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ORIGINAL PAPER

The Effects of Copper (II) Ions on *Enterococcus hirae* Cell Growth and the Proton-Translocating F_oF_1 ATPase Activity

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Abstract *Enterococcus hirae* grow well under anaerobic conditions at alkaline pH (pH 8.0) producing acids by glucose fermentation. Bacterial growth was shown to be accompanied by decrease of redox potential from positive values (~+35 mV) to negative ones (~−220 mV). An oxidizer copper (II) ions (Cu^{2+}) affected bacterial growth in a concentration-dependent manner (within the range of 0.05 mM to 1 mM) increasing lag phase duration and decreasing specific growth rate. These effects were observed with the wild-type strain ATCC9790 and the *atpD* mutant strain MS116 (with absent β subunit of F_1 of the F_oF_1 ATPase) both. Also ATPase activity and proton–potassium ions exchange were assessed with and without *N*,*N*′-dicyclohexylcarbodiimide (DCCD), inhibitor of the F_oF_1 ATPase. In both cases (DCCD ±), even low Cu^{2+} concentrations had noticeable effect on ATPase activity, but with less visible concentration-dependent manner. Changes in the number of accessible SH-groups were observed with *E. hirae* ATCC9790 and MS116 membrane vesicles. In both strains Cu^{2+} markedly decreased the number of SH-groups in the presence of K^+ ions. The addition of ATP increased the amount of accessible SH-groups in ATCC9790 and decreased this number in MS116; Cu^{2+} blocked ATP-installed increase in SH-groups number in ATCC9790. H^+–K^+-exchange of bacteria was markedly inhibited by Cu^{2+}, but stronger effects were detected together with DCCD. Moreover, discrimination between Cu^{2+} and other bivalent cation—Ni^{2+} was shown. It is suggested that Cu^{2+} ions inhibit *E. hirae* cell growth by direct affect on the F_oF_1 ATPase leading to conformational changes in this protein complex and decrease in its activity.

Keywords Cu^{2+} · Bacterial growth · Proton transport · F_oF_1 ATPase · SH-groups · *Enterococcus hirae*

Introduction

Enterococcus hirae grow well under anaerobic conditions which is accompanied by acidification of the medium and changes in environment redox potential (E_h). It is known that positive values of E_h inhibit the anaerobic bacteria growth (for reviews, see [10, 38]) while E_h negative values are required for bacterial growth [4, 7, 22]. The latter can be inhibited by oxidizers, which maintain E_h on positive level [4, 15], and stimulated by reducers, which decrease E_h to negative values [14]. Moreover, E_h affects proton-motive force by changing pH gradient across the membrane [31].

Low concentrations of oxidizing Cu^{2+} ions are required for *Escherichia coli* and the other bacteria (for reviews, see [11, 29, 32]) whereas in considerably higher concentrations they can cause a number of toxic cellular effects inhibiting *E. coli* cell growth [15, 39], which can be explained by alteration of H^+ flux through the F_oF_1 ATPase as installed by Kirakosyan and Trchounian [15] and inhibition of hydrogenase activity associated with the F_oF_1 ATPase as determined by Kirakosyan et al. [16], by increase in surface charge density as shown by Volodina et al. [39] as well as by changes in membrane permeability as suggested for this bacterium by Lebedev et al. [18]. The change in H^+-permeability and the other properties of bacterial membrane may be related to membrane proteins re-organization or changed functional activity, and that might depend on proteins thiol groups' state and distribution. In accordance

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with these ideas, it is suggested that Cu^{2+} can break disulfides in membrane proteins [18], increasing the number of accessible SH-groups [16]. However, a role of E_h in bacterial growth, effects of oxidizers and reducers on bacterial growth would be clarified; the appropriate mechanisms for Cu^{2+} uptake and intracellular handling are still not clear.

Kirakosyan et al. [16] have shown that in *E. coli* membrane vesicles, prepared from anaerobically grown cells at slightly alkaline pH, Cu^{2+} ions alone increase the level of SH-groups but block ATP-stimulated increase of these groups when added together with ATP. The obtained effects might be the result of Cu^{2+} action on E_h or a direct effect of these ions on proteins in bacterial membrane, probably on the F_oF_1 ATPase. It is suggested that the energy of ATP can be transferred through a dithiol–disulfide interchange between the F_oF_1 ATPase and the other membrane proteins [5, 16], which are forming the protein–protein associations within the membrane [6, 22, 36]. And the marked increase in the number of F_oF_1 ATPase SH-groups by ATP but not by ADP has been shown with membrane vesicles [23]. Furthermore, Cu^{2+} can break these interactions thus increasing the level of accessible SH-groups. The latter is proposed to be another way which also depends on E_h. In our laboratory it has been established [15] that the addition of 0.1 and 2 mM Cu^{2+} into the *E. coli* growth medium results in a delayed decrease of E_h although a drop in E_h is less for 2 mM than for 0.1 mM of these ions. All these findings can be taken into consideration to explain the action mechanisms of oxidizers effects on *E. coli* growth.

A closed relationship of the F_oF_1 ATPase with other membrane proteins is also assumed in case of *E. hirae* [37]. This idea results from two findings at least: H^+–K^+-exchange with the fixed stoichiometry through the F_oF_1 ATPase and via the K^+ uptake system [24, 37] and *N*,*N*′-dicyclohexylcarbodiimide (DCCD)-inhibited ATPase activity depended on K^+ ions [37]. Moreover, the marked increase in the number of F_oF_1 ATPase SH-groups by ATP and nicotinamide adenine dinucleotides has been determined with *E. hirae* membrane vesicles [27]. However, mechanisms of such relationship and thiol groups' role in the *E. hirae* F_oF_1 ATPase activity remain unclear. At present there are no data on Cu^{2+} effects with *E. hirae*.

Our present data show that Cu^{2+} affect *E. hirae* cell growth by increasing lag phase duration and decreasing specific growth rate. These ions have noticeable effect on H^+–K^+-exchange and ATPase activity and lower the number of accessible SH-groups in a concentration-dependent manner; discrimination between Cu^{2+} and Ni^{2+} is shown. The results with data obtained by using the *E. hirae atpD* mutant strain MS116 with defective F_oF_1 ATPase might indicate direct effects of Cu^{2+} on this ATPase.

Materials and Methods

Bacterial Strains and Growth, Whole Cells, Membrane Vesicles, Redox Potential

The wild-type strain *E. hirae* ATCC9790 [17] and the *atpD* mutant strain MS116 (having absent β subunit of F_1 of the F_oF_1 ATPase) [3, 37] were used in this study. MS116 strain expresses the F_oF_1 ATPase to the level as wild-type one [3], but it has significantly lowered H^+ efflux [2] and ATPase activity [3, 37]. The strains were kindly supplied by Prof. H. Kobayashi (Department of Biochemistry, Chiba University, Chiba 263, Japan).

Bacteria were grown under anaerobic conditions at 37°C in a 0.2% glucose containing growth medium (1% tryptone, 0.5% yeast extract, 1% K_2HPO_4; pH 8.0) as described earlier [2, 24, 26, 27, 37]. The medium pH was measured by a pH-meter with selective pH-electrode (HJ1131B, Hanna Instruments, Portugal) and adjusted by means of 0.1 M NaOH or HCl. Bacterial growth was assessed by measuring optical density (OD) changes in bacterial suspension using a Spectro UV–vis Auto spectrophotometer (Labomed, USA) at wavelength of 600 nm. To study the effects of Cu^{2+}, 0.05 mM, 0.1 mM, or 1 mM $CuCl_2$ were added in bacterial suspension, when mentioned. The latent (lag) growth phase duration was determined as described before [14]. The specific growth rate was calculated over the interval, where the logarithm of OD for the culture increased linearly with time, and it was expressed as lg2 (lg2 = 0.693)/doubling time.

Whole cells [2, 15, 25] were prepared and right-side-out membrane vesicles [16, 27, 37] were isolated as described except that the buffers lacked K^+.

E_h was measured by a platinum (Pt) (EPB-1, GSEEE, or PT42BNC, Hanna Instruments, Portugal) or titanium-silicate (Ti–Si) (EO-02, Electrometer Equipment State Enterprise, Gomel, Belarus) electrode as described elsewhere [4–7, 14, 16, 22]. When the Pt-electrode was used together with a Ti–Si-electrode, which, unlike the former, is insensitive to molecular oxygen and molecular hydrogen, no significant differences in electrode readings were detected.

It was detected that E_h value in the conditions used was changed on 35–40 mV only by ~8–10-fold alteration of bacterial count. It was also determined that E_h value was not changed more than on 25–30 mV by ~sixfold change of Cu^{2+} concentration within the concentration range used. So the significant decrease of E_h during bacterial growth (see "Results and Discussion" section) does not depend on either bacterial count or Cu^{2+} ions count change.

Accessible SH-Groups and ATPase Assays

Accessible SH-groups of membrane vesicles were determined by Ellmann's reagent (5,5′-dithiobis-2-nitrobenzoic

acid) [30] using spectroscopic method as described [16, 23, 27] and glutathione as a standard. Membrane vesicles were treated with the reagent and OD was measured after ~1.5–2 h (OD became constant), corrections were made for blanks without membrane vesicles and with different reagents used. The level of SH-groups was expressed in nmol per mg protein. Using this reagent gave the same data for the number of SH-groups as the other reagents as shown [16, 23, 28].

ATPase activity was measured by amount of inorganic phosphate (P_i) liberated after adding 5 mM ATP (Tris salt) [22, 28]. P_i was determined by the spectrophotometric method of Taussky and Shorr [35], corrections were made for blanks without ATP or membrane vesicles. ATPase activity was expressed in nmol P_i per μg protein in 1 min.

The assay mixture was of 200 mM Tris-phosphate (pH 8.0) containing 0.4 mM $MgSO_4$, 1 mM NaCl and 1 mM KCl; for ATPase activity determination 50 mM Tris–HCl (pH 8.0), containing 0.4 mM $MgSO_4$ with or without 100 mM KCl was used. When used, membrane vesicles were pre-incubated with Cu^{2+} or DCCD for 10 min.

Proton and Potassium Ions Transport Study

H^+ and K^+ fluxes through the bacterial membrane in whole cells were measured using appropriate selective electrodes (HJ1131B, Hanna Instruments, Portugal; and PVC membrane type, Cole Parmer Instruments Co., USA, or E-031, "Niko Analit" Sci.-Prod. Co., Russia) as described elsewhere [7, 15, 22, 26]. Electrode readings were calibrated by titration of the assay medium (200 mM Tris-phosphate buffer (pH 8.0) containing 0.4 mM $MgSO_4$, 1 mM NaCl and 1 mM KCl) with 0.01 N HCl and 0.02 mM KCl. Ions transport was determined after addition of glucose into the assay medium. 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). For DCCD inhibition studies, cells were treated with this reagent at 0.1 mM for 10 min prior assays; during treatment with DCCD bacterial count was not changed. Note, Cu^{2+} ions had no effects on proton and potassium electrode readings.

Other and Chemicals

Bacterial count was determined by counting colony-forming units grown on solid media with glucose (after plating of diluted bacterial suspension). Protein was measured by the method of Lowry et al. [20] using bovine serum albumin as a standard. All assays were routinely carried out under anaerobic conditions and all measurements were done at 37°C. At least three independent measurements were made; standard errors were not more than 3% if not represented. The Student's validity criteria (p) was calculated to show statistically reliable difference between changed values and control [16].

Trypton, yeast extract were from Roth (Germany), ATP, DCCD, Ellmann's reagent, glucose and glutathione were from Sigma (USA) and other reagents of analytical grade were used in experiments. For metal ions appropriate chloride salts were used.

Results and Discussion

Bacterial Growth and Redox Potential in the Presence of Copper (II) Ions

The wild-type strain *E. hirae* ATCC9790 and the *atpD* mutant strain MS116 with defective F_oF_1 ATPase are known to grow well under anaerobic conditions at pH 8.0 [2, 3, 26, 37].

The addition of Cu^{2+} into the bacterial growth medium resulted in an increased lag phase duration and decreased

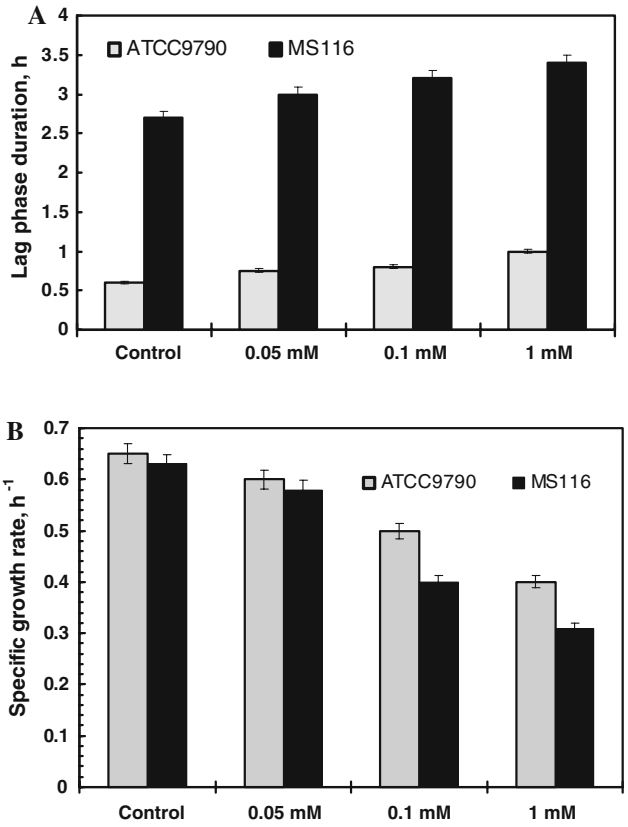

Fig. 1 Effects of Cu^{2+} ions in different concentrations on the *E. hirae* ATCC9790 and MS116 cell growth. **a** Lag phase duration, **b** specific growth rate. $CuCl_2$ of 0.05 mM to 1 mM was added (if specified, see x-axis) to the growth medium before inoculation of bacteria; control was bacterial growth in the growth medium without $CuCl_2$ added. For the others, see "Materials and Methods" section

specific growth rate (Fig. 1). With low concentrations of Cu^{2+} (0.05 mM) no statistically reliable bacterial growth differences were observed (in comparison with control samples, $P > 0.05$). In contrast, a higher concentration of Cu^{2+} (0.1 mM and 1 mM) notably prolonged lag phase duration and significantly decreased the specific growth rate ($P < 0.05$).

The influence of Cu^{2+} on MS116 cell growth was less noticeable than that on ATCC9790 (see Fig. 1). The lag phase duration with this *atp* mutant strain is ~4.5-fold higher than that with wild-type strain but specific growth rate are almost the same (see Fig. 1). These findings point out that the F_oF_1 ATPase is not essential for *E. hirae* growth at alkaline pH. This contradicts with a common idea that the F_oF_1 ATPase of bacteria is a main membrane enzyme of bioenergetic meaning that is responsible for generation of H^+-motive force under anaerobic conditions (for review, see [36]). However, this seems to be in favor with data of Kobayashi with co-workers [17, 24] that *E. hirae* can grow at alkaline pH in the presence of a protonophore which almost completely dissipates the H^+-motive force. However, the requirement of H^+-motive force for *E. hirae* growth and mechanisms of its generation at alkaline pH if any are not clear yet. In addition, the obtained results with Cu^{2+} ions are in accordance with those of Kirakosyan et al. [16] reported for *E. coli*.

Furthermore, the suppression of *E. hirae* growth at alkaline pH can be resulted by Cu^{2+} direct effect on bacterial membrane or on E_h.

During *E. hirae* ATCC9790 growth E_h measured with Pt-electrode drops from positive values (35 ± 10 mV) to negative ones (−220 ± 10 mV) (Fig. 2a). In the case of MS116 the initial E_h value is 5 ± 10 mV which drops to negative values (−170 ± 10 mV) as the culture passes to the stationary growth phase (Fig. 2b). This drop of E_h indicates that there are many reduction processes taking place at anaerobic growth [14, 38]. At the stationary phase, after 24 h of growth, E_h increases, but does not reach the initial values (not shown).

Changes in E_h during *E. hirae* growth were also observed in the presence of Cu^{2+}. These ions were shown to affect E_h values changes in a concentration-dependent manner (Fig. 2). The effects of Cu^{2+} were more expressed in case of wild-type strain *E. hirae* ATCC9790 if comparing with MS116 (Fig. 2a, b). To have clear data with Cu^{2+}, we have detected that copper (I) ions had a little bit less noticeable effect on E_h change (see Fig. 2c). This proves that Cu^{2+} more than Cu^+ ions have significant action on E_h changes during *E. hirae* growth, although Cu^+ rather than Cu^{2+} could be accumulated in spite of some balance between influx and efflux systems for these ions [32]. These results may be related to changes in the number of SH-groups which depends on E_h and redox-regulated

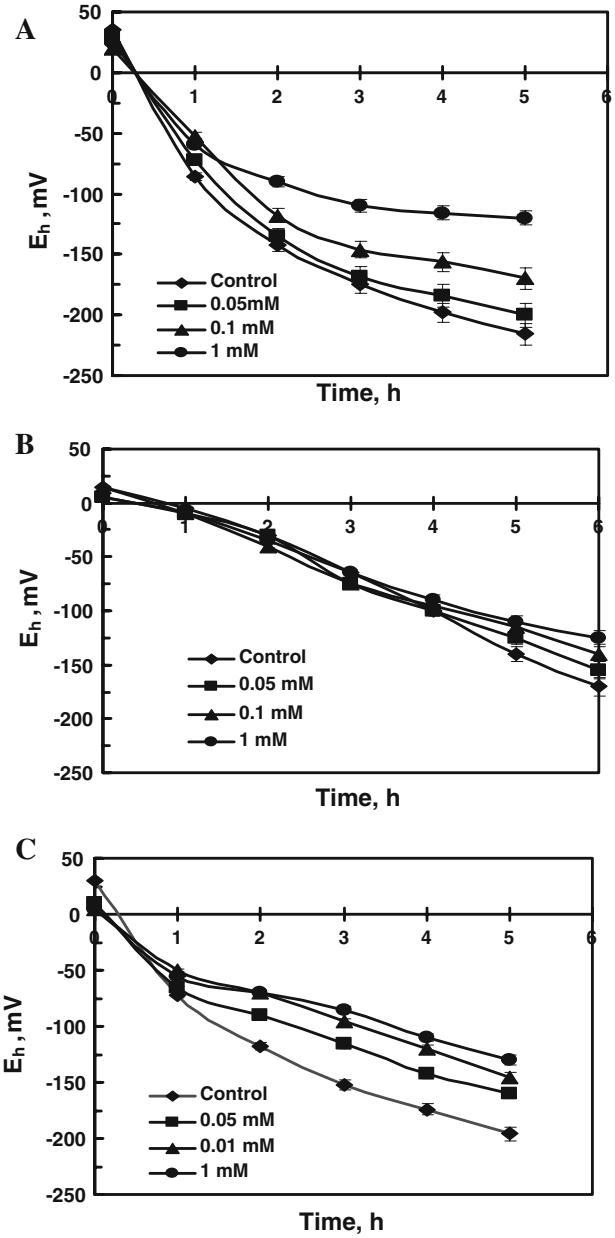

Fig. 2 Changes in redox potential during *E. hirae* ATCC9790 growth in the presence of Cu^{2+} (**a**) and Cu^+ ions (**c**), and MS116 growth in the presence of Cu^{2+} ions (**b**). Redox potential was measured with a Pt-electrode. For details, see the legends in Fig. 1 and "Materials and Methods" section

H^+-translocating ATPase activity [8, 38]. Besides, changes in E_h decrease upon bacterial growth are suggested could be related with changes in bacterial growth [14, 31].

Effects of Cu^{2+} on Accessible SH-Groups Number, Its Changes, and ATPase Activity

Accessible SH-groups were determined in membrane vesicles from *E. hirae* ATCC9790 and MS116 both. As it is

supposed there is an energy transduction within the bacterial membrane between the F_oF_1 ATPase and secondary transport systems [16, 36, 37], and if the accessible SH-groups in *E. hirae* membrane vesicles are the F_oF_1 ATPase cysteine residues [27], their number can be affected by ATP. In fact, the *E. hirae* F_oF_1 ATPase is a typical F_oF_1 ATPase but its gene structure is not identical to that of other bacterial F_oF_1 ATPases [33], and there are no data about cysteine residues localization in the subunits of *E. hirae* F_oF_1 ATPase. However, the findings with *E. coli* that there are two cysteine residues in the b subunit of F_o sector of the F_oF_1 ATPase, which are probably accessible to ATP and are responsible for an appropriate changes in SH-groups number by ATP [23] may be employed in the study with *E. hirae*. Indeed, in this study, ATP (3 mM) was shown to markedly (~1.3-fold) increase the number of accessible SH-groups in *E. hirae* ATCC9790 membrane vesicles in the presence of 100 mM K^+ ($P < 0.05$) (Fig. 3.). Such effect was not detected in MS116 membrane vesicles (Fig. 3). These results confirm data of Poladyan and Trchounian [27] that the increase in the number of SH-groups by ATP might be associated with the F_oF_1 ATPase; some effect of ATP itself is not ruled out.

Note, this bacterium has the other—Na^+-transporting ATPase belonging to the vacuolar (V)-type ATPase [25], and both genes for F- and V-type ATPases are functionally expressed in one bacterial cell [34]; however it is likely that the F_oF_1 ATPase is mainly responsible for the changes in the number of SH-groups (see Fig. 3) which are accessible in our growth and assay conditions.

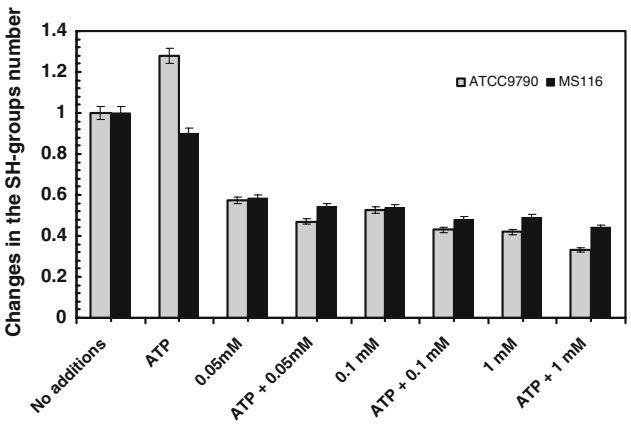

Fig. 3 Effects of Cu^{2+} and ATP on the number of accessible SH-groups in *E. hirae* ATCC9790 and MS116 membrane vesicles. The assay medium contains 100 mM K^+, 3 mM ATP and/or 0.05 mM to 1 mM of $CuCl_2$ were added into the assay medium when specified. The levels of SH-groups in these strains were 1; relative changes by Cu^{2+} and ATP were calculated. For details, see "Materials and Methods" section

We have determined the effect of Cu^{2+} on SH-groups number in ATCC9790 and MS116 membrane vesicles both. The addition of Cu^{2+} decreased the number of SH-groups in a concentration-dependent manner which was less at a higher concentration (Fig. 3). In addition, Cu^{2+} inhibited the effect of ATP in *E. hirae* ATCC9790 membrane vesicles by blocking the ATP-stimulated increase in the number of SH-groups (see Fig. 3). We have shown that only when ATP was added there was a marked difference between SH-groups numbers in ATCC9790 and MS116 (see Fig. 3). The addition of elevated concentrations of Cu^{2+} had no significant effect on SH-groups number difference independent on ATP (see Fig. 3). We can assume that the F_oF_1 ATPase has a major role in these processes especially at a low Cu^{2+} concentration. These ions might cause some conformational change in the F_oF_1 ATPase disturbing its activity. The results obtained seems to be in favor with an idea that oxidizing Cu^{2+} might protect the activity of membrane enzymes from inactivation by SH-reagents presumably by forming a disulfide [19]; decrease in SH-groups level was observed. The breakage of disulfides in membrane proteins when Cu^{2+} ions are reduced on cell surface [18] is less probable with *E. hirae*. Moreover, the results are in agreement with data reported earlier by Kirakosyan et al. [14] who showed that in the case of *E. coli* the addition of ATP increase the number of SH-groups, but when ATP is added together with Cu^{2+} the latter block the ATP-stimulated increase in these groups number as in the case of *E. hirae* ATCC9790.

Furthermore, we can suggest that Cu^{2+} might affect directly the F_oF_1 ATPase. The action of Cu^{2+} on ATPase activity of *E. hirae* membrane vesicles was assessed with and without DCCD (0.1 and 0.2 mM), inhibitor of the F_oF_1 ATPase [1, 27, 36], in the presence of 100 mM K^+. Abrams and Baron [1] had shown many years ago that when a sample of 0.25 mg dry weight of *enterococcal* membranes (membrane preparations were obtained by osmotic lysis of *E. hirae* protoplasts) were incubated with 0.2 mM DCCD for 15 min, the inhibition of membrane ATPase was 89%. This was the maximum inhibition that can be obtained since some of the membrane ATPase is resistant to inhibition by DCCD (Fig. 4) and, moreover, the other ATPase different from the F_oF_1 ATPase might express its activity [34]. In our experiments even with 0.2 mM DCCD used, the inhibition of ATPase activity on ~60% was detected (see Fig. 4). This difference might be due to different growth and assay conditions, the F_oF_1 ATPase level, DCCD preparations and different treatment with DCCD applied. In all cases (DCCD ±) even low Cu^{2+} concentrations had noticeable effect on ATPase activity, but with less visible concentration-dependent manner. Thus, the effect of Cu^{2+} on the number of SH-groups and the F_oF_1 ATPase activity can be explained by destruction

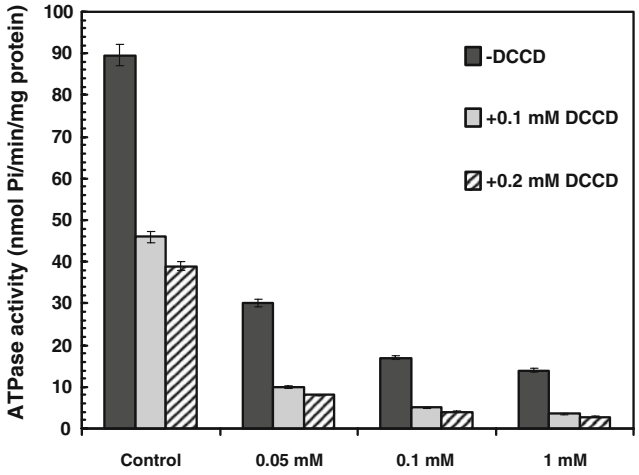

Fig. 4 ATPase activity of membrane vesicles of *E. hirae* ATCC9790 in K^+ containing medium. 0.1 and 0.2 mM DCCD was present in the assay medium when indicated. For details, see "Materials and Methods" section

of dithiol–disulfide interchange in membrane proteins, especially with the F_oF_1 ATPase.

Effect of Cu^{2+} on Proton-Coupled Transport

When grown in a glucose-containing medium, *E. hirae* excrete protons and accumulate potassium ions and such H^+–K^+-exchange has been studied earlier [26]. The F_oF_1 ATPase associated with potassium uptake by TrkA-like system namely KtrI [13] is suggested to be responsible for such an exchange in bacteria [36, 37].

We have shown that DCCD and Cu^{2+} affected each of these ions transport markedly decreasing the fluxes of these ions (Table 1). Moreover, stronger effects were observed when these substances were present together in the medium. The influence of Cu^{2+} on ion fluxes had a concentration-dependent manner (see Table 1). The effect of Cu^{2+} on H^+-coupled transport could be explained by a direct effect of Cu^{2+} on F_oF_1 ATPase those might cause some conformational changes in F_oF_1 destroying dithiol–disulfide interchange between the F_oF_1 ATPase and TrkA-like (KtrI) system. Direct action of Cu^{2+} on the latter system is less probable.

Discrimination Between Cu^{2+} and Ni^2

If Cu^{2+} ions have specific effect on bacterial cells, especially on the number of SH-groups and ATPase activity, there must be discrimination between Cu^{2+} and the other bivalent cations, for instance, nickel (II) ions (Ni^{2+}). The latter is required for all organisms as an enzyme cofactor, and nickel-containing enzymes can account for several percent of the cellular proteins in bacteria such as *E. coli* [12, 21]. Free Ni^{2+} can be toxic to cells by binding non-specifically to biological macromolecules or by displacing other metals from their native binding sites. It is also known that Ni^{2+} can be absorbed on *E. hirae* cell walls [9]. To study effects of Ni^{2+} ions, $NiCl_2$ (0.05, 0.1, and 1 mM) was added in ATCC9790 wild-type strain bacterial suspension. It was established that Ni^{2+} ions did not cause so significant changes in the number of SH-groups of *E. hirae* membrane vesicles if compared with Cu^{2+}: the decrease in SH-groups number by 0.05 mM Ni^{2+} was ~fourfold lower than that with the same concentration of Cu^{2+} (Fig. 5a). Furthermore, Ni^{2+} ions did not also affect ATPase activity:

Table 1 Proton and potassium ions initial fluxes across the membrane for *E. hirae* ATCC9790 wild-type strain at different Cu^{2+} concentrations

Assay conditions[a]	Ion fluxes (mM/min)[b]					
	Total		DCCD-sensitive (0.1 mM)[c]		DCCD-sensitive (0.2 mM)[c]	
	H^+	K^+	H^+	K^+	H^+	K^+
Control	1.90 ± 0.06	0.60 ± 0.03	1.01 ± 0.03	0[d]	0.71 ± 0.02	0
0.05 mM Cu^{2+}	1.70 ± 0.04	0.51 ± 0.02	0.80 ± 0.03	0	0.52 ± 0.04	0
	$P > 0.05$[e]	$P > 0.05$	$P < 0.05$		$P < 0.05$	
0.1 mM Cu^{2+}	1.60 ± 0.03	0	0.72 ± 0.04	0	0.40 ± 0.01	0
	$P < 0.05$		$P < 0.01$		$P < 0.05$	
1 mM Cu^{2+}	1.40 ± 0.04	0	0.50 ± 0.02	0	0.20 ± 0.01	0
	$P < 0.01$		$P < 0.01$		$P < 0.01$	

[a] The bacterial whole cells were washed in distilled water and transferred into the assay medium, 20 mM glucose was added (see "Materials and Methods"); control was without $CuCl_2$ added whereas when specified $CuCl_2$ of 0.05 mM to 1 mM was added before inoculation of bacteria

[b] Calculated per 10^{10} cells/ml

[c] The difference between fluxes values in parallel experiments in the absence and presence of 0.1 mM and 0.2 mM DCCD mentioned (see "Materials and Methods" section)

[d] "0" means the absence of K^+ influx at all

[e] *P* was calculated for difference between the values of experimental samples and appropriate control

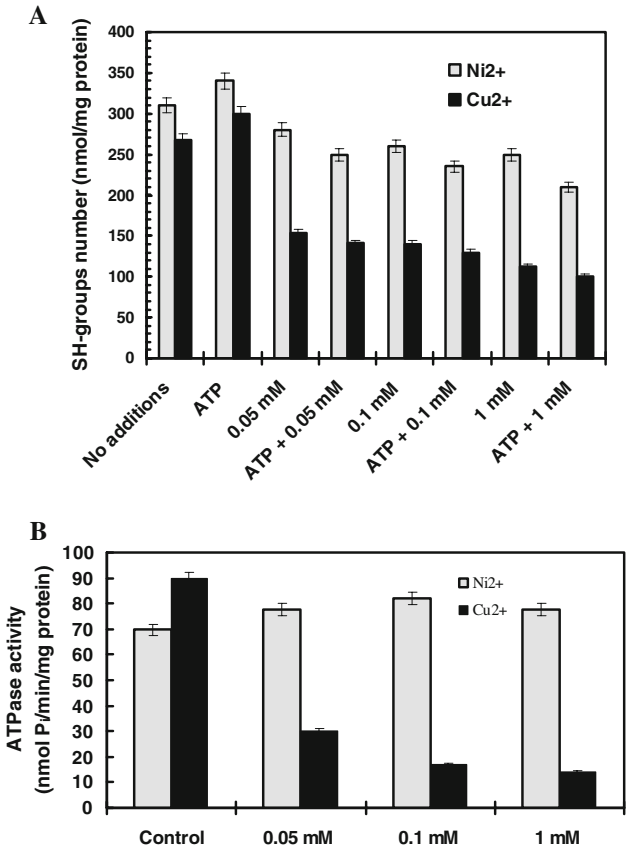

Fig. 5 Changes in the number of SH-groups (**a**) and ATPase activity (**b**) of membrane vesicles of *E. hirae* ATCC9790 in K^+ containing medium in the presence of Ni^{2+} ions. $NiCl_2$ was added into the assay medium when indicated. For details, see the legends in Figs. 3 and 4, and "Materials and Methods" section

if even low Cu^{2+} ions concentrations (0.05 mM $CuCl_2$) had noticeable effect on ATPase activity ($P < 0.01$), much higher Ni^{2+} concentrations (1 mM $NiCl_2$) did not have marked influence on ATPase activity (Fig. 5b).

Thus, the effect of Cu^{2+} is specific among bivalent metal ions, not for one protein, as we have shown that the same concentration of Ni^{2+} ions has lower effect on SH-groups number but has no marked effect on ATPase activity (comp. data on Fig. 5).

Conclusions

In this study, Cu^{2+} ions as oxidizers are shown to inhibit *E. hirae* cell growth at alkaline pH affecting lag phase duration and decreasing specific growth rate. These effects have a concentration-dependent manner, those are less visible in *atpD* mutant MS116 (absence of the β subunit of F_1) and are suggested to be intermediated through E_h (see Fig. 2).

In *E. hirae* ATCC9790 wild-type strain membrane vesicles from anaerobically grown cells, ATP increases the level of accessible SH-groups (see Fig. 3) confirming data reported previously [27]. The addition of Cu^{2+} ions decreases the number of SH-groups in a concentration-dependent manner and these ions block the ATP-stimulated increase in the number of these groups (see Fig. 3). The obtained effects on ATPase activity and SH-groups number are specific for Cu^{2+} (see Fig. 5). These results novel for *E. hirae* are in accordance with those obtained with *E. coli* [14] except opposite effects on the number of SH-groups. Therefore, it is most likely to propose action mechanisms for *E. hirae*.

Such Cu^{2+} ions influence may be resulted by action of these ions on E_h (see Fig. 2a) or by their direct effect on membrane proteins. Cu^{2+} ions are defined to affect directly the F_oF_1 ATPase modulating its activity (see Fig. 4) and to change H^+-efflux and H^+-coupled transport (see Table 1). Cu^{2+} is suggested can cause some conformational changes in the F_oF_1 ATPase. Revealing action mechanisms with *E. hirae*, in contrast to *E. coli,* oxidizing Cu^{2+} might participate in formation a disulfide [19], changing interaction of the F_oF_1 ATPase with Trk-like (KtrI) system [26, 36, 37]; decrease in SH-groups level is observed (see Figs. 3, 5). The breakage of disulfides in membrane proteins when Cu^{2+} ions are reduced on cell surface [18] is less probable.

So Cu^{2+} might affect SH-groups of the F_oF_1 ATPase in *E. hirae* through disturbing a disulfide–dithiol interchange between this ATPase and the other membrane proteins that is installed by ATP and changing activity of this ATPase.

The effects of oxidizing Cu^{2+} ions on *E. hirae* cell growth and activity of the F_oF_1 ATPase have a significance to regulate bacteria in environment and their application in biotechnology.

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