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The Cu(II)-reductase NADH dehydrogenase-2 of *Escherichia coli* improves the bacterial growth in extreme copper concentrations and increases the resistance to the damage caused by copper and hydroperoxide

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Abstract

NADH dehydrogenase-2 (NDH-2) from *Escherichia coli* respiratory chain is a membrane-bound cupric-reductase encoded by *ndh* gene. Here, we report that the respiratory system of a *ndh* deficient strain suffered a faster inactivation than that of the parental strain in the presence of *tert*-butyl hydroperoxide due to endogenous copper. The inactivation was similar for both strains when copper concentration increased in the culture media. Furthermore, several *ndh* deficient mutants grew less well than the corresponding parental strains in media containing either high or low copper concentrations. A mutant strain complemented with *ndh* gene almost recovered the parental phenotype for growing in copper limitation or excess. Then, NDH-2 gives the bacteria advantages to diminish the susceptibility of the respiratory chain to damaging effects produced by copper and hydroperoxides and to survive in extreme copper conditions. These results suggest that NDH-2 contributes in the bacterial oxidative protection and in the copper homeostasis.

Keywords: Copper metabolism; Hydroperoxide toxicity; Cupric-reductase; NADH dehydrogenase; Respiratory chain; *Escherichia coli*

Copper is an essential nutrient required for many biochemical and physiological functions, acting as a cofactor for several enzymes [1]. However, in some conditions, copper is also a toxic element able to catalyze free radicals formation, leading to alteration of nucleic acids, lipids, and proteins [2,3]. Therefore, the viability of cells depends on a perfect regulation of transport, storage, and distribution of this metal. Several aspects in the copper homeostasis have been studied in different organisms [4–6], but the mechanisms involved are not totally elucidated. Copper reduction seems to be a central mechanism related to the metal metabolic processes. Rae et al. [7] have reported that there are no free copper ions in *Sacchromyces cerevisiae*, since they are totally bound mainly as Cu(I) to proteins and low molecular weight substances to avoid the metal toxicity. It is also known that *Escherichia coli* contains a P-type ATPase that pumps Cu(I) but not Cu(II), based on the inhibition of the transport into vesicles in the absence of a strong reducer [4,8].

Typically iron or copper, as redox-active metal ions, interact with peroxides converting them to harmful reactive radicals [9–13]. Our previous studies in *E. coli* as a model system for *tert*-butyl hydroperoxide (t-BOOH)[1] toxicity

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[1] *Abbreviations used:* t-BOOH, *tert*-butyl hydroperoxide; NDH-1 and NDH-2, NADH dehydrogenase 1 and 2, respectively.

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have shown that electron flow through the respiratory chain supports the reduction of membrane-bound cupric ions to Cu(I) [14]. This Cu(I) is then oxidized by hydroperoxide, with simultaneous inactivation of the respiratory oxidases. Two sites of electron transfer to membrane-bound copper, coincident with two major *loci* of copper-mediated damage by hydroperoxide, have been identified in the respiratory chain [15], as shown in Fig. 1. One site of copper reduction is localized between NADH and quinone (Site I), and the other between quinone and the cytochromes (Site II). In Site I, the damaged component is one of the NADH dehydrogenases (NDH), the NDH-2 encoded by *ndh* gene. Our studies have also shown that purified NDH-2 has cupric-reductase activity and contains Cu(I) tightly attached [16,17]. In *E. coli*, both NDH-1 and NDH-2 are used to a significant extent during aerobic respiration [18], but the physiological role of the non-coupling NDH-2 remains unclear.

In the present work, we performed in vivo experiments to analyze the effect of copper and t-BOOH on *E. coli ndh* deficient mutants and their parental strains. We have studied the respiratory chain stability to t-BOOH using cells grown with different copper concentrations. We also have compared the capacity of the cells to grow on minimal media containing either copper or metal chelator supplements. We have found that NDH-2 gives the bacteria advantages to diminish the susceptibility of the respiratory chain to damaging effects produced by Cu(II)/t-BOOH and to survive in extreme copper conditions, suggesting that the enzyme contributes in the oxidative protection of the respiratory chain and in the copper homeostasis.

Materials and methods

Bacterial strains and growth conditions

The *E. coli* strains used in this study are listed in Table 1. Cells were grown aerobically at 37 °C, in minimal salt medium M9 [23] supplemented with 0.1% tryptone and 0.5% glycerol (M-medium). In all cases, growth curves were followed by absorbance at 560 nm for at least three independent times. To study copper toxicity, cells grown in M-medium up to mid-exponential phase were plated for isolated colonies on M-medium agar containing increasing amounts of $CuSO_4$. The plates were incubated at 37 °C for 48 h before determining the CFUs. Additionally, cells were tested in either liquid or solid M-medium supplemented with different quantities of $CuSO_4$, but in the presence of 100 mM sodium citrate (pH 7.5). Citrate cannot be metabolized by the strains tested and it functions as an intermediate strength metal chelator, which stabilized the Cu(II) in solution. Other

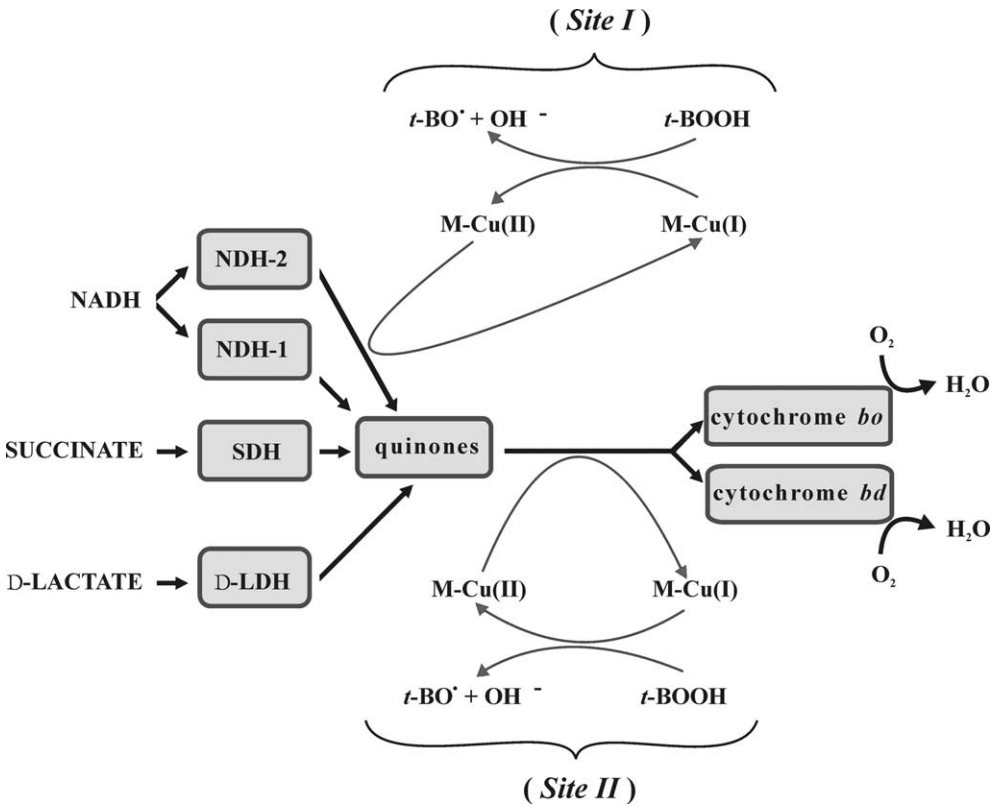

Fig. 1. Scheme of the aerobic respiratory chain of *E. coli*. The sites of electron transfer to membrane-bound copper, involved in hydroperoxide-induced damage, are also indicated (adapted from [15]). SDH, succinate dehydrogenase; D-LDH, D-lactate dehydrogenase; M-Cu, membrane–bound copper; t-BO·, *t*-butylalkoxy radical.

Table 1
Strains and plasmid of *E. coli* used in this study

Strains and plasmid	Relevant genotype	Source or reference
AN387	*rpsl*, *gal*	[19]
ANN001	AN387 *ndh*::Tc	T. Friedrich
LVS003	ANN001 pIY9	This work
GR70N	F^-, *thi*, *rpsl*, *gal*	[20]
MWC215	GR70N *ndh*::Cm, Man^+	[18]
GR19N	F^-, *thi*, *rha*, *lacZ gal*, *cyd*	[21]
MWC233	GR19N *ndh*::Km, Man^+	[18]
pIY9	pSF2124 double *lacp ndh*$^+$ Ap	[22]

group of experiments were carried out with metal unsupplemented M-medium containing different amounts of sodium citrate (pH 7.5).

Membrane preparation and protein determination

Bacteria were harvested in mid-exponential phase of growth by centrifugation at 4 °C. Membranes were prepared from spheroplasts by a procedure adapted from Evans [24] and stored −70 °C in 20 mM Tris/HCl (pH 7.5) containing 1 mM $MgCl_2$, as previously described [14]. Membrane proteins were measured by the method of Lowry et al. [25].

Membrane treatments with copper and t-BOOH

To evaluate the oxidative damage on the respiratory chain, membranes were loaded with $CuSO_4$, and then incubated with or without t-BOOH in the presence of a reduced substrate (NADH, D-lactate, or succinate) as follows. Membranes (0.3 mg ml^{-1}) were preincubated for 10 min at 37 °C in 50 mM sodium phosphate buffer (pH 7.5) with 6 μM $CuSO_4$. They were spun down, rinsed, and resuspended in equal volume of the buffer. Individual aliquots of the Cu(II)-loaded membranes were incubated for different periods of time in the absence (controls) and in the presence of 0.5 mM t-BOOH. Controls and t-BOOH incubations contained also reduced substrates. D-Lactate and succinate were added once at an initial concentration of 10 mM, whereas NADH was added at 0.2 mM; its consumption was followed in parallel at 340 nm and before its depletion, the addition of 0.2 mM NADH was repeated as necessary during the incubation time. After incubations, samples were rinsed and resuspended in the buffer for determining enzymatic activities.

Determination of membrane oxidase and dehydrogenase activities

Membrane NADH, succinate, and D-lactate oxidase activities were assayed by oxygen consumption with a Clark electrode (Gilson Oxygraph) in stirred 2 ml chambers at 37 °C. Reaction mixtures contained appropriated dilutions of the treated membranes in 50 mM sodium phosphate buffer (pH 7.5) and 0.3 mM NADH, 10 mM succinate, or 10 mM D-lactate, respectively.

Membrane NADH, succinate, and D-lactate dehydrogenase activities were determined following the reduction of the artificial electron acceptor MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] at 570 nm. Reactions were performed at 37 °C in 0.5 ml of membrane suspensions in 50 mM sodium phosphate buffer (pH 7.5) containing 50 μg ml^{-1} MTT and 0.3 mM NADH, 10 mM succinate, or 10 mM D-lactate, respectively. To measure the dehydrogenase activities, all the oxidases were inactivated with 3 mM KCN, which was included to the reaction mixtures before the addition of MTT.

Cells treatment with t-BOOH and oxygen consumption assay

Cells grown in M-medium or M-medium containing 0.05 mM or 0.2 mM $CuSO_4$ were harvested in mid-exponential phase by centrifugation at 4 °C, washed and resuspended in 50 mM sodium phosphate buffer (pH 7.5) containing 0.5% glycerol to give an absorbance of 0.4 at 560 nm. The suspensions were then incubated at 37 °C with agitation for up to 2 h in the absence (controls) and in the presence of 1 mM t-BOOH. At different times, the oxygen consumptions of the cells were determined using 2 ml aliquots by a Clark electrode at 37 °C.

Results

NDH-2 inactivation by Cu(II)/t-BOOH and attenuation of the damage produced in the respiratory chain

The wild-type respiratory chain is damaged by t-BOOH in a copper-dependent manner, being NDH-2 and quinones the main injured components (see scheme on Fig. 1) [14,15]. To further understand the effect of copper and t-BOOH on the respiratory chain, we measured NADH, succinate and D-lactate oxidase and dehydrogenase activities in treated wild-type membranes. $CuSO_4$ concentration was used within the range in which it did not inhibit the respiratory oxidases by itself, according to the protein concentration in the mixture [14]. The NADH oxidase is inactivated faster than the other oxidases (Fig. 2), resulting totally inactive at 40 min, meanwhile D-lactate and succinate oxidases maintained 50% of activity. In addition, NADH dependent dehydrogenase is inactivated faster than D-lactate and succinate dehydrogenases, which retained approximately 85–90% of the activity at least for 2 h (data not shown). As expected, in parallel controls incubated only with copper, the oxidases or the dehydrogenases remained active during the study. It should be noted that in our assay conditions, NDH-1 is not active. Therefore, the NADH dependent oxidase and dehydrogenase activities correspond to NDH-2 [15]. In other conditions, in which NDH-1 activity could be measured, only a slight inacti-

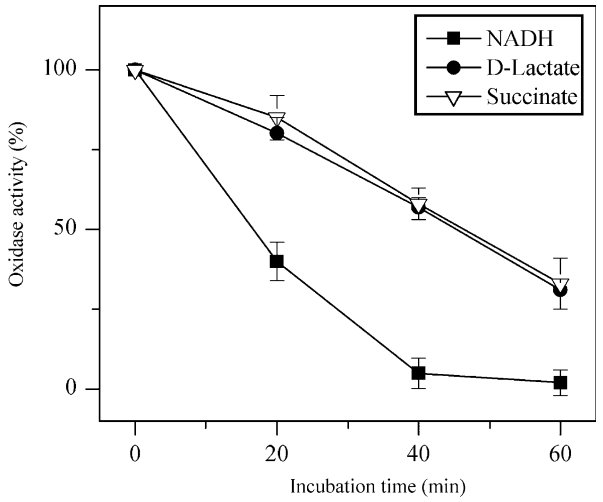

Fig. 2. Copper-mediated inactivation of the respiratory oxidases in AN387 membranes by exposure to t-BOOH and different substrates. Cu(II)-loaded membranes were incubated with t-BOOH in the presence of different substrates (see Materials and methods). At the indicated incubation times, the corresponding membrane oxidases (NADH, D-lactate, or succinate) were measured. Activity values are expressed as percentages of controls without t-BOOH in each time. Data are the average ± SD of four experiments.

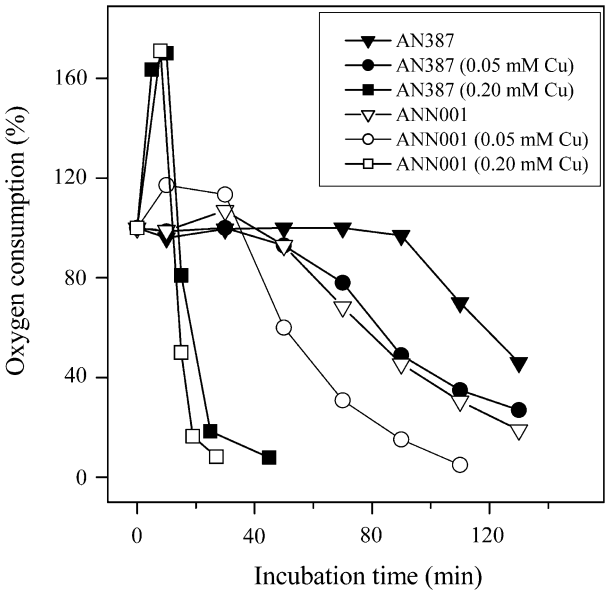

Fig. 3. Copper-dependent damage induced by t-BOOH in the respiratory chain of AN387 and ANN001 intact cells. Bacteria grown in M-medium or M-medium with $CuSO_4$ (concentrations as indicated) were harvested and incubated in buffer containing glycerol and t-BOOH (see Materials and methods). Aliquots of the incubations were taken at different times to measure the oxygen consumption of cells. Relative consumption rates were calculated as percentages of parallel controls without t-BOOH. Data are representative of at least three independent experiments.

vation on NADH dehydrogenase was induced by Cu(II)/t-BOOH using a *ndh* deficient strain [15].

The rapid inactivation of NDH-2 presented here could have a significance in the respiratory chain physiology. To analyze this effect in vivo, we measured the oxygen consumption of intact cells after treatment with Cu(II)/t-BOOH, comparing a *ndh* deficient strain (ANN001) with its parental strain (AN387). When the cells were grown in M-medium without copper supplement, AN387 respiratory chain was fully active up to 90 min, while ANN001 respiratory chain was 55% inactivated (Fig. 3). When the cells were grown with 0.05 mM $CuSO_4$, both strains were more affected than in the unsupplemented condition, but the mutant ANN001 was inhibited faster than AN387 (Fig. 3). On the other hand, with the supplement of 0.2 mM $CuSO_4$, respiratory activity was suppressed independently of the presence of NDH-2 within 20 min, with a strong oxidative burst in the first 10 min (Fig. 3). It should be considered that: (a) a copper concentration in the culture media up to 0.2 mM did not affect the rate of growth in any of the strains tested (see below); (b) absolute rate of oxygen consumption for both strains were similar at the beginning of the incubation with t-BOOH (around 0.1 nmoles O_2 min^{-1} 10^6 $cells^{-1}$); (c) the copper supplement produces a slight effect on the oxygen consumption rate of control samples (incubated in the absence of t-BOOH), with an overall decrease of 10% in the oxygen consumption after 2 h; and (d) t-BOOH by itself cause damage due to the endogenous pool of membrane-bound copper [14].

The above results indicate that *ndh* deficient strain is more sensitive than the wild-type to Cu(II)/t-BOOH and that NDH-2 is one of the first component damaged in the respiratory system. Therefore, the enzyme could act as a free radicals scavenger by preserving the activity of the other respiratory components through its own inactivation.

NDH-2 protects the cells in medium containing high copper concentrations

To study the respiratory chain stability against t-BOOH, we must utilize a Cu(II) concentration in the culture media up to 0.2 mM which did not affect the growth curves. The copper concentrations higher than 0.5 mM affected the growth, mainly of ANN001 strain. To further analyse that point and considering the idea that the Cu(II)-reductase NDH-2 may be involved in copper metabolism, we checked the effect of the high copper levels on AN387 and ANN001. To do that, we determined the number of CFUs on plates with increasing copper concentrations. The *ndh* deficient strain grew up to 0.65 mM $CuSO_4$, whereas the wild-type was able to grow even on 1 mM $CuSO_4$ (not shown). Independently of the strain tested, when the copper concentration increases the colonies become pigmented and smaller.

Besides, we studied the effect of the addition of copper to liquid M-medium containing 100 mM citrate (Fig. 4). We used citrate as a suitable chelator to modulate the free metal concentration available for the cells. In the media containing citrate, the copper concentrations added were in the millimolar range, due to its chelating effect.

ANN001 grew poorly or did not grow at copper concentrations in which AN387 grew normally (Fig. 4A, B, and D). To validate that the observed effects were due to the absence of NDH-2, we complemented the strain ANN001 with the plasmid pIY9 (multicopy plasmid carrying *ndh* gene). The resulting strain, LVS003, showed a partial recovery when it was exposed to high copper concentrations compared with the *ndh* deficient mutant ANN001, being similar to AN387 (Fig. 4C and D). Comparative results were obtained when we assayed AN387, ANN001, and LVS003 on agar plates containing Cu(II) and citrate

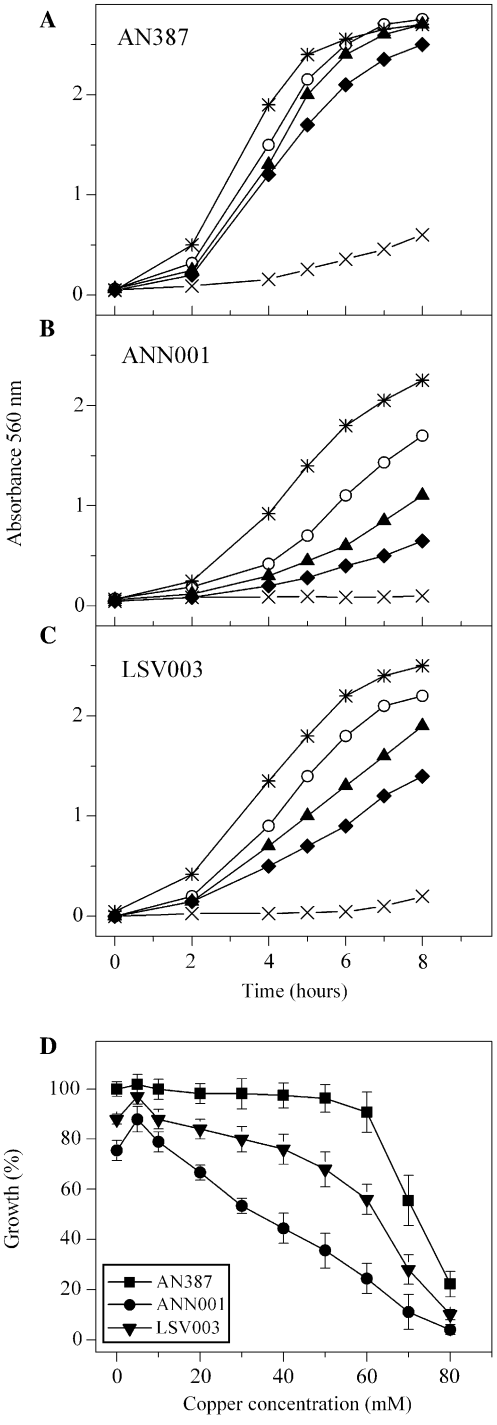

Fig. 4. Effect of copper excess on the bacterial growth of strains AN387, ANN001, and LVS003. (A), (B), and (C) growth curves of the indicated strains followed in M-medium (*) or M-medium in the presence of 100 mM sodium citrate (○) and 100 mM sodium citrate plus 40 (▲), 60 (♦), and 80 (×) mM $CuSO_4$. (D) Relative growth at 8 h of the indicated strains as a function of copper concentration. Values are expressed as percentages of the controls in M-medium and they represent the average ± SD of three experiments.

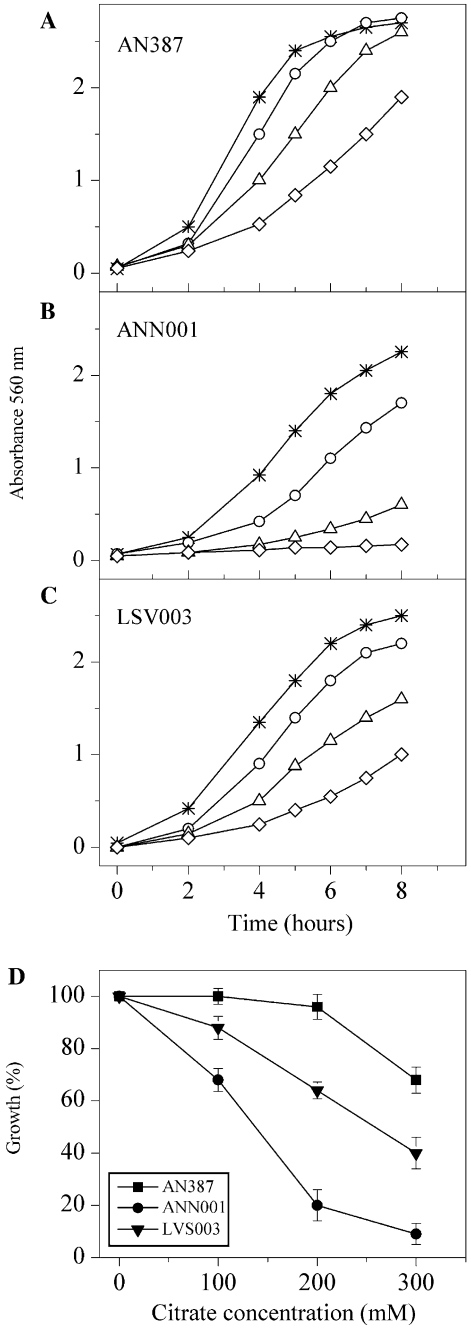

Fig. 5. Effect of the metal chelator sodium citrate on the bacterial growth of strains AN387, ANN001, and LVS003. (A), (B), and (C) growth curves of the indicated strains followed in M-medium (*) or M-medium containing: 100 (○), 200 (Δ), and 300 (◇) mM sodium citrate. (D) Relative growth at 8 h of the indicated strains as a function of citrate concentration. Values are expressed as percentages of the controls in M-medium and they represent the average ± SD of three experiments.

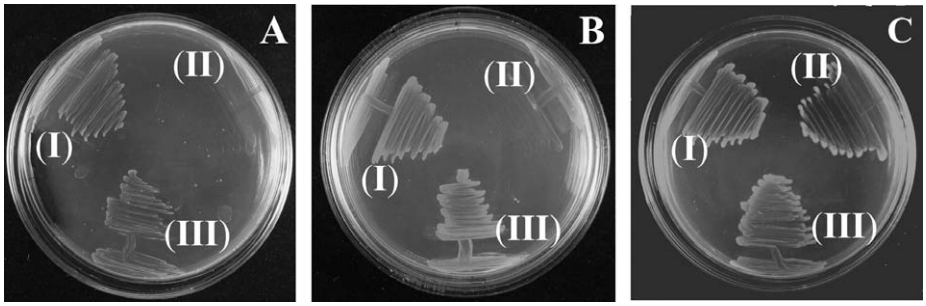

Fig. 6. Recovery of bacterial growth by metals in media with high sodium citrate concentration. Cells from stationary phase M-medium culture were streaked on M-medium agar plates containing 300 mM citrate, without (A), or with the supplement of 2 mM $FeCl_3$ (B) or 2 mM $CuSO_4$ (C). Photographs correspond to 24 h of growths. The experiment was repeated at least four times with similar results. AN387 (I), ANN001 (II), and LVS003 (III).

(not shown). It should be noted that LVS003 presents membrane NADH oxidase and dehydrogenase activities that were slightly higher than the wild-type activities (not shown).

The differential growth observed with ANN001/AN387 on plates containing copper and citrate was confirmed with the *ndh* mutant/parental pairs of strains MCW215/GR70N and MCW233/GR19N, but the effect was not so strong (not shown).

NDH-2 enhances cellular growth in medium containing very low copper concentrations

Fig. 4 shows that the growth of ANN001 was slightly affected by the addition of 100 mM citrate to the culture media. A partial recovery of the growth was achieved with a copper supplement up to 5 mM, denoting a possible copper limitation in the former condition. To further investigate that, we compared the growth of *E. coli* deficient in NDH-2 and their parental strains in media with increasing concentrations of citrate. The *ndh* deficient mutant (ANN001) grew more slowly than its isogenic strain (AN387) in liquid medium supplemented with citrate (Fig. 5A, B, and D). At 300 mM citrate, the growth of ANN001 was almost negligible, while the growth of AN387 is slightly reduced (Fig. 5). Bacteria showed similar effects on growth when they were tested on M-medium plates containing 300 mM citrate (Fig. 6A). The addition of Cu(II) but not of Fe(III) enables the mutant to recover a growth similar to that of the parental strain (Fig. 6). To validate that the observed effect was due to the absence of NDH-2, we used the complemented strain LVS003 which grew better than ANN001 in medium supplemented with citrate, in similar way to the wild-type strain (Fig. 5C and D, and Fig. 6). We also assayed the pairs MCW215/GR70N and MCW233/GR19N corroborating the data obtained with ANN001/AN387 (not shown).

The only addition of a Cu(II) salt to the culture media recovers the growth of the *ndh* deficient mutants, which reinforces the idea that copper limitation is the cause of the differential growth of the strains in the presence of citrate.

Discussion

Our previous studies on *E. coli* membranes demonstrated that either the NDH-2 or quinone from respiratory chain undergo irreversible inactivation due to t-BOOH mediated by cyclic oxido-reduction of cupric ions bound to the membrane [15] (see Fig. 1). Here we showed that the presence of NDH-2 protects the respiratory chain from hydroperoxides, when copper concentration in the culture media is not excessively high (Fig. 3). When we measured the oxidase activities in membranes exposed to t-BOOH and copper, we demonstrated that NADH oxidase (performed essentially by NDH-2 in our assay conditions) is inactivated faster than the other oxidases of the respiratory chain. This indicates that total inhibition of NDH-2 occurred earlier than the inactivation of quinones. Therefore, NDH-2 is the first component damaged in the respiratory chain and it may act as a free radicals scavenger to maintain the functions of the other components. It is known that NDH-2 produces $O_2^{\cdot-}$ and H_2O_2 in the respiratory chain [26], which is in line with the finding that Cu(II)-reduction by the enzyme is partially sensitive to SOD [16]. Moreover, in *Azotobacter vinelandii*, a type-2 NADH dehydrogenase is involved in the protection of the nitrogenase complex, an enzyme extremely sensitive to O_2 [27]. On the other hand, Berthon [28] proposed that a site-specific damage mechanism may function as an antioxidant system, since the Cu(I) bound to certain ligand molecules reacts with peroxides by generating free radicals, which interact with the ligands and form innocuous compounds. In this context, the copper-containing NDH-2 of *E. coli* and its homologues may have a role in respiratory protection at high ambient oxygen concentrations.

Although NDH-2 does not appear to be essential for copper metabolism, it may be important for the cellular growth under certain copper concentration ranges. The present work demonstrates that NDH-2 allows *E. coli* to grow both in high and low copper concentrations. This paradox is not totally clarified yet. One explanation may be the involvement of the copper reduction in the internalisation and/or expulsion of the metal in bacteria, since Cu(I) is the ionic species that participates in both processes

[6]. The enzyme as a Cu(II)-reductase [16] could be associated to mechanisms for copper transport, improving the growth in extreme metal conditions. With regards to the expulsion of the metal, we observed an increased copper accumulation in *E. coli* due to the absence of NDH-2 (Rodríguez-Montelongo, unpublished work).

Several pairs of *ndh* mutant/parental strains showed a differential phenotype in the presence of low or high copper concentrations in the media. However, specially in media with copper excess, the strongest difference was obtained with the pair ANN001/AN387. Additionally, the complementation of ANN001 with the *ndh* gene partially recovered wild-type phenotype, even though the enzyme was fully active in LVS003 membranes. Further investigations should be done to elucidate if ANN001 contains other mutations that increases the differences.

Moreover, we demonstrated that neither membranes nor purified NDH-2 exhibited NADH: Fe(III)-reductase activity [16]. Thus, the enzyme appears to be a metal-ion reductase specific for copper. NDH-2 could be unrelated to iron deficiency, which is consistent with the fact that the *ndh* deficient mutants did not recover growth capacity on iron supplemented citrate media (Fig. 6).

NDH-2 activities may be significant for both the cellular copper metabolism and the protection against oxidative stress. Further studies should be done to understand the mechanisms involved.

Acknowledgments

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