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Redox sensing by *Escherichia coli*: Effects of copper ions as oxidizers on proton-coupled membrane transport

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Abstract

Escherichia coli is able to grow under anaerobic fermentation conditions upon a decrease in redox potential (E_h). Indeed, upon a transition of *E. coli* MC4100 wild-type culture to stationary growth phase a decrease in E_h from the positive values (~+100 mV) to the negative ones (~−520 mV) was observed, the acidification of the medium and the H_2 production were obtained. An oxidizer, copper ions (Cu^{2+}) affected a bacterial growth in a concentration-dependent manner (of 0.1 mM to 10 mM) increasing latent (lag) growth phase duration, delaying logarithmic (log) growth phase and decreasing specific growth rate. Acidification of the medium and the *N*,*N*′-dicyclohexylcarbodiimide (DCCD)- and azide-sensitive proton–potassium exchange by bacteria were inhibited, H_2 production upon growth and under assays disappeared with Cu^{2+} (0.1 mM). These effects were observed with *hycE* but not *hyfR* and *hyc(A–H)* mutants and under aerobic conditions. Cu^{2+} also increased membrane proton conductance. Copper ions are suggested to affect directly the F_0F_1-ATPase associated with potassium uptake transport system and/or formate hydrogenlyase composed with hydrogenase 4. A role of the F_0F_1-ATPase in redox sensing under fermentation is proposed.

Keywords: Copper ions; Redox potential; Proton and potassium transport; The F_0F_1-ATPase; H_2 production; Fermentation; Bacterial growth; *E. coli*

1. Introduction

Escherichia coli grows well under anaerobic conditions upon a decrease in external oxidation–reduction potential (E_h) [1,2]. Under these conditions, this bacterium performs a mixed-acid fermentation of sugars (glucose) when formic acid is oxidized to carbon dioxide and molecular hydrogen [3]. Meantime, CO_2 is likely to be used for the anaerobic metabolic processes [4], whereas the fermentative gas, H_2, produced by formate hydrogenlyase (FHL) [3] is released.

At bacterial growth under these conditions, a shift in E_h to negative values is observed [2,5] that seems to be determinant for the *E. coli* anaerobic fermentation growth: an impermeable oxidizer (ferrycyanide) [5] and a reducer (DL-dithiothreitol) [6] affect the growth in a different manner. *E. coli* is likely to sense E_h independently of oxygen concentration and of the oxidizers or reducers [5–7]. This could suggest a role of membrane redox proteins, however, the mechanisms of such a redox sensing or taxis [5–9] are unclear.

E_h might determine an electron transfer within bacterial membrane and proton-motive force [10]. It is suggested that the effect of E_h on proton-motive force under anaerobic conditions is induced by the change in pH gradient across the membrane [6,10]. The latter resulted from an alteration in the cytoplasmic pH by fermentation acids [11]; the membrane potential is changed slightly [6,10]. Such dependence is due to change in the membrane proton permeability without modification of the proton-translocating F_0F_1-ATPase activity [10,12]. The change of the membrane proton permeability might depend on with the oxidation–reduction state and distribution of thiol groups in membrane proteins [13,14] when a dithiol–disulfide interchange is proposed [15]. The number of accessible SH-groups in *E. coli* membrane vesicles has been shown recently [14] to be increased by ATP or by formate suggesting an interaction of the F_0F_1-ATPase with hydrogenase 4 (Hyd-4) or less probably hydrogenase 3 (Hyd-3), components of FHL, under fermentation conditions [16–18]. This would lead to formation of a protein–protein complex within which the energy could be

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transferred via a dithiol–disulfide interchange [19]. The latter is likely to be an interesting energy-transferring mechanism in fermentation conditions that is of significance for the growth. At the same time, a correlation between E_h and the state and distribution of thiol groups on the bacterial membrane was found [5,13]. Therefore, a change in the state and distribution of thiol groups can be involved in the mechanism of redox sensing in *E. coli*.

Aside from the fact that copper ions (Cu^{2+}) are required for *E. coli* [20,21], in a low concentration, these ions are able to promote the growth [22]; however, in a higher concentration, they are toxic, disturbing the membrane by inducing permeability [23]. The latter effect is suggested to result from the break of disulfides in membrane redox proteins when Cu^{2+} are reduced on cell surface or by reducers. Cu^{+} rather than Cu^{2+} could then be accumulated, but the mechanisms for homeostasis and resistance, which involve a balance between influx and efflux systems, are suggested [20]. At the same time, pathways of Cu^{2+} uptake or its reduction and intracellular copper handling are still not identified [20,21]; however, effects of Cu^{2+} on the membrane might be important for a mechanism of redox taxis.

In the present work, the effect of Cu^{2+} on *E. coli* growth and proton-coupled membrane transport are shown. These ions increase a latent (lag) growth phase duration, delay a logarithmic growth phase as well as decrease specific growth rate; decrease in E_h is less, proton–potassium exchange is altered and H_2 production is inhibited by Cu^{2+}.

2. Experimental

2.1. Bacterial strains, bacterial growth and preparation

The *E. coli* strains used in this study are detailed in Table 1.

Bacteria were grown under anaerobic or aerobic conditions at 37 °C in peptone medium (pH 7.5) with 0.2% glucose as described earlier [5,18,25]. Bacterial growth was monitored and lag growth phase duration was determined as described previously [6]. Specific growth rate was calculated over the interval, where the logarithm of absorbance of the culture at 600 nm increased linearly with the time and is expressed as 0.693/doubling time. Fermentation under the anaerobic growth conditions used was detected as before [25].

Preparation of bacteria for the assays was as described [5,17,18]; the assay mixture was of 200 mM Tris–phosphate (pH 7.5) containing 0.4 mM $MgSO_4$, 1 mM NaCl and 1 mM KCl (indicated if changed). For DCCD inhibition studies, cells were incubated with the reagent at 0.1 mM for 10 min.

2.2. Determination of redox potential and H_2 production

The value of E_h in bacterial suspension was measured using platinum electrode (Hanna Instruments, Portugal) as described [5,6,17,18].

H_2 production rate was determined as a difference between the initial rates of decrease in E_h for platinum and titanium silicate (Enterprise of Measuring Instruments, Gomel, Belarus) electrodes [5,6,17,18]. In contrast to titanium silicate electrode, a platinum electrode is sensitive to H_2 and O_2, allowing detection of H_2 production under anaerobic fermentation conditions. The latter is expressed in mV E_h/min/mg of dry weight of bacteria. This difference has become obvious since H_2 production confirmed also by a chemical assay and the Durham tube method [17,18]. It would be interesting to note, by the latter method, that CO_2 and H_2 could be detected; however, CO_2 is likely not to be evolved and to be used for the anaerobic metabolic processes by *E. coli* [4], while H_2 is extruded outside. Hence, the Durham tube method was used in confirmation with the other one.

2.3. H^+, K^+ transport assays

H^+ and K^+ fluxes through the bacterial membrane in whole cells were measured using appropriate selective electrodes (Hanna Instruments, Portugal; Cole Parmer Instruments Co., USA) as described elsewhere [2,5,25]. Ion fluxes are expressed as the change in external activity of the ion in mmol/min/10^{12} cells. Small changes in external pH and K^+ activity were recorded using a potentiometer, and they were calibrated by titration with 0.01 N HCl and 0.02 mM KCl.

2.4. Determination of membrane proton conductance

The membrane proton conductance (C_m^{H+}) was determined by the acid-pulse technique described by Maloney [26] and employed before [27]. Cells were washed with 300 mM KCl and transferred into the assay mixture containing 300 mM KCl, 50 mM potassium thiocyanate and 10 μM valinomycin. After incubation for 1–2 h and establishment of a steady-state pH, 0.01 N HCl in small portions (acid pulse) were added to change pH for ~0.1 unit, then H^+ flow kinetics was measured as

Table 1
E. coli strains used in this study

Strain	Genotype[a]	Protein absent	Source or reference
HD700	MC4100 *Δ(hycA–H)*	Hyd-3	S. C. Andrews (School of Animal and Microbial Sciences, The University of Reading, Reading, UK) [24]
HD705	MC4100 *Δ(hycE)*	HycE[b] of Hyd-3	S. C. Andrews [24]
JRG3618	MC4100 *Δ(hyfR)*	Hyd-4	S. C. Andrews [18]
MC4100	*araD139Δ(argF-lac)169 ptsF relA1 fibB5301 rpsL150*	wild-type	A. Bock (Laboratory of Microbiology, Munich University, Munich, Germany) via S.C. Andrews [24]

[a] *hyc* operon codes Hyd-3 and *hyf* operon codes Hyd-4 [16,24].
[b] Large subunit of Hyd-3 [24].

detailed above. C_m^{H+} was expressed in μmol H^+/s per unit of pH and the dry weight of bacteria.

Note, the addition of a protonophore, carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), 2 μM, in 30 s after the acid pulse did not change pH indicating the achievement of electrochemical equilibrium in the H^+ distribution on each side of the membrane. The change in buffer capacity was very little after the acid pulse, and this had no effect on the H^+ flow.

2.5. Others and reagents

Bacterial titre and dry weight of bacteria were determined as described [5,6,18,25]. The average data are presented from two or three independent measurements; the standard error does not exceed 5% (if not indicated). Agar, CCCP, $CuCl_2$, DCCD, peptone, NaN_3 and valinomycin were from Sigma (USA), and other reagents were of analytical grade.

3. Results and discussion

3.1. Bacterial growth inhibition with Cu^{2+}

It is a matter of interest that the anaerobic growth of *E. coli* is coupled with a shift of E_h from the positive to the negative values, which probably determine the growth [2,5,25]. Indeed, *E. coli* MC4100, wild-type strain (Table 1), grew well under anaerobic conditions, resulting in a shift in E_h from the positive values (~+100 mV) at the beginning of a lag growth phase to the negative ones (~−520 mV) (Fig. 1) upon transition to a stationary phase. The drop of E_h gives evidence of the amplification of reduction processes, which are apparently characteristic of metabolism under anaerobic conditions. Under these conditions, upon the fermentation of glucose, the medium was acidified and the production of H_2 was detected (not shown).

The addition of 0.1 mM and 2 mM Cu^{2+} ions ($CuCl_2$) into the growth medium resulted in a delayed decrease of E_h although a drop in E_h was less for rather 2 mM than 0.1 mM (Fig. 1). Duration of a lag growth phase was considerably increased and growth rate was decreased in the presence of Cu^{2+} in a concentration-dependent manner (for concentrations of 0.1 mM to 10 mM used) (Fig. 2). It should be noted that a higher concentration value inhibiting *E. coli* growth was reported by Jun et al. [22]. This could be due to different growth conditions and bacterial strains.

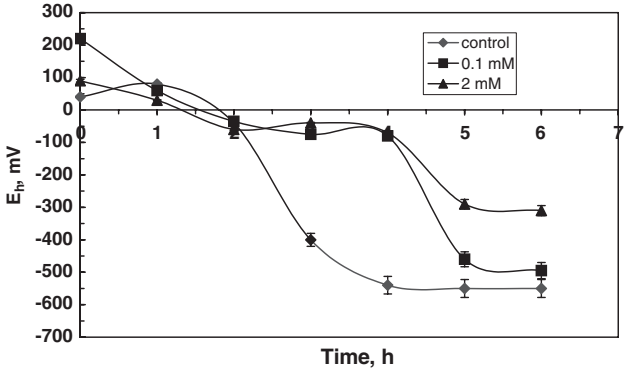

Fig. 1. Decrease of E_h during *E. coli* MC4100 wild-type cells anaerobic growth in the peptone media with various concentrations of $CuCl_2$ indicated. For the others, see Experimental.

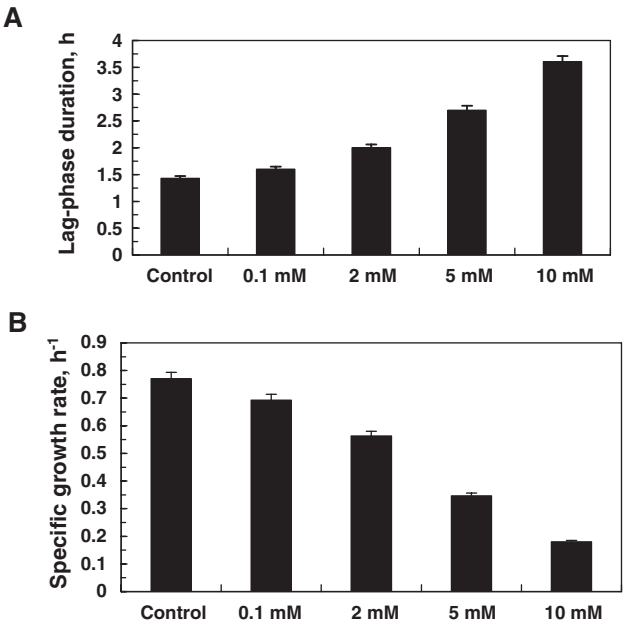

Fig. 2. Effects of Cu^{2+} in different concentrations on the growth characteristics of *E. coli* MC 4100: (A) lag growth phase duration; (B) specific growth rate. The conditions were the same as in Fig. 1; concentrations of $CuCl_2$ added into the growth medium were indicated; for the others, see Experimental.

The suppression of bacterial growth in the presence of Cu^{2+} may be resulted by action of the reagent on E_h or by direct effect of these ions on bacterial membrane. The first of these possibilities seems to be not likely since E_h measured with titanium silicate electrode did not decrease markedly (not shown). Anyway, the results are in accordance with data of Kirakosyan et al. [6] and Riondet et al. [28] indicating that a decrease in E_h has brought about a longer lag phase and a slower growth rate and even led to growth failure.

3.2. Effects of Cu^{2+} on proton-coupled transport

E. coli requires Cu^{2+} [20,21], however in a higher concentration these ions have bactericide action [22] (see Fig. 2). The latter could be due to direct effect of the reagent on thiol groups having a role in the affinity and activity of transport systems and membrane-associated enzymes [14,21].

Cu^{2+} in a low concentration (0.1 mM) resulted in change of medium acidification by proton secretion and of potassium accumulation by *E. coli* MC4100 (Table 2). Proton–potassium exchange carried out by *E. coli* has been shown to be sensitive to DCCD and azide as well as to have a fixed stoichiometry of $2H^+$ for K^+ for DCCD-inhibited fluxes [25]. Moreover, the F_0F_1-ATPase associated with potassium uptake TrkA system is responsible for such an exchange [19]. Indeed, H^+ efflux and K^+ influx by *E. coli* wild-type cells at slightly alkaline pH upon applying glucose (10 mM) were inhibited by DCCD (Table 2) and azide (not shown), and this exchange had a stoichiometry of

Table 2
Effects of Cu^{2+} on proton and potassium ions fluxes through the membrane of *E. coli* wild-type and mutant cells grown in anaerobic (fermentative) or aerobic conditions[a]

Assay conditions	Ion fluxes (mmol/min per 10^{12} cells)					
	Fermentation				Aerobic respiration	
	MC4100 (wild-type)		HD705 (*hycE*)		MC4100 (wild-type)	
	H^+	K^+	H^+	K^+	H^+	K^+
Control (no additions)	5.45±0.10	0.23±0.03	5.50±0.06	0.19±0.02	3.50±0.08	0.21±0.02
+DCCD (0.1 mM)	5.10±0.04	0.08±0.01	5.25±0.03	0.09±0.01	2.80±0.04	0.15±0.01
+$CuCl_2$ (0.1 mM)	3.60±0.21	0.14±0.01	4.56±0.23	0.22±0.00	3.30±0.12	0.18±0.01
+DCCD (0.1 mM)+$CuCl_2$ (0.1 mM)	1.74±0.12	0.12±0.01	5.50±0.15	0.18±0.02	2.85±0.03	0.14±0.01

[a] Glucose in concentration of 10 mM added in the assay medium; for the others, see Experimental.

$2H^+$ for K^+ (could be calculated from data represented in the Table 2) fixed for variable K^+ activity in the assay medium (not shown). However, these fluxes were markedly decreased with 0.1 mM Cu^{2+} (Table 2), and the stoichiometry became non-fixed (not shown). Interestingly, DCCD and Cu^{2+} adding together had a more strong effect on H^+ efflux (Table 2) requiring a further study.

In the *E. coli* HD705 mutant with deleted *hycE* gene (Table 1), the H^+ efflux and K^+ influx both were inhibited with DCCD (Table 2); however, the stoichiometry for DCCD-inhibited fluxes is changed (not shown). This is in confirmation of data reported before [18,29–31] indicating that the *hycB* gene only and the *hyf* operon products are involved in $2H^+/K^+$-exchange.

In *hycE* mutant, proton-potassium exchange is carried out by the F_0F_1-ATPase and via the TrkA system, which are operating in association of each other as suggested [29,30]. This mode of H^+–K^+-exchange is characteristic for *E. coli* wild-type strain grown in anaerobic but not in aerobic conditions [19,25] although proton fluxes having lower values (Table 2). Note that in aerobic conditions the F_0F_1-ATPase is functioning to synthesize ATP and H^+ are secreted through electron transfer chain of *E. coli*, which is also DCCD-inhibited (Table 2) [25]. It was of the most interest that, in *hycE* mutant but not in aerobically grown wild-type cells, Cu^{2+} decreased the H^+ efflux but had negligible effect on K^+ influx (Table 2). These indicate that Cu^{2+} might affect the F_0F_1-ATPase but not the TrkA system.

It should be noted that Cu^{2+} were not effective in changing H^+ fluxes through the membrane measured without glucose applied (not shown), in spite of active H^+ efflux changed (Table 2). Moreover, in *E. coli* MC4100 cells, the proton conductance measured (see Experimental) was of 0.33 μmol H^+/s per pH unit and g of dry weight, and Cu^{2+} increased proton conductance in ~2-fold confirming the suggestion of Lebedev et al. [23]. This might be counted for a decrease in H^+ secretion (see Table 2) and effects of DCCD with Cu^{2+}, although the bacterial membrane without the F_0F_1-ATPase (*atp* operon deleted) has a less proton conductance (K. Akopyan, personal communication), and the relationship between passive and active pathways in circulation of H^+ through the membrane is not studied well.

Therefore, the effects of Cu^{2+} in a low concentration on proton-coupled transport in fermenting cells could be explained by a direct effect of Cu^{2+} on the F_0F_1-ATPase. Some conformational change in F_0F_1 leading to modulation of activity by Cu^{2+} is possible. For the functioning of F_0F_1-ATPase in association with the TrkA system under fermentation conditions, a dithiol–disulfide interchange is suggested to transfer energy from F_0F_1 to TrkA [15,19], hence the change in accessible SH-groups under defined conditions has been determined [14]. The effect of Cu^{2+} on proton-coupled transport might be because of affecting such a dithiol–disulfide interchange. This could be in accordance with a break of disulfides in membrane proteins when Cu^{2+} ions are reduced on cell surface or by reducer that is suggested by Lebedev et al. [23]. Direct action of Cu^{2+} on the TrkA protein is ruled out. In addition, under aerobic conditions, the absence of the effect of Cu^{2+} on K^+ uptake (Table 2) might point out a distinguish mode for proton-coupled transport. The results are agreed with an idea that oxygen has no effect on Cu^{2+}-induced change in membrane properties; otherwise, oxygen is able to protect reduction of these ions on the membrane [32].

3.3. Effects of Cu^{2+} on H_2 production

E. coli has been shown to produce H_2 by FHL, the second pathway of which composed of Hyd-4 is major at alkaline pH [17,18]. This FHL is suggested to function in a proton-coupled

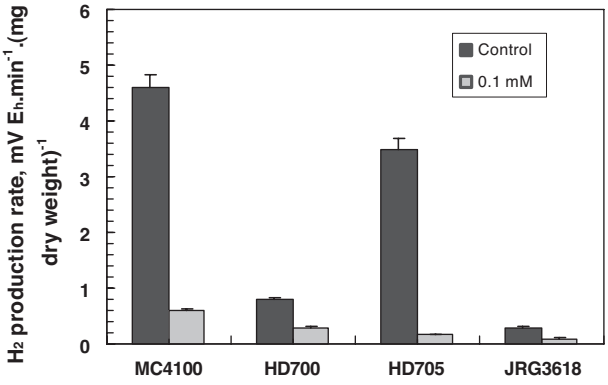

Fig. 3. Effects of Cu^{2+} (0.1 mM) on the molecular hydrogen production by *E. coli* wild-type and mutant cells grown under anaerobic conditions. For mutants, see Table 1. H_2 production was assayed with cells washed and transferred into the assay mixture, 10 mM glucose was added; for the others, see Experimental.

manner when the F_0F_1-ATPase associates with the enzyme [19]. In fact, H_2 production by a wild-type strain after the addition of glucose was inhibited with Cu^{2+} (Fig. 3). Such an inhibition was observed (Fig. 3) with *hycE* mutant (Table 1). In this mutant, Hyd-4 is responsible for H_2 production [18,29,31]. To confirm this, H_2 production was shown to be insignificant in *hyc(A–H)* (HD700) and *hyfR* (JRG3618) mutants (Fig. 3) in which Hyd-4 did not function [18,29,31]. This could also point out an interaction between the *hyf* operon and the genes determining the synthesis of other hydrogenases that is discussed in different papers [16,17,24].

The results indicate the action of Cu^{2+} on FHL composed of Hyd-4 that could be again explained by an effect of Cu^{2+} on the F_0F_1-ATPase, which is associated with formate hydrogenlyase under fermentative conditions [19]. Direct effect of Cu^{2+} on the *E. coli* FHL is not ruled out. Interestingly, inhibitory action of copper ions on hydrogenase activity in arche-bacteria has been shown by Sapra et al. [33]; however, the enzyme, which may have different forms, is not yet identified.

4. Concluding remarks

It is clear that the oxidation–reduction processes play an exclusive role in the habitability of bacteria. The majority of these processes implemented on bacterial membrane depend on E_h [6,12,17,18]. In this respect, Cu^{2+} as oxidizers are shown to affect the growth of *E. coli* as well as their effects on the growth is suggested can be intermediated through E_h. If so, a concentration-dependent effect should be observed (Fig. 2).

Moreover, Cu^{2+} are likely to affect directly the F_0F_1-ATPase and to change proton permeability of the bacterial membrane (see Results and discussion). This could be the break of disulfides in membrane proteins [23]; inhibition of a dithiol–disulfide interchange between proteins proposed [15,19] is possible. The latter is a mechanism for transfer of energy in functioning of the F_0F_1-ATPase, which could associate with secondary transport system (TrkA) and/or anaerobic redox enzyme (FHL) under fermentation conditions [19]. This suggestion seems to be in favor with an idea that the F_0F_1-ATPase itself is a redox mechanism [2,12,14,34]. If so, this enzyme might be a target having a significant role or involving in redox taxis of bacteria.

Interestingly, Cu^{2+} have specific effects on yeast cells eliciting changes in the membrane permeability with release of K^+ and amino acids [35], however the effects with *E. coli* appear to be different.

Besides, oxidizers and reducers modifying the redox environment and affecting bacterial fermentation [5,6,24] are being used in many biotechnological processes [36] and understanding of the mechanisms of this could lead to directed regulation of cellular metabolism.

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