Experimental conditions affect the site of tetrazolium violet reduction in the electron transport chain of *Lactococcus lactis*

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The reduction of tetrazolium salts to coloured formazans is often used as an indicator of cell metabolism during microbiology studies, although the reduction mechanisms have never clearly been established in bacteria. The objective of the present study was to identify the reduction mechanisms of tetrazolium violet (TV) in *Lactococcus lactis* using a mutagenesis approach, under two experimental conditions generally applied in microbiology: a plate test with growing cells, and a liquid test with non-growing (resting) cells. The results showed that in both tests, TV reduction resulted from electron transfer from an intracellular donor (mainly NADH) to TV via the electron transport chain (ETC), but the reduction sites in the ETC depended on experimental conditions. Using the plate test, menaquinones were essential for TV reduction and membrane NADH dehydrogenases (NoxA and/or NoxB) were partly involved in electron transfer to menaquinones. In this case, TV reduction mainly occurred outside the cells and in the outer part of the plasma membrane. During the liquid test, TV was directly reduced by NoxA and/or NoxB, probably in the inner part of the membrane, where NoxA and NoxB are localized. In this case, reduction was directly related to the intracellular NADH pool. Based on these findings, new applications for TV tests are proposed, such as NADH pool determination with the liquid test and the screening of mutants affected in menaquinone biosynthesis with the plate test. Preliminary results using other tetrazolium salts in the plate test showed that the reduction sites depended on the salt, suggesting that similar studies should be carried out with other tetrazolium salts so that the outcome of each test can be interpreted correctly.

INTRODUCTION

Tetrazolium salts are colourless compounds which become coloured when reduced to formazans. Traditionally used as indicators of cell metabolism in eukaryotes and prokaryotes, they function as artificial electron acceptors and thus detect dehydrogenase activities. The production of coloured formazan is irreversible and can be quantified using spectrophotometry.

Two types of tetrazolium salts are used in the context of microbiological studies, either in nutritive media where cells are growing or in non-nutritive media where cells do not grow and are thus in a resting state. The first type includes MTT, INT, CTC, TTC and TV, which form water-insoluble formazans (see the footnote on this page for the full names of the salts). These salts are positively charged and considered as cell permeable (Berridge et al., 2005). Their positive charge is thought to facilitate cellular uptake due to the membrane potential. They are generally used as indicators of cell redox activity in order to test resistance to solvents (Hayashi et al., 2003), drug susceptibility (Raut et al., 2008) or specific substrate utilization (Bochner & Savageau, 1977; Lin et al., 2008). They also enable the discrimination of viable cells in populations of respiring...
bacteria (Rodriguez et al., 1992) and anaerobic bacteria (Bhupathiraju et al., 1999; Smith & McFeters, 1997). They can help to enumerate microbial colonies in solid culture media, and to detect microbial contamination in food (Beloti et al., 1999). These salts have also been used as indicators of cell surface redox potential (Sollod et al., 1992) and extracellular redox activity (Griebe et al., 1997). Tetrazolium salts of the second type, which include XTT, MTS, WST-1 and WST-5, form water-soluble formazans. They are negatively charged and largely cell impermeable. They are generally used in conjunction with intermediate electron acceptors (quinones or their derivatives) that facilitate reduction reactions. These salts have been used in microplate assays to measure bacterial proliferation (Tsukatani et al., 2008) and in colorimetric assays to test antimicrobial susceptibility (Tsukatani et al., 2008; Tunney et al., 2004).

The mechanisms of tetrazolium salt reduction are well documented in eukaryotes, but have been little investigated in bacteria. Studies on eukaryotic cells have shown that reduction can occur extracellularly or intracellularly, depending on the capacity of the tetrazolium salt to penetrate into the cell. Extracellular reduction involves the membrane electron transport chain (ETC), while intracellular reduction involves dehydrogenases (Bernas & Dobrucki, 1999; Berridge et al., 2005). In prokaryotes, the main studies have concerned the Gram-negative respiring bacterium Escherichia coli. Smith & McFeters (1997) used a liquid test with resting cells and various inhibitors of the ETC to show that CTC and INT were mainly reduced before ubiquinone by two ETC enzymes, succinate dehydrogenase and NADH dehydrogenase. By contrast, Bochner & Savageau (1977) reported that a mutant of E. coli that was unable to synthesize ubiquinone was deficient in TTC reduction in a plate test with growing cells, indicating that TTC reduction occurs at or after the coenzyme-quinone site of the ETC. These different conclusions may be due to differences in the cell permeability of the tetrazolium salts used. However, the three salts, CTC, INT and TTC, are all supposed to enter cells. Another explanation is that the ability of the tetrazolium salt to enter cells is dependent on the experimental conditions employed, i.e. cells growing on a solid medium in one case and resting cells in a liquid medium in the other. The ability of bacteria to reduce tetrazolium salts is strongly species-dependent. Beloti et al. (1999) showed that most of the bacteria isolated from pasteurized milks that did not reduce TTC were Gram-negative. On the other hand, Tsukatani et al. (2008) showed that Gram-positive bacteria generally reduced tetrazolium salts that form water-soluble formazan better than Gram-negative bacteria. Only a few uses of tetrazolium salts have been reported for lactic acid bacteria (LAB), probably because the acid produced by LAB inhibits tetrazolium salt reduction. In this respect, TTC was used to distinguish Lactococcus lactis subsp. cremoris from L. lactis subsp. lactis in a medium containing arginine because only L. lactis subsp. lactis produces ammonia, which can counteract the acid inhibition of the dye reduction (Turner et al., 1963). Also, a recent study using tetrazolium salts that form water-soluble formazans showed that LAB were less efficient in reducing WST-5 than other Gram-positive bacteria, but reduced the dye as well as Gram-negative bacteria (Tsukatani et al., 2008).

L. lactis is widely used as a starter in the dairy industry and is considered as a model of lactic acid bacteria. This acidifying Gram-positive bacterium has been shown to exert a strong reducing activity (Brasca et al., 2007). It does not respire, because its haem biosynthetic pathway is incomplete. However, L. lactis possesses all the components of the ETC, i.e. membrane dehydrogenases (Brooijmans et al., 2007; Gaudu et al., 2002; Melo et al., 2004), menaquinones (Morishita et al., 1999; Rezaiki et al., 2008) and a unique cytochrome oxidase. Cytochrome d oxidase, encoded by cydA and cydB, is only active when haem is provided in the growth medium (Bolotin et al., 2001; Wegmann et al., 2007). It is noteworthy that L. lactis menaquinones were recently shown to reduce extracellular divalent cations as well as oxygen (Rezaiki et al., 2008). Because the reaction reflects electron transfers, lacticocci are certainly able to reduce tetrazolium salts.

Despite the frequent use of tetrazolium salts in microbiology, the mechanisms of tetrazolium salt reduction have not clearly been identified in bacteria, and the impact of experimental conditions has never to our knowledge been investigated. This lack of knowledge may lead to the incorrect use and interpretation of tetrazolium salt tests. To address scientific questions using the most appropriate salt and method, it is therefore necessary to clarify the mechanisms and sites of reduction for each tetrazolium salt.

During the present study, we investigated the mechanisms of TV reduction by L. lactis. TV is the constitutive dye of Biolog microplates, which have been used to achieve numerous research discoveries in microbiology (Bochner, 1989; Bochner & Savageau, 1977; Bochner et al., 2001, 2008). TV is a monotetrazolium salt that is reduced to a water-insoluble formazan, like MTT, CTC, INT and TTC. It is more difficult to reduce than MTT, CTC and INT, but more easily reduced than TTC (Sollod et al., 1992). The intracellular reduction of MTT, INT, CTC and TTC has been demonstrated (Bernas & Dobrucki, 1999, 2000, 2002, 2004; Berridge & Tan, 1993; Berridge et al., 2005), while the localization of TV reduction has not.

To our knowledge, questions concerning tetrazolium salt reduction have hitherto essentially been addressed using physiological approaches. Only one publication has investigated the cellular site of tetrazolium dye reduction using non-lethal mutants covering the whole genome in the eukaryote Saccharomyces cerevisiae (Herst et al., 2008). Here, we investigated the mechanism of TV reduction by the prokaryote L. lactis using a mutagenesis approach under the two experimental conditions generally employed in microbiology, i.e. on a solid medium with growing cells
(plate test) and in a liquid medium with resting cells (liquid test).

**METHODS**

**Bacterial strains and growth conditions.** *L. lactis* subsp. * cremoris* TIL46 (wild-type strain) is derived from *L. lactis* NCD0763 cured of its 2 kb plasmid (National Collection of Food Bacteria; now part of the NCIMB, Aberdeen, UK). The TIL46 ldh-negative mutant, TIL380, had previously been constructed by single cross-over (Chambellon et al., 2009). Lactococcal strains were grown at 30 °C either in M17 broth (Difco) supplemented with lactose (LM17) or glucose (GM17) at a final concentration of 0.5 % (w/v), or in skimmed milk (Lescure, Surgères, France). When required, an antibiotic (tetracycline or erythromycin) was added at a final concentration of 5 µg ml⁻¹. Bacterial growth was estimated by measuring OD₅₆₀ (Uvikon XL spectrophotometer, Biotek Instruments, path length 1 cm), after the dilution of 0.1 ml culture with 0.9 ml EDTA (5 g l⁻¹, pH 12.0) in the case of milk culture.

**Plate assays with tetrazolium salts.** The base medium used to test the ability of strains to reduce tetrazolium salts consisted of 2.5 vols skimmed milk (Lescure) and 1 vol. 3 % (w/v) agar solution (MilkAgar). The tetrazolium salt TV, MTT, TTC or INT (Sigma) was added to MilkAgar at a final concentration of 0.02 mg ml⁻¹. Two microlitres of an overnight GM17 culture was spotted on a MilkAgar plate containing the appropriate salt and incubated overnight at 30 °C.

**DNA manipulations.** DNA extractions, PCR, plasmid preparation and sequencing were performed as described by Sambrook & Russell (2001). DNA restriction and modification enzymes were purchased from New England Biolabs and used as recommended by the supplier. The primers used in this study were synthesized by Eurogentec and are listed in Table 1. *L. lactis* electrocompetent cells were prepared and transformed as described by Holo & Nes (1989). Plasmid DNA was prepared according to the method of O’Sullivan & Klaenhammer (1993). PCR amplifications were carried out with an Applied Biosystems DNA thermal cycler (Taq DNA polymerase (MP Biomedicals) or DNA polymerase (EcoIS ATAGTTCATTGATATATC PGEcoIS) and then ligated using T4 ligase. Ligation mixtures were used to transform *E. coli* (a TG1 derivative with the host9 : : IS₁ host9 : : IS₁, pH 12.0) in the case of milk culture; P. Renault, INRA, Génétique Microbienne, Jouy-en-Josas, France). Transformants were selected on MilkAgar plates containing TV (MilkAgarTV), using *E. coli* TG1 repA as the probe (Maguin et al., 1992). Transposition was shown to be random, but about 80 % of the tested mutants displayed a tandem insertion.

**Screening of the mutant library for TV-reducing ability.** Mutants were spotted on MilkAgar plates containing TV (MilkAgarTV), using a 96-in replicator, and incubated at 30 °C for 24 h. A violet coloration is indicative of TV reduction to formazan. Mutants that reduced TV more or less than the wild-type (WT) strain TIL46 were selected. To verify the difference in TV reduction between the selected mutants and WT, the TV reduction and growth of each selected mutant were tested individually. Growth was verified on MilkAgarBP plates containing a pH indicator, bromoresol purple (BP). Two microlitres of overnight cultures of each mutant in LM17 supplemented with erythromycin were spotted on MilkAgarBP and MilkAgarBP and incubated at 30 °C for 24 h.

**Construction of a random mutant library.** A random insertion mutant library was constructed in *L. lactis* TIL46 using the thermosensitive plasmid pG⁺ host9 : : ISS1, as described by Maguin et al. (1992, 1996). About 8500 integrants were selected on LM17Ery, because it was calculated that 8000 mutants were needed so that 95 % of the genes would be represented by an integrant (Bolotin et al., 2001; Smit et al., 2005). They were organized in 96-well microtitre plates containing 100 µl LM17 supplemented with erythromycin, and grown overnight at 30 °C. Before storage at −80 °C, 200 µl LM17 containing glycerol was added to each well to obtain a final glycerol concentration of 27 % (v/v). Random integration was verified by Southern blotting using the ECL direct nucleic acid labelling and detection system kit (GE Healthcare). For this purpose, total DNAs of 45 mutants from the library were extracted and digested with either EcoRI or HindIII. The fragments obtained were separated on a 0.7 % agarose gel, blotted, and hybridized with HindIII-cut, digoxigenin-labelled pG⁺ host9 as the probe (Maguin et al., 1992). Transposition was shown to be random, but about 80 % of the selected mutants contained a tandem insertion.

**Determination of disrupted genes in the selected mutants.** To determine the pG⁺ host9 : : ISS1 integration site in the selected mutants, total DNA was extracted, digested with EcoRI or HindIII, and then ligated using T4 ligase. Ligation mixtures were used to transform *E. coli* TG1 repA⁺ (a TG1 derivative with the repA gene integrated into the chromosome; P. Renault, INRA, Génétique Microbienne, Jouy-en-Josas, France). Transformants were selected by plating on Luria–Bertani (LB) medium containing 150 µg erythromycin ml⁻¹. Plasmid DNA of the transformants was extracted and sequenced using pG⁺ host9 : : ISS1-derived primers (Table 1). PG1 and PGHIIS were used for plasmids originating from HindIII

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**Table 1. Primers used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG1</td>
<td>TGGCGGAAGGGGATGTGC</td>
</tr>
<tr>
<td>PG2</td>
<td>ACTGACAGCTTCAAGGA</td>
</tr>
<tr>
<td>PG3</td>
<td>AGAATGTTACAGTCTATCC</td>
</tr>
<tr>
<td>PGHIIS</td>
<td>CTACTGAGATTAGGTTCT</td>
</tr>
<tr>
<td>PGecoIS</td>
<td>ATAGTCATGGATATATC</td>
</tr>
<tr>
<td>noxB</td>
<td>GTCCCCGTGAAATGGAAC</td>
</tr>
<tr>
<td>noxB_XmaI_R</td>
<td>CCCCGGGCAtGTGATAGAAGATAT</td>
</tr>
<tr>
<td>noxA_XmaI_F</td>
<td>CCCCGGGGCACCTACAAATACGATT</td>
</tr>
<tr>
<td>cpoR</td>
<td>TCTCCGATTTTTCCAGC</td>
</tr>
<tr>
<td>sdaBF</td>
<td>ATCCGAGCACCGAGC</td>
</tr>
<tr>
<td>cpoR2</td>
<td>TGATTTCAACGGCTATCG</td>
</tr>
<tr>
<td>menCF</td>
<td>TTAAGAAAGAGGCTAAAGC</td>
</tr>
<tr>
<td>menCR</td>
<td>TGCCAAGTAAAAATTCATGG</td>
</tr>
</tbody>
</table>

* XmaI sites indicated by underlining.
digestion and PG2 and PGEcoI5S were used for plasmids originating from EcoRI digestion. The sequences were compared with the genome of L. lactis MG1363 (Wegmann et al., 2007) using BLAST programs (Altschul et al., 1990) on the BLAST server (National Center for Biomedical Information, Bethesda, MD, USA) to identify interrupted genes.

pG\textsuperscript{+}host9::ISSI excision. A stable menC-negative mutant was obtained from the pG\textsuperscript{+}host9::ISSI-inserted mutant \(\Delta\)menC\(B\) by plasmid excision, as described by Maguin et al. (1996). Excision of the pG\textsuperscript{+}host9::ISSI was confirmed by PCR. Two primers, one upstream (menCF) and one downstream (menCR) of the menC\(B\) gene, were used to amplify the corresponding genomic region. In the wild-type strain, the amplicon was 1276 bp long, while it was 1807 bp long in the excised mutant, because of the presence of ISSI (711 bp). When the pG\textsuperscript{+}host9::ISSI did not excise, there was no amplification.

Construction of a noxA-noxB negative mutant (\(\Delta\)noxA\(B\)). The noxA and noxB genes were inactivated together in L. lactis TIL46 by double crossing-over, resulting in the \(\Delta\)noxA\(B\) mutant. Gene sequences were obtained from the published MG1363 sequence (Wegmann et al., 2007). An internal 848 bp DNA fragment of the noxB gene (F1), and an 852 bp DNA fragment of the downstream region of noxA (F2) were PCR-amplified with the noxBF and noxB\_Xmal\_R and the noxA\_Xmal\_F and cpoR primers, respectively (Table 1). The F1 and F2 fragments were introduced independently into the subcloning vector pGEM-T Easy (Promega). F1 was first digested with PstI and a blunt end was generated by the action of T4 DNA polymerase. F1 was subsequently digested by Xmal. F2 was digested by Apal and Xmal and cloned into the thermosensitive vector pG\textsuperscript{+}host9 (Maguin et al., 1992). This construction was then digested with Xmal and MolI to allow the cloning of F1 next to F2. The resulting pG\textsuperscript{+}host9 containing F1 and F2 was verified by PCR amplification with PG2 and PG3, and then transformed into TIL46 with erythromycin selection. After integration and excision of the plasmid by temperature shifts (Maguin et al., 1992), noxA-noxB inactivation was confirmed by sequencing with the sdbBF and cpoR2 primers (Table 1).

Quantification of the TV reduction ability of L. lactis

On a solid medium with growing cells (plate test). For each strain, 250 \(\mu\)l of an overnight GM17 culture was plated on a MilkAgarTV plate. After incubation for 24 h at 30 °C, formazan was quantified in both L. lactis cells and MilkAgarTV. L. lactis cells were swabbed from the surface of the MilkAgarTV three times using 2 ml water. The optical density of the suspension was measured at 480 and 600 nm. L. lactis cells from 4 ml of the suspension were harvested by centrifugation (4000 \(\times\) g, 5 min) and resuspended in 1 ml DMSO to solubilize the formazan. After centrifugation (15,000 \(\times\) g, 10 min), the \(A_{510}\) of the supernatant was measured. Another set of experiments was carried out using the same procedure as described above, but with the addition of 200 U superoxide dismutase (SOD, Sigma) to the TV solution.

For quantification, a calibration curve was established with a TV-formazan solution prepared as described by Smith & McFeters (1997). Briefly, 5.2 mM TV was reduced with 10 mM sodium hydrosulfit. The precipitate was washed twice with MilliQ water and solubilized in DMSO or 95 % ethanol. The absorption spectrum of the solutions showed a maximum at 510 nm. The \(A_{510}\) of TV-formazan solutions from 0 to 0.1 mM was measured to draw the calibration curve. The slope of the curve was used to determine the amount of formazan in the samples.

**Determination of the intracellular NADH concentration of cells grown in milk.** The intracellular NADH concentration was determined using an in vitro procedure based on the rapid inactivation of cell metabolism followed by direct NADH extraction from culture samples, and measurement by HPLC. The selected mutants were grown in skimmed milk (Lescure) until the culture reached an \(OD_{480}\) of 2. Culture samples of 5 ml were immediately frozen in liquid nitrogen and stored at −80 °C. NADH was extracted as described by Leboaas et al. (1993) and Garrigues et al. (1997). A variable volume (100–200 \(\mu\)l) of 10 M KOH was added to the culture samples during thawing to obtain a final pH of 12.5. For each culture, two samples were treated with KOH. Fifteen microlitres of 1 mM NADH in DMSO was added to one sample while the same volume of DMSO was added to the second sample. The KOH-treated cultures were incubated for 10 min at room temperature. After centrifugation (15,000 \(\times\) g, 10 min, 4 °C), the supernatant was cleared of proteins using an Ultrafree-0.5 centrifugal filter device consisting of a BioMax membrane with a 10 kDa molecular mass cut-off point (Millipore). The amount of NADH in the ultrafiltrate was immediately determined by ion-pairing HPLC, as described by Ganzer et al. (2006). Briefly, the HPLC system (Waters) consisted of a 717 plus autosampler, a 600 quaternary pump, a 486 UV detector and Millennium software. Determination of the quantity of NADH in samples took account of the percentage recovery of NADH added to each sample, which was around 75 %.

**RESULTS**

Selection of TIL46 mutants affected in their ability to reduce TV on MilkAgarTV and identification of interrupted genes

MilkAgarTV screening of the 8544-mutant library constructed in L. lactis TIL46 revealed 30 mutants with TV reduction abilities that differed from those of the WT strain TIL46. Individual plating of the 30 mutants on MilkAgarTV and MilkAgarBP media confirmed six mutants that were actually affected regarding TV reduction, the others being affected in their growth. Five of the six selected mutants did not reduce TV, and one reduced TV more than the WT (Fig. 1).

Sequencing of the DNA regions in which pG\textsuperscript{+}host9::ISSI was integrated in the five non-reducing mutants showed that the disrupted genes were all involved in menaquinone biosynthesis. The inactivated genes were menB in one mutant and \(\Delta\)menC in the four others. In the high-reducing mutant, the interrupted gene was \(ldh\), encoding lactate dehydrogenase.

The results were confirmed using TIL380, a TIL46 \(ldh\)-negative mutant (Chambellon et al., 2009) and the \(\Delta\)menC-
negative mutant from which pG\textsuperscript{+}host9::ISSI was excised. These two stable mutants were named Δldh and ΔmenC and used for further experiments.

To complete this mutant selection, we constructed a mutant inactivated for the membrane NADH dehydrogenases NoxA and NoxB (ΔnoxAB), since they are thought to be involved in the reduction of menaquinones in \textit{L. lactis} (Gaudu et al., 2002; Melo et al., 2004). When spotted on MilkAgarTV, this mutant reduced TV less than the WT strain (Fig. 2, picture).

**TV reduction by growing cells on solid medium**

Each strain, i.e. WT, Δldh, ΔmenC and ΔnoxAB, was spread and grown on a MilkAgarTV plate. After incubation for 24 h at 30 °C, \textit{L. lactis} cells totally covered the MilkAgarTV and displayed the same visual TV reduction as when spotted (Fig. 2). The strains were ranked from the most reducing to the least reducing as follows: Δldh, WT and ΔnoxAB. ΔmenC did not reduce MilkAgarTV. In order to quantify formazan, \textit{L. lactis} cells were recovered from MilkAgarTV. The cell suspension was coloured in the case of WT, Δldh and ΔnoxAB, but not for ΔmenC. It is noteworthy that a considerable amount of formazan remained in the upper part of MilkAgarTV plates after the WT, Δldh and ΔnoxAB cells had been removed. No formazan was observed deeper in the medium, indicating that TV reduction occurred on the surface of MilkAgarTV. Formazan was quantified both in \textit{L. lactis} cells and in the coloured part of MilkAgarTV for the four strains. In the case of \textit{L. lactis}, formazan was strongly associated with the cells. Indeed, after centrifugation, the bacterial pellets were coloured, while the supernatant was colourless. Moreover, we failed to solubilize formazan from cells using 95 % ethanol, even after sonication and shaking for one night. Only DMSO, which is known to partially solubilize cell membranes, was able to solubilize formazan. In the case of MilkAgarTV, formazan included in the agar was easily solubilized with ethanol, suggesting that it had been produced outside the cell and precipitated close to the bacteria. The quantification of formazan contained in both \textit{L. lactis} cells and MilkAgarTV revealed that 65–75 % of total formazan was localized in MilkAgarTV (Fig. 2). The total production of formazan by \textit{L. lactis} TIL46 was decreased by 50 % by NoxAB inactivation (\(P<0.001\)), while it was almost totally suppressed by MenC inactivation. By contrast, formazan production increased 1.6-fold when LDH was inactivated (\(P<0.001\)). The addition of SOD to the medium had no effect on TV reduction, indicating that the reduction did not involve superoxide ions.

**TV reduction by resting cells in liquid medium**

WT, Δldh, ΔmenC and ΔnoxAB cells from milk cultures were incubated in an aqueous TV solution (liquid medium). TV reduction was rapid, and a maximum of 15 min was sufficient for the reaction to be completed. All the formazan thus formed pelleted with cells after centrifugation, while the supernatants were colourless. Attempts to solubilize formazan from the cell pellet using 95 % ethanol failed, even after sonication, treatment with lysozyme (30 mg ml\(^{-1}\)) and Triton (2 %). Only DMSO was able to solubilize formazan from \textit{L. lactis} cells, suggesting that formazan was inside the cell membranes. Fig. 3 shows the amount of formazan formed by each strain. Formazan production by the WT was increased threefold by LDH inactivation (\(P<0.001\)). Surprisingly, it was only partly affected by MenC inactivation (\(P<0.05\)), while it was almost totally suppressed by NoxAB inactivation (\(P<0.001\)). The results of these liquid tests differed markedly from those observed with the plate test, especially in the case of MenC and NoxAB inactivations. In no cases did the addition of SOD affect TV reduction.

**NADH pool in the selected mutants**

To verify that an increase in TV reduction was related to a larger intracellular NADH pool, this pool was quantified in milk cultures (Fig. 4). The NADH pool was threefold larger when LDH was inactivated compared to WT (\(P<0.001\)), while it was not affected by MenC inactivation (\(P>0.05\)) and only slightly increased by NoxAB inactivation (\(P>0.05\)).
MenC were inactive. Moreover, TV reduction by menaquinones appeared to be the sole site of reduction, as no reduction occurred when MenB or MenC were inactive. In the plate test, menaquinones appeared to be the sole site of reduction, as no reduction occurred when MenB or MenC were inactive. In both cases, we showed that the ETC was actually involved in TV reduction by L. lactis, but the sites of reduction depended on the experimental conditions. The essential factors for TV reduction were the sites of reduction, as no reduction occurred when MenB or MenC were inactive. Moreover, TV reduction by menaquinones did not involve an extracellular superoxide intermediate, as the addition of superoxide dismutase did not affect reduction. Similarly, menaquinones have been shown to reduce Cu²⁺ directly, while they reduced Fe³⁺ via the superoxide produced from oxygen reduction (Rezaiki et al., 2008). Menaquinones that reduced TV appeared to be partially reduced by the membrane NADH dehydrogenases NoxA and/or NoxB, as NoxAB inactivation led to a 50 % decrease in TV reduction; thus another membrane dehydrogenase has to be responsible for the other 50 % contribution to menaquinone reduction. Succinate dehydrogenase, also called fumarate reductase (FRD), is a component of the ETC in E. coli but seems to be lacking in L. lactis. Only the frdC gene, which encodes the FRD flavoprotein subunit, was found in the genome (Wegmann et al., 2007), ruling out a role for FRD in menaquinone reduction in L. lactis. According to Gaudu et al. (2002), the membrane glycerol-3-phosphate reductase GlpD may be the alternative dehydrogenase. Since NoxA and/or NoxB transfer electrons from the intracellular NADH pool to menaquinones, a larger intracellular pool of NADH in Aldh could explain the greater capacity of this strain to reduce TV. In agreement with the 50 % contribution of NoxA and/or NoxB to menaquinone reduction, a threefold increase in the NADH pool led to a 1.5-fold increase in TV reduction by Δaldh. Fig. 5(A) shows a schematic representation of the TV reduction mechanism in the plate test with growing cells.

In the liquid test, in contrast to the plate test, NoxA and/or NoxB appeared to be the principal site of TV reduction. Indeed, NoxAB inactivation suppressed 85 % of TV reduction, while MenC inactivation only decreased TV reduction by 15 %. In ΔnoxAB, the menaquinones that are responsible for residual TV reduction are probably reduced by the other membrane dehydrogenase (GlpD). This dehydrogenase appeared to be unable to reduce TV directly, otherwise ΔnoxAB would have been able to reduce more TV. Under these experimental conditions, and since NoxA and/or NoxB were almost totally responsible for TV reduction, reduction was proportional to the NADH pool. Indeed, a threefold increase in the NADH pool in Δaldh led to a threefold increase in TV reduction. Fig. 5(B) shows a schematic representation of the TV reduction mechanism in the liquid test with resting cells.

These different reduction sites in the ETC under both experimental conditions are probably linked to the cellular localization of TV reduction. In the liquid test, TV reduction occurred entirely in the cell membrane, probably in the inner part where NoxA and NoxB are localized. In the plate test, we observed that 65–75 % of TV reduction occurred outside the cell, and only 25–35 % in the cell membrane, probably in the outer part because NoxA and/or NoxB did not reduce TV directly. In both tests, there was no evidence of intracellular reduction by cytoplasmic dehydrogenases. Indeed, formazan was not released after cell breakdown due to chemical, mechanical or enzymic treatments. The localization of TV reduction is probably affected by the different ability of TV to enter cells under...
the two conditions. Both the nature of the growth medium (liquid vs solid) and the physiological state of bacteria (growing vs resting) could affect the cell permeability of TV. On the one hand, the mobility of nutrient ions and other soluble molecules is known to be higher in liquid media (Miller & Davey, 1965); on the other hand, the passive diffusion of hydrophobic molecules may be greater in resting cells than in growing cells.

Contrary to what we had expected, the reduction sites identified here for TV could not be generalized to all other positively charged salts. MTT, TTC and INT reduction by the four selected strains (WT, Δldh, ΔmenC and ΔnoxAB) was tested on plates and only the results with TTC were the same as those with TV, although the coloration was weaker. MTT was markedly reduced by all strains and INT was similarly reduced by all strains except ΔnoxAB, where reduction was only partial. Because of the chemical differences between salts, it is not possible to predict the reduction of different tetrazolium salts by the same strain, even if they are all positively charged.

New applications for tetrazolium tests could be based on the conclusions of this study. Firstly, we demonstrated that tests with tetrazolium salts, and especially TV, could be used for studies of LAB despite their acidifying property. In *L. lactis*, the TV plate test could be used to screen mutants affected in menaquinone biosynthesis. This application could be extended to other LAB in order to distinguish species that produce menaquinones from those that do not. The TV reduction test in liquid medium could be useful to estimate the NADH pool in cells. This method could facilitate studies of the impact of different growth conditions or gene inactivation on the NADH pool. Indeed, the TV reduction test in a liquid medium is simple, rapid and inexpensive when compared to the standard methods used to measure NADH in bacteria, which require special equipment, are often difficult to perform and are very time-consuming (Ganzera et al., 2006; Garrigues et al., 1997; Lebloas et al., 1993).

To conclude, using a mutagenesis approach, we have demonstrated that the ETC sites of TV reduction by *L. lactis* are membrane NADH dehydrogenases in a liquid test and menaquinones in a plate test. Similar studies using other tetrazolium salts will be necessary to understand the differences in reduction capacity observed between tetrazolium salts.

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**REFERENCES**


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