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.

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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

Abbreviations: CTC, 5-cyano-2,3-ditolyl tetrazolium chloride; INT, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride; MTS, 5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulphophenyl)-2*H*-tetrazolium inner salt; MTT, 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2*H*-tetrazolium bromide; TTC, 2,3,5-triphenyl-2*H*-tetrazolium chloride; TV, 2,5-diphenyl-3-2-naphthyl tetrazolium chloride or tetrazolium violet; WST-1, sodium 5-(2,4-disulphophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2*H*-tetrazolium inner salt; WST-5, disodium 2,2'-dibenzothiazolyl-5,5'-bis[4-di(2-sulfoethyl)carbamoylphenyl]-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium salt; XTT, sodium 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)-carbonyl]-2*H*-tetrazolium inner salt; ETC, electron transport chain; LAB, lactic acid bacteria, SOD, superoxide dismutase.

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-positive. 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, lactococci 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* NCDO763 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 OD₄₈₀ (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 2720, using *Taq* DNA polymerase (MP Biomedicals) or DNA polymerase Phusion (High-Fidelity PCR Master Mix, Finnzymes). Samples for sequencing were prepared using the PRISM Ready Reaction Dye Deoxy terminator cycle sequencing kit (Applied Biosystems) and sequences were determined with an automatic sequencer (Abi Prism 310 DNA sequencer, Applied Biosystems).

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 *Eco*RI or *Hind*III. The fragments obtained were separated on a 0.7 % agarose gel, blotted, and hybridized with *Hind*III-cut, digoxigenin-labelled pG⁺host9 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-pin 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, bromocresol purple (BP). Two microlitres of overnight cultures of each mutant in LM17 supplemented with erythromycin were spotted on MilkAgarTV and MilkAgarBP and incubated at 30 °C for 24 h.

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 *Eco*RI or *Hind*III, 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 PGHiS were used for plasmids originating from *Hind*III

Table 1. Primers used in this study

Primer	Sequence (5'–3')
PG1	TGGCGAAAGGGGGATGTGCT
PG2	ACTGACAGCTTCCAAGGA
PG3	AGAATGTTACAGTCTATCC
PGHiS	CTACTGAGATTAAGGTCT
PGEcoIS	ATAGTTTCATTGATATATC
noxBF	GTCCTTTGTAAATTGAAC
noxB_XmaI_R	CCCCCGGGCAATGTTGATGAAGAATAT*
noxA_XmaI_F	CCCCCGGGCACCTACAATAACGATT*
cpoR	TCTCCGATTTTCCAGC
sdaBF	ATTCGAGCAGCACCAGC
cpoR2	TGATTTCAAACGCTATCG
menCF	TTAAGAAAAGAGCTCAAAGC
menCR	TGCGAAGTAAAATTCACCTTG

**Xma*I sites indicated by underlining.

digestion and PG2 and PGEcoIS 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 Biological Information, Bethesda, MD, USA) to identify interrupted genes.

pG⁺host9::ISS1 excision. A stable *menC*-negative mutant was obtained from the pG⁺host9::ISS1-inserted mutant $\Delta menC_3$ by plasmid excision, as described by Maguin *et al.* (1996). Excision of the pG⁺host9::ISS1 was confirmed by PCR. Two primers, one upstream (*menCF*) and one downstream (*menCR*) of the *menC* gene, were used to amplify the corresponding genomic region. In the wild-type strain, the amplicon was 1276 bp long, while it was 1987 bp long in the excised mutant, because of the presence of ISS1 (711 bp). When the pG⁺host9::ISS1 did not excise, there was no amplification.

Construction of a *noxA*-*noxB* negative mutant ($\Delta noxAB$). The *noxA* and *noxB* genes were inactivated together in *L. lactis* TIL46 by double crossing-over, resulting in the $\Delta noxAB$ 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_XmaI_R* and the *noxA_XmaI_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 *XmaI*. F2 was digested by *ApaI* and *XmaI* and cloned into the thermosensitive vector pG⁺host9 (Maguin *et al.*, 1992). This construction was then digested with *XmaI* and *MslI* to allow the cloning of F1 next to F2. The resulting pG⁺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 *sdaBF* 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 μ 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 g, 5 min) and resuspended in 1 ml DMSO to solubilize the formazan. After centrifugation (15 000 g, 10 min), the A_{510} of the supernatant was measured (Uvikon XL spectrophotometer, Biotek Instruments, path length 1 cm). The coloured MilkAgarTV was transferred to a 50 ml tube, melted in a microwave (2 $\times$ 5 s) and kept at 50 °C. After homogenization, 30 ml 100% ethanol was added to solubilize the formazan. After the mixture had been shaken and centrifuged (15 000 g, 15 min), the volume and A_{510} of the supernatant were determined.

In a liquid medium with resting cells (liquid test). *L. lactis* cells from 2 ml of the milk culture at OD₄₈₀ 2 were harvested by centrifugation (4000 g, 5 min) after solubilizing caseins with a 10⁻¹-dilution in 100 mM Tris buffer pH 8.0 containing 20 g sodium citrate l⁻¹. The cells, in a resting state, were resuspended in 4 ml water. One millilitre of resting cells was immediately incubated for 15 min with 0.5 mM TV, in the dark. The cells were then harvested by centrifugation (15 000 g, 10 min) and resuspended in 1 ml DMSO to solubilize the formazan. After centrifugation (15 000 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 hydrosulfite. 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 OD₄₈₀ of 2. Culture samples of 5 ml were immediately frozen in liquid nitrogen and stored at -80 °C. NADH was extracted as described by Leblias *et al.* (1993) and Garrigues *et al.* (1997). A variable volume (100–200 μ 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 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 Ganzera *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⁺host9::ISS1 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 *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 *menC*-

Fig. 1. Plate tests of TIL46 (wild-type strain, WT) and selected mutants from the library screening: (A) on MilkAgarTV; (B) on MilkAgarBP.

negative mutant from which $pG^+host9::ISS1$ was excised. These two stable mutants were named Δldh and $\Delta menC$ and used for further experiments.

To complete this mutant selection, we constructed a mutant inactivated for the membrane NADH dehydrogenases NoxA and NoxB ($\Delta noxAB$), since they are thought to be involved in the reduction of menaquinones in *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 , $\Delta menC$ and $\Delta noxAB$, was spread and grown on a MilkAgarTV plate. After incubation for 24 h at 30 °C, *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 $\Delta noxAB$. $\Delta menC$ did not reduce MilkAgarTV. In order to quantify formazan, *L. lactis* cells were recovered from MilkAgarTV. The cell suspension was coloured in the case of WT, Δldh and $\Delta noxAB$, but not for $\Delta menC$. It is noteworthy that a considerable amount of formazan remained in the upper part of MilkAgarTV plates after the WT, Δldh and $\Delta noxAB$ 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 *L. lactis* cells and in the coloured part of MilkAgarTV for the four strains. In the case of *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 *L. lactis* cells and MilkAgarTV revealed that 65–75 % of total formazan was localized in MilkAgarTV (Fig. 2). The total production of formazan by *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 , $\Delta menC$ and $\Delta noxAB$ 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 *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.

	WT	Δldh	$\Delta noxAB$	$\Delta menC$
<i>L. lactis</i> cells	45 ± 8	104 ± 13***	27 ± 1*	7 ± 1***
Agar	144 ± 33	189 ± 27***	69 ± 12**	IS***
Total	188 ± 26	293 ± 38***	96 ± 11***	IS***

Fig. 2. Amounts of formazan obtained from plated cultures. The quantities of formazan determined in swabbed cells and in agar fractions are given in nmoles (means ± SD; $n=3$). IS, insignificant amount of formazan. Student's *t* test between data for WT and mutants: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

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 ($P>0.05$).

Fig. 3. Amounts of formazan formed by *L. lactis* cells recovered from 1 ml of milk culture with an OD₄₈₀ of 2, after incubation for 15 min in an 0.5 mM TV solution. Data are means $\pm$ SD ($n=3$). Student's *t* test between data for WT and mutants: *, $P<0.05$; ***, $P<0.001$.

DISCUSSION

Although it is assumed that tetrazolium salts are reduced by electrons originating from intracellularly metabolized substrates via the ETC, the sites of reduction in the chain are not clearly established in bacteria. Using a mutagenesis approach, we determined the sites of TV reduction in *L. lactis*, using both a plate test with growing cells and a liquid test with resting cells. 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 menaquinones in the plate test with growing cells, and the NADH dehydrogenases NoxA and/or NoxB in the liquid test with resting cells.

In the plate test, menaquinones appeared to be the sole site of TV 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 Cu^{2+} directly, while they reduced Fe^{3+} 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 Δldh 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 about a 1.5-fold increase in TV reduction by Δldh . 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 $\Delta noxAB$, 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 $\Delta noxAB$ 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 Δldh 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

Fig. 4. NADH pool in *L. lactis* cells of 1 ml of milk culture with an OD₄₈₀ of 2. Data are means $\pm$ SD ($n=3$). Student's *t* test between data for WT and mutants: ***, $P<0.001$.

Fig. 5. Schematic representation of the mechanism of TV reduction: (A) in the plate test with growing cells; (B) in the liquid test with resting cells. TV, tetrazolium violet; F, formazan; MK, oxidized menaquinones; MK^{••}, semiquinones; MKH₂, reduced menaquinones; NoxAB DH, membrane NADH dehydrogenases NoxA and NoxB; DH, dehydrogenase.

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 , $\Delta menC$ and $\Delta noxAB$) 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 $\Delta noxAB$, 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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