Non-enzymic copper reduction by menaquinone enhances copper toxicity in \textit{Lactococcus lactis} IL1403

Helge K. Abicht,¹ Yulia Gonskikh,¹,² Simon D. Gerber¹ and Marc Solioz¹

¹Department Clinical Research, University of Bern, Murtenstrasse 35, 3010 Bern, Switzerland
²Department of Plant Physiology and Biotechnology, Tomsk State University, Lenin Prospect 36, 634050 Tomsk, Russia

\textit{Lactococcus lactis} possesses a pronounced extracellular Cu\textsuperscript{2+}-reduction activity which leads to the accumulation of Cu\textsuperscript{+} in the medium. The kinetics of this reaction were not saturable by increasing copper concentrations, suggesting a non-enzymic reaction. A copper-reductase-deficient mutant, isolated by random transposon mutagenesis, had an insertion in the \textit{menE} gene, which encodes \textit{O}-succinylbenzoic acid CoA ligase. This is a key enzyme in menaquinone biosynthesis. The \textit{ΔmenE} mutant was deficient in short-chain menaquinones, and exogenously added menaquinone complemented the copper-reductase-deficient phenotype. Haem-induced respiration of wild-type \textit{L. lactis} efficiently suppressed copper reduction, presumably by competition by the \textit{bd}-type quinol oxidase for menaquinone. As expected, the \textit{ΔmenE} mutant was respiration-deficient, but could be made respiration-proficient by supplementation with menaquinone. Growth of wild-type cells was more copper-sensitive than that of the \textit{ΔmenE} mutant, due to the production of Cu\textsuperscript{+} ions by the wild-type. This growth inhibition of the wild-type was strongly attenuated if Cu\textsuperscript{+} was scavenged with the Cu(I) chelator bicinchoninic acid. These findings support a model whereby copper is non-enzymically reduced at the membrane by menaquinones. Respiration effectively competes for reduced quinones, which suppresses copper reduction. These findings highlight novel links between copper reduction, respiration and Cu\textsuperscript{+} toxicity in \textit{L. lactis}.

INTRODUCTION

Due to the ability of copper ions to cycle between Cu\textsuperscript{2+} and Cu\textsuperscript{+} at biologically relevant redox-potentials, copper has become a cofactor for over 30 known enzymes. Examples are cytochrome oxidases involved in respiration or superoxide dismutases, which are essential to combat oxidative stress (Karlin, 1993). Oxidative stress can be caused by copper ions which participate in Fenton-type reactions and thereby generate reactive oxygen species (Magnani & Solioz, 2007). Copper ions can also damage [Fe–S]-cluster proteins by displacing the iron (Macomber & Imlay, 2009). Therefore, cells have elaborated sophisticated systems for copper homeostasis.

How copper enters bacteria remains largely unexplored. Inside the cell, specialized proteins are responsible for copper handling, such as insertion into cuproenzymes or delivery to copper-responsive regulators [for recent reviews, see Solioz et al. (2010) and Magnani & Solioz (2007)]. To the extent it has been studied, these copper homeostatic proteins bind Cu\textsuperscript{+} and it is generally assumed that Cu\textsuperscript{+} prevails in the reducing environment of the cytoplasm, without the need for a copper reductase (Solioz et al., 2011). A key element of copper homeostasis in all bacteria is the copper-transporting ATPase. It pumps excessive copper out of the cytoplasm. The ionic species transported by bacterial Cu-ATPases appears to be Cu\textsuperscript{+} in most cases, but Cu\textsuperscript{2+}-transporting ATPases have also been described in some species (Argüello et al., 2007; Chilappagari et al., 2009).

In the oxidizing, extracellular environment, copper is present in the form of cupric ions, Cu\textsuperscript{2+}. More than 30 years ago, it had been recognized that \textit{Escherichia coli} cultures reduce Cu\textsuperscript{2+} to Cu\textsuperscript{+} and that Cu\textsuperscript{+} was between fivefold and tenfold more toxic than Cu\textsuperscript{2+} (Beswick et al., 1976). In aerobic cultures, the Cu\textsuperscript{+} thus generated was steadily reoxidized by oxygen to less toxic Cu\textsuperscript{2+}, whereas anaerobic cultures were severely affected by the accumulating Cu\textsuperscript{+}. Only relatively recently, the copper-reducing activity of \textit{Escherichia coli} has been molecularly characterized. It has been shown that approximately 70% of the copper reduction activity by cells was due to quinones and 10% to copper reduction by the NADH dehydrogenase, NDH-2 (Rapisarda et al., 1999, 2002;
Volentini et al., 2011). The Gram-positive bacteria Enterococcus hirae and Lactococcus lactis have also been reported to exhibit extracellular Cu²⁺ reductase activities (Wunderli-Ye & Solioz, 1999; Rezaïki et al., 2008). It has been shown that quinones can be mediators of cupric ion reduction in lactococci, but the physiological role and the molecular details of these activities have not been fully clarified.

We here describe the isolation of a copper-reductase-deficient mutant, ΔmenE, which is deficient in short-chain menaquinones. The mutant could be complemented by exogenous menaquinone. When cells were supplied with haem, they respired and copper reduction was strongly reduced by competition of the oxidase for reducing equivalents. Growth of wild-type cells was more copper-sensitive than that of the ΔmenE mutant, demonstrating the higher toxicity of Cu⁺ versus Cu²⁺. Cu⁺ toxicity was curbed by the Cu(I)-chelator bincinchoninic acid. Menaquinone-dependent Cu²⁺ reduction is thus a key process in copper toxicity in L. lactis.

METHODS

Reagents and chemicals. All reagents were of analytical grade and were obtained from Sigma–Aldrich unless indicated differently. Bicinchoninic acid (BCA) was from Pierce. Short-chain menaquinone (MK-4, comprising four isoprenyl residues) was obtained from ICN Biochemicals and haemin from Sigma-Aldrich.

Strains and culture conditions. L. lactis IL1403 was routinely grown in M17 (Terzaghi & Sandine, 1975) or N medium (10 g peptone l⁻¹, 5 g yeast extract l⁻¹, 56 mM Na₂HPO₄, 1 % glucose), either fermentatively in Falcon tubes or half-micro cuvettes, or respiratory, in 5 ml cultures supplied with 2 g haemin ml⁻¹ in 50 ml Falcon tubes at 30 °C with rotary shaking at 165 r.p.m. Where required, haem was added from a 0.5 mg haemin ml⁻¹ stock solution in 50 mM NaOH. Growth was monitored by measuring the attenuation of tenfold dilutions in M17 medium at 750 nm with a Lambda 16 spectrophotometer (PerkinElmer). One absorption unit corresponded to (9.22 ± 1.18) × 10⁸ cf.u. ml⁻¹, as assessed by serial dilutions and plating. The c.f.u. were determined by spreading serial dilutions on M17 agar plates and counting the colonies after 24 h incubation at 30 °C.

Isolation of a menE mutant. L. lactis IL1403 was transformed with pGH9:ISS1 (Maguin et al., 1996), which contains a temperature-sensitive origin of replication and a transposon insertion sequence with an erythromycin resistance cassette, as described previously (Gerber & Solioz, 2007). Transformed cells were grown fermentatively at 30 °C to stationary phase in the presence of 10 µg erythromycin ml⁻¹. From these cultures, suitable dilutions were spread on 15 × 22 cm nylon filters on plates of solid M17 medium, containing 10 µg erythromycin ml⁻¹. Following incubation overnight at 37 °C to allow for plasmid curing, colonies were stained for copper reductase activity as follows: filters were transferred onto filter paper soaked with 50 mM sodium citrate, 1 % glucose, 1 mM CuSO₄, 1 mM BCA, pH 6.5 and incubated at room temperature. Cu-reductase-deficient colonies were identified by the lack of purple staining after 3–5 min and were directly subcloned for further use. Deficiency in Cu²⁺ reduction was confirmed by quantitative copper reductase assays as described below. Genomic DNA of the mutant was isolated by alkaline lysis (Ausubel et al., 1995) and single-primer PCR was performed as described previously (Karlyshev et al., 2000), using primer sg32 (5′-ATCCGGTTTCTTGGTACG-3′) directed against the ISS1 insertion element of pGH9:ISS1. Sequencing of the PCR product led to the identification of the transposons insertion sites. The primer pairs sg36 (5′-CAGTTGGCATCTGCTTAG-3′) and sg32 as well as sg37 (5′-ACATCGGACCCTTGTTG-3′) and sg34 (5′-AGAGCATCGAACCCTGGAAG-3′) were used to verify the ΔmenE mutant.

Quantitative copper reductase assay with whole cells. Cu²⁺ reduction by whole cells was assessed essentially as described previously (Wunderli-Ye & Solioz, 1999). Briefly, L. lactis was grown fermentatively to early stationary phase in M17 medium. Before measurement, cultures were adjusted to an optical density at 600 nm (OD₆₀₀) of 0.6 with M17 medium and 10 ml were centrifuged for 5 min at 3700g at 4 °C. Cells were washed once in 200 mM sodium-N-morpholinoethanesulfonic acid (Na-MES), 20 mM sodium citrate, 5 mM MgCl₂ (pH 6) at 4 °C and finally resuspended in 1/10 volume of the same buffer containing 10 % (w/v) glycerol at 4 °C. To determine Cu²⁺ reduction, 50 μl washed cells were added to 950 μl reduction assay buffer (200 mM Na-MES, 20 mM sodium citrate, 5 mM MgCl₂, 0.1 mM BCA, pH 6), supplied with CuSO₄ as required, and the absorption of the Cu²⁺–BCA complex was monitored at 355 nm with a Lambda 16 photometer (PerkinElmer) at 30 °C. Cu²⁺ reduction was increased by addition of 1 % glucose. An absorption coefficient (ε) of 4.6 × 10⁴ M⁻¹·cm⁻¹ was used for the Cu(I)BCA₂ complex (Brenner & Harris, 1995). MK-4 was added from a 2 mM stock solution in ethanol.

Menaquinone extraction and TLC. Cultures (500 ml) of M17 medium containing 2 μg haemin ml⁻¹ were inoculated with 5 ml fresh overnight cultures of the respective strains and grown in baffled 1 l Erlenmeyer flasks at 30 °C with shaking at 160 r.p.m. to early stationary phase. Cells were harvested by centrifugation at 5000g for 10 min at 18 °C and washed with 100 ml PBS. Aliquots of 0.5 g wet cells were stored at −20 °C and used for hydrophobic extraction essentially as described previously (Rezaïki et al., 2008). Cells were suspended in 2 ml cold acetone and sonicated at 4 °C for 15 min in a sonicator bath. To this suspension, 10 ml cold acetone and 0.5 g Na₂SO₄ were added and the mixture incubated in tubes by end-over-end shaking for 30 min at 4 °C. The organic phases were recovered after centrifugation at 3400g for 15 min at 4 °C and the acetone was evaporated under vacuum. Residual water was removed by freeze drying. Extracted materials were dissolved in 50 μl heptane. Aliquots of the samples corresponding to 0.1 g wet cells were resolved by TLC on silica gel plates (Merck, Darmstadt, type 60, without fluorescence indicator), using heptane:diethyl ether (85:15; v/v) as the mobile phase. The silica gel plates were stained with iodine vapour and photographed with a Canon Digital IXUS 55 camera.

Respiration measurements. To determine oxygen consumption, cells were grown fermentatively to stationary phase in M17 medium containing, where required, 2 μg haemin ml⁻¹ (cytochrome bd oxidase starts to be expressed in early stationary phase). Cultures were washed in PBS and resuspended in PBS at an OD₆₀₀ of 2. Oxygen consumption was monitored with a Clark electrode in a 1 ml cuvette. Cell aliquots of 50 μl were measured in PBS at 30 °C with stirring at 750 r.p.m.

RESULTS

Copper reduction by L. lactis exhibits nonsaturable kinetics

Copper homeostasis in Gram-positives has extensively been studied in the model organism Enterococcus hirae.
Copper at low copper concentrations (Navarrete avidly bind copper, which probably reduced the available copper. The cell walls of Gram-positive bacteria are known to possess copper reductase activity which reduces extracellular Cu\(\text{2+}\) ions with an IC\(50\) of 1 µM copper, the observed reductase activity was low, but increased strongly at higher copper concentrations. Since L. lactis cells do not have significant energy stores, glucose had to be added as an energy source to observe copper reductase activity. Up to 0.3 µM copper, the observed reductase activity was low, but increased strongly at higher copper concentrations. The cell walls of Gram-positive bacteria are known to avidly bind copper, which probably reduced the available copper at low copper concentrations (Navarrete et al., 2011). The reductase activity reached a maximum of 1.57 ± 0.03 nmol min\(^{-1}\) (10\(^8\) c.f.u.)\(^{-1}\) at 3 mM copper and dropped off at even higher copper concentrations. This was most likely to be due to copper toxicity. It has previously been shown that the extracellular copper reductase activity of Enterococcus hirae was inhibited by Hg\(\text{2+}\) ions with an IC\(50\) of 1 µM (Wunderli-Ye & Solioz, 1999). An IC\(50\) of 0.7 µM was determined for mercury inhibition in L. lactis, suggesting that copper reduction proceeds by similar pathways in the two organisms. The increasing rate of the copper reduction by L. lactis with increasing substrate concentrations over five orders of magnitude suggested non-saturable enzyme kinetics. This is supported by a poor fit of the data to Michaelis–Menten kinetics and a highly non-linear Eddie–Hofstee plot (Fig. 1, inset); in this plot, Michaelis–Menten kinetics would result in a straight line with a negative slope. Thus, copper reduction by L. lactis appeared to be a non-enzymic reaction.

### Isolation of reductase-deficient mutants

To identify the molecular nature of copper reduction by L. lactis, mutants deficient in this activity were selected from a random transposon mutagenesis library, generated with plasmid pGH9:ISS1 as described previously (Maguin et al., 1996). Reductase-deficient mutants were identified by colony staining with BCA, which stains wild-type colonies pink within 3–5 min. Out of 2 x 10\(^4\) transposants, five strains which exhibited reduced staining were isolated. Quantitative copper reductase assays on these mutants, performed essentially as described in Fig. 1, showed that in four of the mutants, the copper reductase activity was still 40–80% of the wild-type activity. Only one mutant, CO205, had its reductase activity reduced to 15% of wild-type activity. This mutant is analysed in detail here. The transposon insertion site of this mutant was identified as the menE gene. MenE encodes O-succinylbenzoic acid CoA ligase, the fourth-last enzyme in menaquinone biosynthesis (Myers & Myers, 2004).

#### Characterization of the ΔmenE mutant

Quinone profiles of the wild-type and the ΔmenE mutant were examined by acetone extraction of cells, followed by TLC (Fig. 2a). Wild-type extract yielded a spot with an R\(_f\) of 0.31 which was missing in extracts from the ΔmenE strain. MK-4, which was used as a standard, migrated with an R\(_f\) of 0.41. The spot missing in the ΔmenE mutant most probably corresponded to MK-3, which has been described as the short-chain menaquinone of L. lactis (Rezaiki et al., 2008). It thus appeared likely that the lack of MK-3 in the ΔmenE mutant led to the loss of copper reductase activity. MK-3 is not commercially available but MK-4 complemented the MK-3 deficiency. MenE mutant cells in the presence of 500 µM copper and glucose exhibited only a marginal copper reductase activity, corresponding to 15 ± 7% of wild-type activity (Fig. 2b). Upon addition of 30 µM MK-4, the copper reductase activity was restored to 50 ± 10% of wild-type activity within a few seconds. In contrast, adding the same amount of MK-4 to wild-type cells had essentially no effect. This suggests that the ΔmenE mutant is deficient in copper reduction due to a quinone deficiency and that this deficiency can be complemented by exogenously added MK-4. This established a link between quinones and copper reduction.

### Common menaquinone pool in respiration and copper reduction

L. lactis is considered to be a facultative anaerobe, but it respires when supplied with haem or protoporphyrin IX (Bolotin et al., 2001; Gaudu et al., 2002). Under these conditions, the cells express a cytochrome bd terminal oxidase [ubi-(mena-)quinol:O\(_2\) oxidoreductase], encoded...
by the cydAB operon (Duwat et al., 2001). MK-3 has been reported to be required for respiration by cytochrome bd oxidase (Rezáei et al., 2008). If cytochrome bd oxidase and copper reduction both draw electrons from the same quinone pool, they are expected to compete with each other. Fig. 3 shows that in cells grown in the presence of haem, and thus respiration-competent, the copper reductase activity was reduced to 33% compared with cells grown in the absence of haem. Growing the ΔmenE mutant in the presence of haem had no effect, e.g. the copper reductase activity remained at the same low level observed with cells grown without haem. The reduced copper reductase activity in respiring cells suggests that respiration effectively competes with copper reduction for electrons from the quinone pool. The rates of the two reactions, namely a respiratory rate of 1.1 nmol min⁻¹ (10⁸ c.f.u.)⁻¹ and a copper reduction rate of 0.47 nmol min⁻¹ (10⁸ c.f.u.)⁻¹ at 4 mM copper, are compatible with this hypothesis.

Respiration and copper reduction in L. lactis appeared to rely on the same quinone pool, which was depleted in the ΔmenE mutant. It was thus of interest to look at the respiration of ΔmenE cells. Fig. 4(a) shows that wild-type cells grown without the addition of haem only showed a basal oxygen consumption which was not stimulated by glucose. Presumably, this basal activity, which was observed in all experiments, was due to H₂O-forming, cytoplasmic NADH oxidases, for which there are three genes in L. lactis IL1403, namely noxC, noxD and noxE (Neves et al., 2002). As expected, wild-type cells grown in the presence of haem showed glucose-stimulated respiration. The ΔmenE mutant was respiration-deficient even when grown in the presence of haem, due to the lack of the required menaquinone pool. If haem-grown ΔmenE cells were incubated with 30 μM MK-4, they acquired respiratory competence within seconds; respiration rates similar to those of the wild-type were obtained after prolonged (up to 1 h) incubation with haem. Clearly, copper reductase activity as well as respiration could be reconstituted with exogenously added MK-4 in the ΔmenE mutant.

The respiration deficiency of ΔmenE cells was also apparent by the growth yield. Wild-type cells grown in the presence of haem, or haem plus MK-4, achieved a final OD₇₅₀ of 3.8 (Fig. 4b). The ΔmenE cells, on the other hand, only reached an OD₇₅₀ of 1.4, or about one-third of the growth yield, when grown in the presence of haem (similar to wild-type

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**Fig. 2.** Menaquinone content and copper reductase activity of ΔmenE mutant cells. (a) TLC of menaquinones extracted from whole cells. Extracts of an equivalent of 0.1 g wet cells were resolved on silica gel as described in Methods. Lanes: 1, extract of wild-type cells; 2, extract of ΔmenE mutant cells; 3, 1 μg MK-4. (b) Copper reduction by L. lactis wild-type and ΔmenE cells was measured by following the formation of a Cu⁺–BCA complex at 355 nm in the presence of 500 μM of both CuSO₄ and BCA. At the arrows, glucose and MK-4 were added as indicated to final concentrations of 1% and 30 μM, respectively.

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**Fig. 3.** Effect of respiration on copper reductase activity. The copper reductase activity of wild-type L. lactis grown in the absence of haem (wild-type – haem) or the presence of haem (wild-type + haem) was measured as in Fig. 2. Also shown is the copper reductase activity of ΔmenE cells grown in the presence of haem (ΔmenE + haem). At the arrow, glucose was added to a final concentration of 1%.
grown without haem; not shown), but grew to an OD 750 of 2.2 when haem and MK-4 were supplied. Apparently, the menaquinone deficiency of the $D$ menE mutant can also be complemented with exogenous MK-4 during cell growth, albeit less efficiently than in the resting cells used for respiration measurements. Taken together, these findings support the existence of a common menaquinone pool for copper reduction and respiration.

Copper reduction enhances copper toxicity

The reduction of Cu$^{2+}$ to Cu$^+$ by wild-type $L$. lactis cells raised the question of the effect of this reaction on copper toxicity. $L$. lactis wild-type cultures grown in the absence of copper grew to maximal OD 750 of 1.8 (Fig. 5a). When challenged with 1 mM CuSO$_4$, the growth rate was significantly reduced and a final OD 750 of only 0.8 was reached. If, in addition to the copper, 2 mM BCA was added to scavenge Cu$^+$ ions, growth was indistinguishable from that of cells in the absence of copper, suggesting that BCA ameliorated copper toxicity. In the presence of 2 mM CuSO$_4$, there was essentially no growth of wild-type cells, but adding 2 mM CuSO$_4$ plus 4 mM BCA restored the growth rate to near control levels and led to a final OD 750 that was even slightly higher than that of cells grown without any additions. However, small changes in growth yield cannot readily be interpreted as the presence of haem and/or copper changes the metabolism of $L$. lactis such that less lactic acid but more acetoin and diacetyl are being produced (Kaneko et al., 1990). Adding only 4 mM BCA to cells had no detectable effect. Since OD measurements on cultures are subject to artefacts from cell clumping and changes in morphology, we also assessed the c.f.u. under these conditions (Fig. 5b). While the overall picture remained the same, there were some differences between OD and c.f.u. measurements. The final titres of wild-type cells in 1 or 2 mM copper, $8 \times 10^7$ and $4 \times 10^7$ c.f.u. ml$^{-1}$, respectively, were less than 5% of those of control cultures without copper, which reached $1.85 \times 10^9$ c.f.u. ml$^{-1}$. OD measurements thus overestimated growth.

![Fig. 4. Respiration and growth yields of wild-type and $D$menE cells. (a) Wild-type and $D$menE cells were grown in the absence or presence of haem or haem plus MK-4 and respiratory activity was measured with a Clark electrode as described in Methods. At the arrow, glucose was added to a final concentration of 1%. (b) Wild-type cells were grown aerobically in the presence of haem (○) or haem plus MK-4 (●). Similarly, $D$menE cells were grown aerobically in the presence of haem (△) or haem plus 20 μM MK-4 (▲); growth was recorded by measuring OD 750.](image)

![Fig. 5. Effect of scavenging Cu$^+$ with BCA on the growth of wild-type cells. (a) Growth of anaerobic $L$. lactis wild-type cultures followed by OD 750. Cultures were supplemented with the following: ○, no addition; ●, 4 mM BCA; □, 1 mM CuSO$_4$; ■, 1 mM CuSO$_4$, 2 mM BCA; △, 2 mM CuSO$_4$; ▲, 2 mM CuSO$_4$, 4 mM BCA. (b) c.f.u. of the cultures in (a).](image)
Strikingly, when copper and BCA were present, the final c.f.u. values reached 84% (1 mM Cu, 2 mM BCA) and 64% (2 mM Cu, 4 mM BCA) of those of control cultures without copper. The c.f.u. of cultures in the presence of BCA, but not copper, reached control levels at 6 h, but dropped to 49% after three more hours of incubation, indicating long-term BCA toxicity. Clearly, these overall findings support the hypothesis that Cu²⁺ reduction enhances copper toxicity by generating Cu⁺, which appears to be considerably more toxic to cells than Cu²⁺ (Table 1). The non-enzymatic nature of copper reduction would suggest that it is a fortuitous mechanism.

**DISCUSSION**

Since the first description of the extracellular copper reductase activity of *Enterococcus hirae* (Wunderli-Ye & Solioz, 1999), we have made many fruitless attempts to identify the molecular nature of this activity. These included complementation cloning with chemical *Enterococcus hirae* mutants deficient in copper reductase activity (no genome sequence was available at the time), attempts to clone a copper reductase from environmental libraries, proteomics in *L. lactis* to identify a copper reductase via its regulation by copper, and targeted knockout of putative copper reductase genes gleaned from the genome sequence of *L. lactis*, like *ydiD*, *ydiE*, *yaiA*, *yaiB* and *yijD*. The failure of all these attempts raised, but did not prove, the possibility that extracellular copper reduction was not an enzyme-catalysed reaction.

In the present study, we showed that copper reduction by *L. lactis* exhibited non-saturable kinetics, indicating a copper reduction mechanism not involving an enzyme. A ΔmenE mutant that could not produce short-chain menaquinones was unable to reduce Cu²⁺. This phenotype could be complemented by exogenously added MK-4. That quinones participate in Cu²⁺ reduction has previously been shown in *L. lactis* as well as *Escherichia coli* (Rezaïki et al., 2008; Volentini et al., 2011). *L. lactis* primarily uses menaquinones for copper reduction and respiration by cytochrome *bd* oxidase. In contrast, *Escherichia coli* has been shown to mainly use ubiquinones for copper reduction (Volentini et al., 2011). Based on our data, we hypothesize that in *L. lactis*, menaquinones located in the cytoplasmic membrane can directly interact with copper and lead to its reduction. By trapping Cu²⁺ with the Cu(I)-specific chelator BCA in wild-type cultures, we showed that Cu⁺ is considerably more toxic than Cu²⁺. As expected, the ΔmenE strain, which displayed only marginal copper reductase activity, was more copper-resistant than the wild-type.

When wild-type *L. lactis* is supplied with haem in the growth medium, it expresses a cytochrome *bd* oxidase [ubi-(mena-)-quinol:O₂ oxidoreductase], which draws electrons from reduced menaquinones (Bolotin et al., 2001; Duwat et al., 2001; Gaudu et al., 2002; Sijpesteijn, 1970). This results in an almost threefold growth yield. In contrast to the wild-type, the ΔmenE mutant did not become respiration-competent when supplied with haem, as apparent by its reduced oxygen consumption and growth yield. This was attributed to the absence of short-chain menaquinones. MK-4 restored respiration in the ΔmenE mutant during growth as well as when added to non-growing cells. Respiring cells of either the wild-type or the complemented ΔmenE mutant exhibited greatly attenuated copper reductase activity.

These findings support a model in which electrons flow from NADH to menaquinone via a membrane-bound NADH oxidase (Fig. 6). If cytochrome *bd* oxidase is present, these reducing equivalents are used to reduce oxygen to water. In the absence of an oxidase, there will be an excess of reducing equivalents, typical of fermenting organisms (Bongers et al., 2005). Thus, any opportunity to pass electrons on to a substrate will be favourable for the cells. It has been shown that menaquinones contribute substantially to the reduction of the redox potential of milk by *L. lactis* (Tachon et al., 2010). Copper appears to also be a suitable acceptor for excess reducing equivalents, resulting in the observed extracellular copper reductase activity. Metal reduction as a means to support growth is a well-known phenomenon and has been studied extensively in other organisms (Lovley, 2002). In fact, iron respiration has been proposed to be one of the earliest forms of respiration that evolved on Earth (Vargas et al., 1998).

Based on the redox potentials, electron transfer from NADH to O₂ with a ΔE° of 1.13 V is far more favourable for reduction than Cu²⁺ to Cu⁺ (Table 1). This is indicated by the fact that the ΔmenE strain, which harbours only marginal copper reductase activity, is considerably more toxic than Cu²⁺. However, since copper reduction by *L. lactis* is not an enzyme-catalysed reaction, it is a fortuitous mechanism.

**Table 1. Growth of *L. lactis* wild-type and the ΔmenE mutant with or without copper and BCA**

<table>
<thead>
<tr>
<th>Condition*</th>
<th>c.f.u.†</th>
<th>OD750†</th>
<th>Growth rate (h⁻¹)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>ΔmenE</td>
<td>Wild-type</td>
</tr>
<tr>
<td>Control</td>
<td>8.28 ± 10⁸</td>
<td>7.95 ± 10⁸</td>
<td>1.58 ± 0.17</td>
</tr>
<tr>
<td>Cu</td>
<td>0.86 ± 10⁸</td>
<td>0.74 ± 10⁸</td>
<td>0.78 ± 0.13</td>
</tr>
<tr>
<td>Cu/BCA</td>
<td>0.44 ± 10⁸</td>
<td>1.19 ± 10⁸</td>
<td>1.41 ± 0.11</td>
</tr>
</tbody>
</table>

*CuSO₄ (Cu) and BCA were added at 1 mM.
†Values were determined from cultures grown anaerobically in N medium to stationary phase.
‡Exponential-phase doubling times were determined from OD750 measurements.
than from NADH to Cu$^{2+}$, where $\Delta E^\circ$ is only 0.47 V (cf. Fig. 6). Also, the respiratory rate of 1.1 nmol min$^{-1}$ (10$^8$ c.f.u.)$^{-1}$ is higher than the rate of copper reduction, which is 0.47 nmol min$^{-1}$ (10$^8$ c.f.u.)$^{-1}$ at 4 mM copper. This would explain the efficient competition of oxygen respiration with ‘copper respiration’. However, either reaction is considerably exergonic and could provide energy to the cell. Cytochrome $bd$ oxidases are not known to be proton-pumping enzymes, but the cycling of menaquinones could, in a mechanism called the Q-cycle, generate a proton-motive force, which in turn contributes to ATP synthesis by the F$_{1}$F$_{0}$-ATPase (Brandt and Trumpower, 1994). The reduction of menaquinone, on the other hand, is accomplished by the two membrane-bound NADH oxidases, $noxA$ or $noxB$, which are part of the electron transport chain as outlined in Fig. 6 (Bongers et al., 2005).

The reduction of copper by menaquinone is most likely a fortuitous mechanism. Firstly, it produces the more toxic Cu$^+$ ions which interfere with growth. Secondly, copper is not normally present in sufficient concentrations in the natural habitats of L. lactis to be used for metal respiration. In milk, where L. lactis most often grows, it substantially reduces the redox potential of the milk, presumably as a way of disposing of excess reducing equivalents (Tachon et al., 2010).

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