific growth rate. While iron(II) ions (Fe^{2+}) had opposite effects which were reflected by suppressing bacterial growth. These ions also affected the changes in E_h values during bacterial growth. It was revealed that ATPase activity with and without *N,N′*-dicyclohexylcarbodiimide (DCCD), an inhibitor of the F_0F_1-ATPase, increased in the presence of even low Fe^{3+} concentration (0.05 mM) but decreased in the presence of Fe^{2+}. It was established that Fe^{3+} and Fe^{2+} both significantly inhibited the proton-potassium exchange of bacteria, but stronger effects were in the case of Fe^{2+} with DCCD. Such results were observed with both wild-type ATCC9790 and *atpD* mutant (with defective F_0F_1) MS116 strains but they were different with Fe^{3+} and Fe^{2+}. It is suggested that the effects of Fe^{3+} might be due to interaction of these ions with F_0F_1 or there might be a Fe^{3+}-dependent ATPase different from F_0F_1 in these bacteria that is active even in the presence of DCCD. Fe^{2+} inhibits *E. hirae* cell growth probably by strong effect on E_h leading to changes in F_0F_1 and decreasing its activity.

1. Introduction

Enterococcus hirae is among human and animal gastrointestinal bacteria having a role in physiology, a clinical relevance and a large application in technology. This bacterium grows under anaerobic conditions fermenting glucose which is accompanied by an acidification of the medium, changes in the proton-motive force and environment oxidation–reduction potential (E_h) [1–3]. It is known that positive values of E_h inhibit the growth of anaerobic bacteria while negative E_h values are required for normal bacterial cell growth [4,5].

In our previous study, the effects of different oxidizers and reducers on *E. hirae* and other anaerobic bacteria have been established [6–8]. Oxidizers can maintain E_h on positive level, and reducers can decrease it to negative values affecting bacterial growth and cellular activity. This is quite interesting for application.

Copper (II) ions (Cu^{2+}) as oxidizers have been shown recently to inhibit *E. hirae* cell growth, affecting the lag phase duration and decreasing specific growth rate [6]. Cu^{2+} also affects ATPase activity, the number of accessible SH-groups and H^+-coupled transport in a concentration-dependent manner. It was suggested that Cu^{2+} might affect E_h or SH-groups of the H^+-transporting F_0F_1-ATPase in *E. hirae* by disturbing a dithiol-disulfide interchange between F_0F_1 and other membrane proteins, thus changing activity of this ATPase. This was in contrast to *Escherichia coli* where the other action mechanism of Cu^{2+} effects has been proposed [8]. Moreover, the effects of Cu^{2+} were stronger than copper (I) (Cu^+) ions which had a less inhibitory effect on *E. hirae* growth [6]. However detailed mechanisms of Cu ions effects are not clear.

Fe is a very abundant element in surroundings as a component of insoluble hydroxides while in biological systems it can be bonded to specific proteins as a cofactor [9]. In some bacteria Fe is required for a number of essential enzymes, catalyzing Fe-S clusters biogenesis [10]. It is mostly contained in the reaction centers of oxidation–reduction enzymes and directly participates in appropriate reactions [10]. At the same time, abundance of free Fe, particularly Fe(II) ions (Fe^{2+}), is lethal for cells [11]. It is known that under anaerobic conditions Fe^{2+} is stable and more soluble than Fe(III) ions (Fe^{3+}). The latter is, therefore, inaccessible for living organisms. Thus, bacteria should evolve various mechanisms to satisfy the requirement in Fe, such as transport systems at first.

Fe^{2+} uptake transport system has been identified in *E. coli* under anaerobic conditions [12]. This is encoded by three *feoABC* genes, the sequence of which reveals the parts homology to ATPases, therefore Fe^{2+} uptake system may be ATP driven. The other ion – Fe^{3+} is also taken up by *E. coli* but together with siderophore [13]. Once transported across the outer membrane, Fe^{3+}-sidero-

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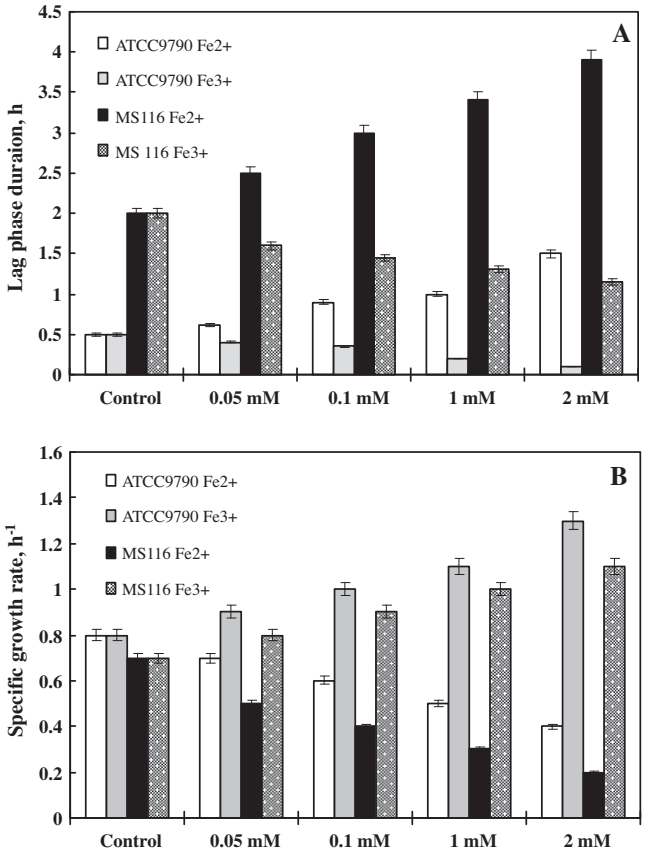

Fig. 1. Effects of Fe^{3+} and Fe^{2+} on *E. hirae* ATCC9790 and MS116 cell growth. (A) Lag phase duration and (B) specific growth rate. Control was bacterial growth in the medium without Fe ions. $FeCl_2$ or $FeCl_3$ of 0.05–2 mM was added (if specified). For details, see Section 2.

phore complex is bonded to specific proteins to pass through the plasma membrane into the cells; those proteins are components of ABC transporters [13]. In *E. coli*, many other Fe transport systems are also known [14,15]. Moreover, it is known that there are two repressor families which control Fe uptake in bacteria, one of those is Fur system [16]. Namely, in *Bacillus subtilis* there are three Fur-like proteins, which regulate Fe uptake and siderophore synthesis and peroxide stress response genes [16,17]. The transport and regulation mechanisms seem to be not clear due to their complexity and multiplicity. Fe homeostasis consists of not only uptake but also of efflux. But there are no data about Fe transport and regulation for *E. hirae* [18]; moreover Fe^{3+} and Fe^{2+} effects on this bacterium are not known yet.

In the present paper it has been revealed that Fe^{3+} within the concentration range of 0.05–2 mM stimulates *E. hirae* growth by decreasing lag growth phase duration and increasing specific growth rate. In contrast, Fe^{2+} had opposite effects by suppressing bacterial growth. Similar patterns were also observed with membrane-associated ATPase activity although H^+–K^+-exchange was inhibited. These effects were more noticeable in the case of Fe^{2+} in the presence of *N,N′*-dicyclohexylcarbodiimide (DCCD), an inhibitor of F_0F_1.

2. Materials and methods

2.1. Bacterial strains and growth, membrane vesicles

The wild-type strain *E. hirae* ATCC9790 [1] and the *atpD* mutant strain MS116 (lacked β subunit in F_1) [19] were used in this study. MS116 mutant strain expresses F_0F_1 in the same level as the wild-type, but it has lowered ATPase activity. The strains were kindly supplied by Prof. H. Kobayashi (Graduate School of Pharmaceutical Sciences, Chiba University, Chiba 263, Japan).

Bacteria were grown under anaerobic conditions at 37 °C in the medium that contained 1% tryptone, 0.5% yeast extract, 1% K_2HPO_4 with addition of 0.2% glucose at pH 8.0 as described earlier [1,2,19]. Bacterial growth was monitored by changes in optical density (OD) of bacterial suspension using a Spectro UV–Vis Auto spectrophotometer (Labomed, USA) at a wave length of 600 nm. 0.05, 0.1, 1 and 2 mM $FeCl_3$ or $FeCl_2$ were added, respectively, when mentioned. The lag phase duration was determined as described before [3,6,20]. The specific growth rate was calculated by dividing 0.693 ($\lg 2 = 0.693$) by the doubling time of OD in the ranges where changes in the logarithm of OD depended on time in a linear manner.

Membrane vesicles were isolated as described earlier [6,20] except that the buffers lacked K^+.

2.2. E_h and pH determination

E_h was measured by a platinum electrode (EPB-1, Electrometer Equipment State Enterprise, Gomel, Belarus; GDEEE, Hanna Instruments, Portugal) as described elsewhere [3,7–8,21]. E_h value was defined to be changed on 35–40 mV by ~8-fold change of bacterial count and was not changed more than on 45 mV by addition of Fe ions within the concentration range used. So the decrease of E_h during bacterial growth does not depend on bacterial or metal ions count change.

The pH values were measured by selective pH-electrode (HJ1131B, Hanna Instruments, Portugal) and were settled by 0.1 M NaOH or HCl.

2.3. ATPase assay

ATPase activity was measured by amount of liberated inorganic phosphate (P_i) after adding 5 mM ATP by Taussky and Shorr method [22]. The corrections were made for blanks without ATP or membrane vesicles. Relative ATPase activity was expressed in nmol P_i per mg protein in 1 min. The assay mixture contains 50 mM Tris–HCl (pH 8.0), 0.4 mM $MgSO_4$ and 100 mM KCl. When it was necessary, membrane vesicles were pre-incubated with Fe ions or DCCD for 10 min.

2.4. Proton and potassium ions transport study

H^+ and K^+ fluxes through the bacterial membrane in the whole cells were studied by monitoring changes in their activity in the medium with the use of appropriate selective electrodes (HJ1131B, Hanna Instruments, Portugal, and PVC membrane type, Cole Parmer Instruments Co., USA) as described elsewhere [2–4,6,23]. Electrode readings data were outputted automatically by LabView computer program (National Instruments Co., USA). Using this program, electrode readings were calibrated by titration with 0.01 N HCl and 0.02 mM KCl. Ion fluxes are expressed as the change in external activity of the ion in mM/min per number of cells in a unit of medium volume (ml). When mentioned, cells were treated with 0.1 mM DCCD or with Fe ions within the concentration range used for 10 min prior the assays.

2.5. Others and reagents

The protein content was measured by the method of Lowry et al. [24] using bovine serum albumin as a standard. All assays were routinely carried out under anaerobic conditions and all measurements were done at 37 °C. The average data are presented

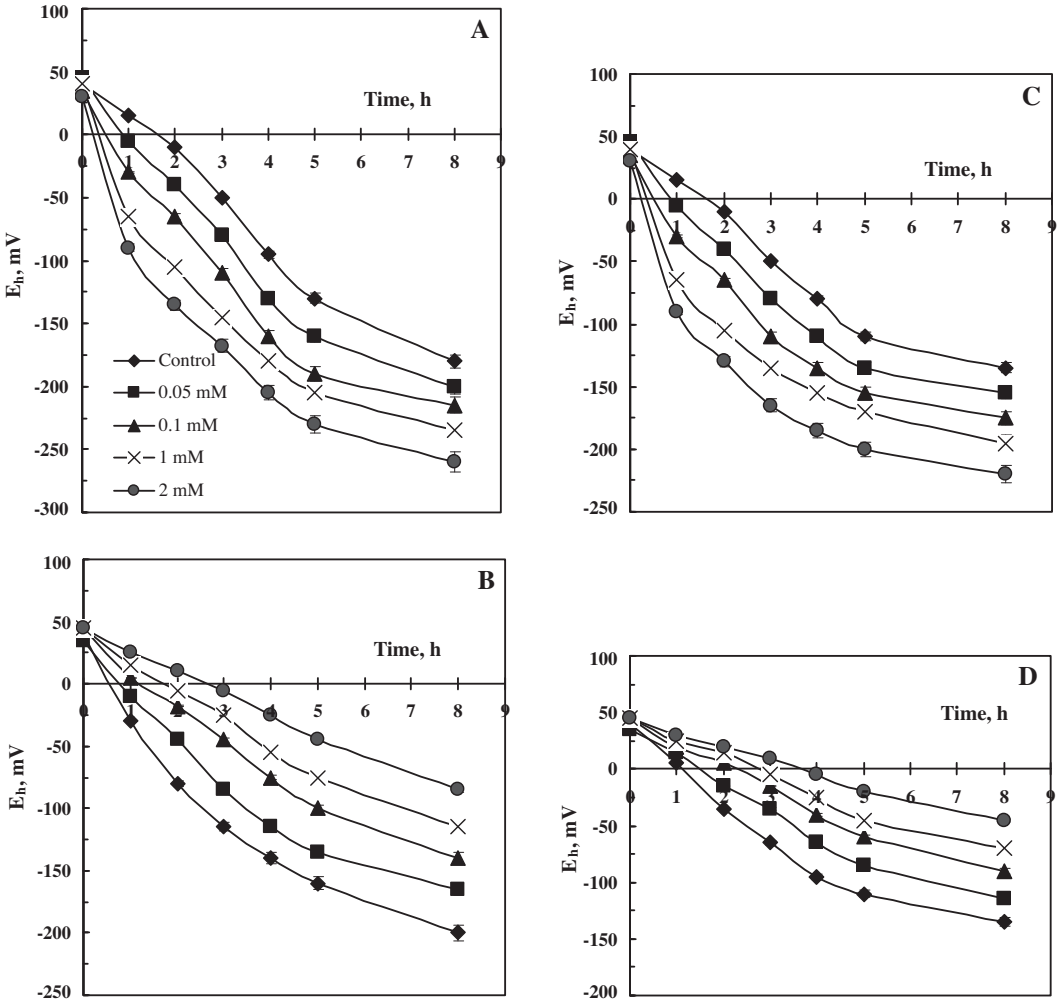

Fig. 2. Changes in redox potential during *E. hirae* ATCC9790 growth in the presence of Fe^{3+} (A) and Fe^{2+} (B) and MS116 growth in the presence of Fe^{3+} (C) and Fe^{2+} (D). For details, see Section 2.

from three independent measurements; standard errors were not more 3% if not indicated. The Student's validity criterion (p) was calculated to show the reliability of the difference between the changes values and the control.

Tryptone, yeast extract and Tris (aminomethan) were from Roth (Germany), agar, ATP (Tris salt) and DCCD were from Sigma (USA), glucose was from *Borisov Medical Preparations Plant* (Belarus) and other reagents of analytical grade were used in the study.

3. Results and discussion

3.1. Effects of Fe ions on E. hirae cell growth and E_h

We have shown that the addition of Fe^{3+} in bacterial growth medium within the range of 0.05–2 mM led to the decrease in the lag growth phase duration and the increase in the specific growth rate for *E. hirae* (Fig. 1). In contrast, Fe^{2+} had opposite effects by suppressing bacterial growth (see Fig. 1). For both Fe ions high concentrations (1–2 mM) had more noticeable influence. Such effects were observed with wild-type ATCC9790 and *atpD* mutant MS116 strains both. But the lag phase duration with MS116 was more than 10-fold higher than that with ATCC9790, although the specific growth rate was almost the same (see Fig. 1B). These findings also point out that F_0F_1 is not essential for *E. hirae* growth at alkaline pH. This is in confirmation of data about *E. hirae atpD* mutant growing under those conditions [1,2,6,19] but it contradicts with a common idea that bacterial F_0F_1 is a main membrane enzyme for bioenergetics responsible for generation of H^+-motive force under anaerobic conditions [25].

During *E. hirae* ATCC9790 growth after 8 h, E_h dropped from positive values (35 ± 5 mV) to negative ones (−200 ± 10 mV) (Fig. 2A). In the case of MS116 the initial E_h value was 25 ± 10 mV which dropped to negative values (−140 ± 10 mV) as the culture passed to the stationary growth phase (Fig. 2C). The rate of these drops is apparently directly related to the rate of bacterial growth: drop of E_h indicates that there are many reduction processes taking place during anaerobic growth of the bacterial cells [5–7,20]. At the stationary phase, after 24 h of growth, E_h markedly increased, but did not reach the initial values (not shown). Then, changes in E_h during ATCC9790 growth were also observed in the presence of Fe^{3+} and Fe^{2+} both (Fig. 2). Interestingly, these ions had contrary effects on E_h in a concentration-dependent manner. In the presence of 2 mM Fe^{3+} E_h strongly dropped (down to −260 ± 15 mV, Fig. 2A) whereas the same concentration of Fe^{2+} was less effective (E_h lowered down to −80 ± 8 mV only, Fig. 2B) compared with that of the control (∼−200 mV). Similar effects were observed with MS116: 2 mM Fe^{3+} caused decreased E_h to −220 ± 15 mV (Fig. 2C) when compared with that of the control (∼−140 mV), whereas in the presence of 2 mM Fe^{2+} E_h value was −40 ± 8 mV only (Fig. 2D).

In our laboratory it has been shown that a reducer of SH-groups, DL-dithiothreitol, stimulated *E. hirae* cell growth and enhanced

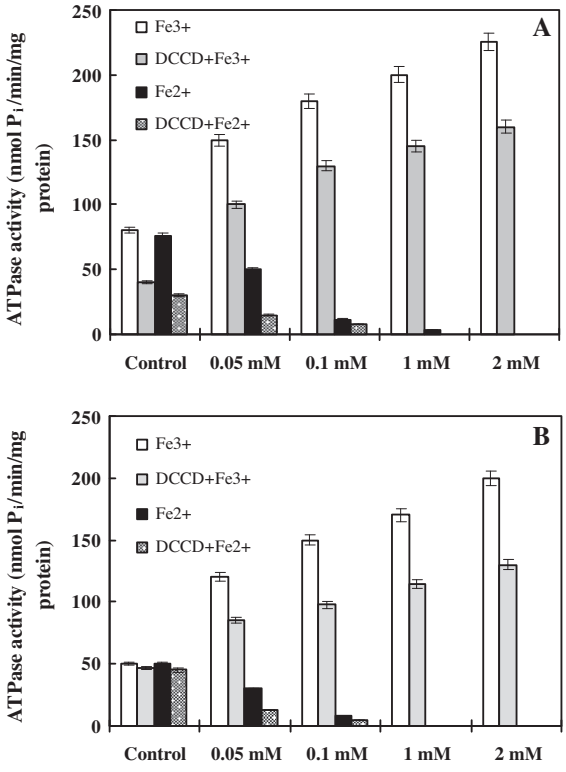

Fig. 3. Changes in ATPase activity of *E. hirae* ATCC9790 (A) and MS116 (B) membrane vesicles in the presence of Fe^{3+} and Fe^{2+} in K^+-containing medium. For details, see Section 2.

acidification of the medium but an oxidant, ferricyanide, suppressed bacterial growth and decelerates oxidation of the medium [3]. Fe^{2+} and Fe^{3+} are reducer and oxidizer, respectively, so just the opposite effects of these ions could be expected. But our results indicate that Fe ions might affect not only E_h, which by-turn can regulate F_0F_1 as it is suggested [26]; a redox regulation of F_0F_1, itself, is of significance. Fe ions may act on membrane-associated proteins thus changing their activity. The influence of Fe^{2+} is similar with results that we observed with Cu^{2+} in the same concentration range [6]. But in contrast to Fe ions, Cu ions with another oxidation state (Cu^+) have no significant action on E_h changes during *E. hirae* growth. This pointed out that, in case of Fe ions, different mechanisms could be evaluated.

3.2. Effects of Fe ions on ATPase activity and proton-coupled transport

E. hirae membrane-associated ATPase activity has been shown to be K^+-dependent [1,23] and inhibited by DCCD, an inhibitor of F_0F_1 [1,6,19]. And H^+ exchange to external K^+ inhibited by DCCD is carried out by this bacterium under certain conditions; this exchange has the fixed stoichiometry of the DCCD-inhibited ion fluxes [3,23]. It was suggested that such ATPase activity and H^+-coupled K^+ transport is the result of F_0F_1 interaction with K^+ transport system, KtrI [25].

The influence of Fe ions on ATPase activity of *E. hirae* ATCC9790 (Fig. 3A) and MS116 (Fig. 3B) membrane vesicles was, therefore, assessed in the presence of 100 mM K^+ with and without 0.1 mM DCCD. In all cases, the changes in ATPase activity were noticeable but they were depended on Fe ions concentration. In the presence of Fe^{3+}, a higher ATPase activity was observed in comparison with the control even together with 0.1 mM DCCD. It is possible that these effects are due to Fe^{3+} binding to F_0F_1 thus modifying its activity. Interestingly, thermophilic *Bacillus* PS3 F_1-ATPase has a binding site for Fe^{3+} and after that the whole complex undergoes structural modifications [27]. But ATPase activity determined in the presence of DCCD and with MS116 with non-functional F_0F_1 indicates another possibility: there might be a Fe^{3+}-dependent ATPase in *E. hirae*, different from F_0F_1, activity of which can be observed either when F_0F_1 is inhibited by DCCD or non-functional.

In contrast, even low Fe^{2+} concentrations had marked effect on ATPase activity (see Fig. 3). When a high concentration (1–2 mM) of Fe^{2+} was present, a residual ATPase activity was defined only. These effects were observed with *E. hirae* both wild-type and *atpD* mutant strains. Similar pattern was shown before with Cu^{2+} [6]. Such influence might be a result of Fe^{2+} effect on E_h (see Fig. 2B and D), which by-turn can regulate F_0F_1 [26] causing changes and decreasing activity.

Furthermore, H^+ exchange to external K^+ in the presence of Fe ions was studied in *E. hirae* at moderate K^+ activity (1 mM) and with or without DCCD [3,23]; two concentrations of Fe ions were used in this study to be more distinct. At first, H^+–K^+-exchange by ATCC9790 was inhibited by DCCD and had the stoichiometry

Table 1
Proton and potassium ions fluxes across the membrane for *E. hirae* at different Fe^{2+} and Fe^{3+} concentrations.

Strain, assay conditions[a]	Ion fluxes (mM/min)[b]							
	Fe^{3+}				Fe^{2+}			
	Total		DCCD-sensitive[c]		Total		DCCD-sensitive[c]	
	H^+	K^+	H^+	K^+	H^+	K^+	H^+	K^+
ATCC9790								
Control	1.8	0.67	0.8	0. 35	1.7	0.65	1	0.42
0.1 mM	$P < 0.05$[d]	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
	1.56	0.5	0.7	0.22	1.2	0.4	0.66	0.27
2 mM	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
	1.38	0.39	0.5	0.16	0.95	0.2	0.35	0.15
MS116								
Control	1.01	0.4	0.21	0.25	1	0.45	0.3	0.28
0.1 mM	$P < 0.05$	$P < 0.05$	$P < 0.01$	$P < 0.05$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
	0.85	0.28	0.15	0.1	0.68	0.19	0.2	0.16
2 mM	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.01$	$P < 0.01$
	0.68	0.19	0.08	0.05	0.5	0.09	0.11	0.08

[a] The bacteria were washed and transferred in Tris–phosphate buffer (pH 8.0) containing 0.4 mM $MgSO_4$, 1 mM NaCl, 1 mM KCl; 20 mM glucose was added.
[b] Calculated per 10^{10} cells/ml.
[c] The difference between fluxes in parallel experiments in the absence and presence of 0.1 mM DCCD.
[d] P was calculated for difference between the values of experimental samples and appropriate control.

of $2H^+/K^+$ which was absent in MS116 (Table 1). It was shown that Fe ions markedly decreased the fluxes of those ions (Table 1). This novel and important finding was added by stronger effects with Fe^{2+} observed when DCCD and Fe^{2+} were present together in the medium. Moreover, these results were more noticeable with wild-type than *atpD* mutant strain (see Table 1). They might be explained by action of Fe ions on F_0F_1 causing conformational changes which may affect its association with KtrI if any. Interestingly to note, similar effects on *E. hirae* have been observed with Cu^{2+} in the same concentration range, and Cu^{2+} and DCCD together markedly decrease the fluxes of H^+ and K^+ [6].

Thus, even low concentrations of Fe ions affect *E. hirae* wild type and *atpD* mutant strains growth and membrane-associated ATPase activity. These ions also cause changes in E_h drop during the growth and in H^+-coupled K^+ transport through the membrane. These effects have a concentration-dependent manner and are quite different for Fe^{3+} and Fe^{2+} but they are almost similar for the wild-type and the mutant with non-functional F_0F_1 used. Importantly, these findings are absolutely novel and have interest to understand the mechanisms of these effects on *E. hirae* and other bacteria for Fe ions itself and oxidizers and reducers as well. They can be useful to regulate bacterial growth and activity during oxidative stress or in the environment which contains heavy metals.

Acknowledgments

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