The redox potential plays a major role in the microbial and sensorial quality of fermented dairy products. The redox potential of milk (around 400 mV) is mainly due to the presence of oxygen and many other oxidizing compounds. Lactococcus lactis has a strong ability to decrease the redox potential of milk to a negative value (−220 mV), but the molecular mechanisms of milk reduction have never been addressed. In this study, we investigated the impact of inactivation of genes encoding NADH oxidases (noxE and ahpF) and components of the electron transport chain (ETC) (menC and noxAB) on the ability of L. lactis to decrease the redox potential of ultrahigh-temperature (UHT) skim milk during growth under aerobic and anaerobic conditions. Our results revealed that elimination of oxygen is required for milk reduction and that NoxE is mainly responsible for the rapid removal of oxygen from milk before the exponential growth phase. The ETC also contributes slightly to oxygen consumption, especially during the stationary growth phase. We also demonstrated that the ETC is responsible for the decrease in the milk redox potential from 300 mV to −220 mV when the oxygen concentration reaches zero or under anaerobic conditions. This suggests that the ETC is responsible for the reduction of oxidizing compounds other than oxygen. Moreover, we found great diversity in the reducing activities of natural L. lactis strains originating from the dairy environment. This diversity allows selection of specific strains that can be used to modulate the redox potential of fermented dairy products to optimize their microbial and sensorial qualities.
tion by L. lactis have never been addressed. In the present study, we investigated the impact of overexpression of genes encoding NADH oxidases (noxE and ahpF) and components of the ETC (menC and noxAB) on the reduction of ultrahigh-temperature (UHT) skim milk by L. lactis. Our results showed that two distinct and complementary mechanisms are involved in milk reduction. The first mechanism is essential and relies on the elimination of dissolved oxygen, principally by the NoxE NADH oxidase. The second mechanism is oxygen independent on the elimination of dissolved oxygen, principally by the NoxE NADH oxidase. The second mechanism is oxygen independent and is due to the ETC, which probably reduces oxidizing compounds other than oxygen. Moreover, we found great diversity in the reducing activities of natural L. lactis strains, which makes control of the Eₚ in fermented dairy products through replication of L. lactis plasmids.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All L. lactis strains used in this study are L. lactis subsp. cremoris strains. L. lactis mutant strains were derived from laboratory strain TIL46 and are listed in Table 1. The noxAB mutant was constructed previously (38), and the menC mutant was obtained previously by insertion mutagenesis and selection for an inability to reduce tetrazolium violet (38). Other wild-type (WT) L. lactis strains used in this study were supplied by CSK (Ipswich, MA) and used as recommended by the supplier. The primers used in this study were synthesized by Eurogentec (Seraing, Belgium) and are listed in Table 2. L. lactis electroporative cells were prepared and transformed as described previously by Holo and Nes (18). Plasmid extraction was performed as described by O’Sullivan and Klaenhammer (32). PCR amplification was carried out with an Applied Biosystems 2720 DNA thermal cycler (Courtaboeuf, France), using the Taq DNA polymerase (MP Biomedicals, Illkirch, France) or Phusion DNA polymerase (high-fidelity PCR master mixture; Finnzymes, Finland).

Construction of the ahpF null mutant. The ahpF mutant was constructed by deleting 435 bp of the ahpF gene using double-crossover gene replacement. A 1,741-bp DNA fragment that included the ahpF gene (1,527 bp) was PCR amplified with primers ahpF-up and ahpF-down containing truncated ahpF; Ery<sup>+</sup> 38 The truncated fragment was then PCR amplified with primers ahpF-up and ahpF-down and subcloned into the pGEM-T Easy vector (Promega, Madison, WI). After digestion of the subcloning vector with EcoRI and SacII restriction sites, respectively (Table 2). A 435-bp deletion inside the PCR product was obtained by digestion with AvaI and ligation of the two outside fragments (649 and 657 bp). The truncated fragment was then PCR amplified with primers ahpF-up and ahpF-down and subcloned into the pGEM-T Easy vector (Promega, Madison, WI). After digestion of the subcloning vector with EcoRI and SacII, the resulting truncated fragment (1,289 bp) was cloned into pG+ host<sup>+</sup> (28), and the recombinant vector was introduced by electroporation into L. lactis.

### Table 1. Strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Source or reference |
|-------------------|--------------------------|---------------------|
| L. lactis strains |                         |                     |
| TIL46             | Wild-type (WT) strain derived from L. lactis NCDO763 cured of its 2-kb plasmid | NCIMB (Aberdeen, UK) |
| noxE-pJIM mutant  | TIL46 noxE mutant obtained by single-crossover integration of pORI::noxE; Ery<sup>+</sup> | This study |
| noxE-pJIM::noxE mutant | TIL46 noxE mutant complemented with pJIM; Ery<sup>+</sup> Cm<sup>+</sup> | This study |
| ahpF mutant       | TIL46 ahpF deletion mutant obtained by double-crossover gene replacement with pG+host<sup>+</sup>::ahpF | This study |
| noxAB mutant      | TIL46 noxAB deletion mutant obtained by double-crossover gene replacement | 38 |
| menC mutant       | TIL46 menC integration mutant obtained by double-crossover gene replacement with pG+host<sup>+</sup>::ISS1 | 38 |
| E. coli TG1 repA+  | TG1 derivative with repA gene integrated into the chromosome, allowing replication of L. lactis plasmids | P. Renault (INRA, Génétique Microbienne, Jouy-en-Josas, France) |

| Plasmids          | Relevant characteristics | Source |
|-------------------|--------------------------|--------|
| pGEM-T Easy       | 3.0-kb linear T-overhang vector for PCR fragment cloning; nonreplicative in Gram-positive bacteria; Ap<sup>+</sup> | Promega, Madison, WI |
| pGEM-TaheE        | pGEM-T Easy containing a 644-bp internal fragment of the noxE gene | This study |
| pORInewlux        | pORInewlux containing a 644-bp internal fragment of the noxE gene | 13 |
| pG+host<sup>+</sup> | 3.8-kb pWW01 carrying a thermosensitive replicon; Ery<sup>+</sup> | 28 |
| pG+host<sup>+</sup>::ahpF | pG+host<sup>+</sup> containing truncated ahpF; Ery<sup>+</sup> | This study |
| pJIM              | pJIM2246, 6.4-kb multicopy plasmid vector; Cm<sup>+</sup> | 34 |
| pJIM::noxE        | pJIM2246 containing noxE of TIL46; Cm<sup>+</sup> | This study |

### Table 2. Primers used in this study

| Primer | Sequence (5’ → 3’<sup>a</sup>) |
|--------|---------------------------------|
| ahpF-up | ATATAATGAATTCGTCTTGGACT          |
| ahpF-down | AATTTCGCCGGCAACGTTTTTA            |
| ahpF-up2 | GTTGAAGAAAGAGATGGGGAAA            |
| ahpF-down2 | CTGACCGAACCTTTATGGCGT            |
| noXR | CAATCTATCAGGCAGAAC              |
| noX2F | TGAATTACATTTAGACC              |
| noX2R | GACGTGAATAGACCAACATGAC           |
| noX3F | CCTGAATCTCAGTTTGAAC             |
| noX3R | ACCCGATGTCCTCCGGATGGTTC         |
| pORI-Ery | GGTTCGGTTTTGTTAAATGAGC             |
| luxAinv | GCCATTTACCCATTTTA                |

<sup>a</sup> Underlining indicates restriction sites of the enzymes indicated in parentheses.
TIL46. The double crossovers leading to the expected gene replacement were screened and obtained as described by Biwas et al. (2). Correct chromosomal deletion of the \( \alpha hpf \) gene was verified using PCR.

**Construction of the noxE null mutant.** The \( \text{noxE} \) gene, encoding the water-soluble NADH oxidase \( \text{NoxE} \), was disrupted in \( L. \text{lactis} \) TIL46 using a single crossover, resulting in the \( \text{noxE} \) mutant (Table 1). An internal 644-bp DNA fragment of \( \text{noxE} \) was PCR amplified with the \( \text{noxE} \) and \( \text{noxR} \) primers (Table 2) and subcloned into the PGEM-T Easy vector (Promega, Madison, WI). The resulting plasmid, pGEM-T-noxE (Table 1), was produced in \( E. \text{coli} \) TG1 (Table 1) and digested by ScaI and AatII. The digest was then cloned into the nonreplicative pORIrewlex vector (13), leading to pORIrewlex (Table 1), which was also produced in \( E. \text{coli} \) and transformed into TIL46 electrocompetent cells. Transformants were selected with erythromycin (5 \( \mu \)g ml\(^{-1} \)). The disruption of \( \text{noxE} \) by pORIrewlex was confirmed by PCR using the external primers pORIery, luxAinv, Nox2F, and Nox2R (Table 2).

**Complementation of the noxE mutant.** Complementation of the \( \text{noxE} \) mutant was achieved by cloning the \( \text{noxE} \) gene into the pJIM2246 multicopy plasmid vector. A 1,728-bp fragment encoding \( \text{noxE} \) with its putative promoter and terminator was amplified by PCR from TIL46 total DNA with primers pJIM33F and noxR (Table 2) and used in the Phusion high-fidelity DNA polymerase. This fragment was cloned into pGEM-T Easy, and the resulting plasmid was produced in \( E. \text{coli} \) TG1 and digested with SpeI and SacI. The digest was then cloned into the nonreplicative pORIrewlex vector pJIM2246. The resulting plasmid, pJIM2246::noxE (Table 1), was produced in \( E. \text{coli} \) TG1 repA\(^{+} \) and used to transform the \( \text{noxE} \) mutant. The resulting strain was designated \( \text{noxE} \)::\( \text{pJIM} \)-\( \text{noxE} \). A control strain was constructed by introducing the empty vector pJIM2246 into the \( \text{noxE} \) mutant (\( \text{noxE} \)::\( \text{pJIM} \)).

**Cell extract preparation.** Cells were harvested from \( L. \text{lactis} \) cultures by centrifugation (4,000 \( \times \)g, 15 min, 4°C). The cell pellets were washed twice with 5 ml 50 mM potassium phosphate buffer (pH 7.0) and stored at \(-20^\circ \text{C} \) until use. The cells were resuspended in 50 mM potassium phosphate buffer (pH 7.0) containing 0.6 g of glass beads and disrupted twice for 45 s at 180 V and 4.5 m/s (Fast Prep orbital mixer; MP Biomedicals, Illkirch, France). Each cell extract was recovered by centrifugation (17,400 \( \times \) g, 20 min, 4°C). The protein concentration was determined using a Coomassie (Bradford) protein assay kit (Pierce, Rockford, IL), and bovine serum albumin (BSA) was used as the standard.

**NOX assay.** NADH oxidase (NOX) activity was determined by using a 1-ml reaction mixture consisting of 50 mM potassium phosphate buffer (pH 7.0), 0.3 mM EDTA, and 0.2 mM NADH. The reaction was initiated by addition of an appropriate amount of freshly prepared cell extract. NADH oxidation was monitored by spectrophotometry at 340 nm and 25°C (UVikon XL; Biotek Instruments, Colmar, France). NOX activity was calculated using the molar extinction coefficient of NADH (6.220 M\(^{-1} \) cm\(^{-1} \)) and was expressed in units per milligram of protein. One unit of NADH oxidase activity is defined as the amount of enzyme which catalyzes the oxidation of 1 \( \mu \)mol NADH per min at 25°C. Control experiments without the cell extract were performed to verify the absence of spontaneous NADH oxidation.

**Milk fermentation and measurement of pH, dissolved oxygen, and redox potential.** All milk fermentations were performed at 30°C in 1-liter fermentors (BioStat Q plus; Sartorius) containing 500 ml UHT skim milk (Lesure, Surgères, France). Milk was inoculated (1%, vol/vol) with a milk culture at an optical density at 480 nm (OD\(_{480} \)) of 1.8. The cultures were kept homogeneous by gentle stirring (100 rpm). Aerobic incubation was performed with air in the headspace, which remained in contact with the atmosphere via a sterile filter so that oxygen could diffuse into the medium during fermentation (low-oxygen conditions). Anaerobic cultures were grown in nitrogen-saturated milk with the headspace continuously sparged with nitrogen. During \( L. \text{lactis} \) growth, acidification and the oxygen concentration were measured using MFCS/win 2.0 software (B. Braun Biotech International). Acidification data were expressed as the change in pH during fermentation (15). The abilities of the different mutant strains to grow in milk and acidify under low-oxygen conditions (gentle stirring at 100 rpm) were examined and compared with the abilities of the WT parent (Fig. 1A and B). The growth of the \( \text{noxE} \) mutant did not include an exponential phase, and both the growth rate and the final biomass were significantly \((P < 0.001) \) reduced compared with the growth rate and the final biomass of the WT strain. The acidification rate was also significantly affected \((P < 0.001) \) but less than the growth rate, suggesting that the \( \text{noxE} \) mutant produced more lactic acid than the wild-type strain. The growth and acidification kinetics were restored when a \( \text{noxE} \) expressing strain harboring \( \text{noxE} \) exhibited an 8-fold increase in activity compared with the WT strain. Finally, inactivation of the \( \alpha hpf \) gene did not affect NOX activity.

**RESULTS**

Analysis of milk reduction mechanism using knockout mutants. (i) NADH oxidase activity. The NADH oxidase (NOX) activities of TIL46 (WT) and the derived \( \text{noxE} \) and \( \alpha hpf \) mutants grown in GM17 were determined using extracts of cells harvested at the end of the exponential growth phase, when NOX activity was optimal (Table 3). Disruption of the \( \text{noxE} \) gene suppressed 95% of the NOX activity. Conversely, the \( \text{noxE} \) mutant complemented with the \( \text{pJIM} \) expression vector harboring \( \text{noxE} \) exhibited an 8-fold increase in activity compared with the WT strain. Finally, inactivation of the \( \alpha hpf \) gene did not affect NOX activity.

(ii) Aerobic growth and acidification in milk. The abilities of the different mutant strains to grow in milk and acidify under low-oxygen conditions (gentle stirring at 100 rpm) were examined and compared with the abilities of the WT parent (Fig. 1A and B). The growth of the \( \text{noxE} \) mutant did not include an exponential phase, and both the growth rate and the final biomass were significantly \((P < 0.001) \) reduced compared with the growth rate and the final biomass of the WT strain. The acidification rate was also significantly affected \((P < 0.001) \) but less than the growth rate, suggesting that the \( \text{noxE} \) mutant produced more lactic acid than the wild-type strain. The growth and acidification kinetics were restored when a \( \text{noxE} \) expressing strain (\( \text{pJIM}::\text{noxE} \)) was introduced into the \( \text{noxE} \) mutant (see Fig. S1 in the supplemental material), indicating that the growth defect was actually due to the absence of NOX activity. The growth and acidification rates of the \( \alpha hpf \) mutant were also strongly reduced \((P < 0.001) \) compared with the growth and acidification rates of the WT (Fig. 1), while the \( \text{menC} \) and \( \text{noxAB} \) mutants grew and acidified slightly slower than the WT strain but the final biomasses and pH reached about the same levels as those of the WT strain (Fig. 1).

(iii) Dissolved oxygen consumption. The abilities of the WT strain and mutants to consume dissolved oxygen were also examined during milk fermentation under low-oxygen conditions (Fig. 2). The WT strain very rapidly consumed dissolved oxygen which catalyzes the oxidation of 1 \( \mu \)mol NADH per min at 25°C. Control experiments without the cell extract were performed to verify the absence of spontaneous NADH oxidation.

Testing of \( \text{noxE} \) and \( \text{noxE} \) mutants complemented with \( \text{pJIM} \) on NOX activity.

| Strain | NADH oxidase activity (U mg\(^{-1} \)) |
|--------|--------------------------------------|
| WT     | 0.30 ± 0.05                          |
| \( \text{noxE} \) mutant | 0.014 ± 0.003 |
| \( \text{noxE} \)::\( \text{pJIM} \) mutant | 0.016 ± 0.005 |
| \( \text{noxE} \)::\( \text{pJIM}::\text{noxE} \) mutant | 2.5 ± 0.8 |
| \( \alpha hpf \) mutant | 0.32 ± 0.15 |

*\( \text{Cell extracts were prepared from GM17 cultures at the end of the exponential growth phase. The data are means ± standard deviations of at least four determinations.} \)
oxygen at the beginning of growth (4 h; OD$_{480}$, 0.8) and main-
tained the pO$_2$ level at zero until the end of growth (8 h;
OD$_{480}$, 3.3). Then the pO$_2$ level rose slightly. Disruption of
noxE strongly decreased the oxygen consumption; the pO$_2$
level declined to only 7% when the biomass reached an
OD$_{480}$ of 1.8, and then it gradually rose. Both the oxygen
consumption rate and the minimum pO$_2$ level were signifi-
cantly affected by noxE inactivation (P < 0.001). The ability of
the noxE mutant to consume O$_2$ was totally restored by
complementation with pJIM:noxE (see Fig. S2 in the supple-
mental material).

In the ahpF mutant culture the pO$_2$ level reached zero later
than it reached zero in the WT strain culture (6 h for the
ahpF mutant versus 4 h for the WT strain), but at the same biomass
as the WT strain culture (OD$_{480}$, 0.8). The noxAB and menC
mutants consumed dissolved oxygen as fast as the wild-type
strain (the pO$_2$ reached zero at 4 h [OD$_{480}$, 0.8]), but when
cultures of these mutants entered the stationary growth phase,
the pO$_2$ level immediately rose to 12% ± 1%, while it rose to
2.5% ± 0.5% for the ahpF mutant and to 1.5% ± 1.5% for the
WT strain (Fig. 2). These results indicate that NoxE is princi-
ppally responsible for O$_2$ consumption at the beginning of
growth and that the ETC (which includes menaquinones and
NoxAB) is involved in oxygen consumption during the station-
ary growth phase.

(iv) Milk reduction under aerobic conditions. The abilities
of the WT strain and the mutants to decrease the redox po-
tential (E$_h$) of milk were also investigated during milk fer-
m entation (Fig. 3). The WT strain started to reduce milk slowly
from 375 ± 30 mV to 300 ± 25 mV, and as soon as the pO$_2$

FIG. 1. Growth (A) and acidification (B) kinetics of L. lactis TIL46 (WT) and the derived noxE, ahpF, menC, and noxAB mutants in UHT skim milk under aerobic (low-oxygen) conditions. The data are means ± standard deviations (n = 3).

FIG. 2. Evolution of dissolved oxygen levels (pO$_2$) in UHT skim milk during growth of L. lactis TIL46 (WT) and the derived noxE, ahpF, menC, and noxAB mutants under aerobic (low-oxygen) conditions. The data are means ± standard deviations (n = 3). A pO$_2$ value of 21% corresponds to milk saturated with air, and a pO$_2$ value of 0% corresponds to milk saturated with nitrogen.

FIG. 3. Evolution of the redox potential (E$_h$) of UHT skim milk during growth of L. lactis TIL46 (WT) and the derived noxE, ahpF, menC, and noxAB mutants under aerobic (low-oxygen) conditions. The data are means ± standard deviations (n = 3).
reached zero (at 4 h), the $E_{h7}$ immediately dropped to $-200$ mV and then stabilized at around $-230 \pm 10$ mV until the end of the exponential growth phase (Fig. 3). The noxE mutant slowly reduced milk, and the $E_{h7}$ only declined to $260 \pm 40$ mV, which is in line with the relatively high oxygen level (pO$_2$, $\sim$10%) in milk. The reducing ability of the noxE mutant was restored by complementation with pJIM::noxE (see Fig. S3 in the supplemental material). The ahpF mutant reduced milk later than the WT, when the pO$_2$ reached zero (after 6 h), but then the $E_{h7}$ dropped to the same level that was observed with the WT ($-213 \pm 5$ mV) (Fig. 3). Finally, the mutants whose ETC was affected (menC and noxAB mutants) reduced milk very gradually to an $E_{h7}$ value of $-65 \pm 50$ mV at the end of growth (8 h; OD$_{480}$, $\sim$3), although the pO$_2$ reached zero at the beginning of growth (4 h; OD$_{480}$, 0.8) (Fig. 2). For all strains, the $E_{h7}$ of milk increased after the end of growth, in line with the increase in the pO$_2$. All of these results indicate that NoxE contributes markedly to the reduction of aerated milk via elimination of oxygen. The ETC is also strongly involved in milk reduction, probably by reducing oxidizing compounds other than the oxygen present in milk.

(v) Milk reduction under anaerobic conditions. The abilities of the WT strain and the mutants to grow, acidify, and reduce milk under anaerobiosis were also examined (Fig. 4). The anaerobic growth rate of TIL46 in milk (Fig. 4A) was slightly reduced ($P = 0.05$) compared with the aerobic growth rate and was similar to the aerobic growth rate of the noxE mutant ($P = 0.17$) (Fig. 1), indicating that NAD regeneration via NoxE (under aerobiosis) stimulates the growth of L. lactis TIL46 in milk. Of course, the negative impact of noxE inactivation on anaerobic growth and acidification was weak because NoxE is inactive in the absence of oxygen. In contrast, the negative impact of ahpF inactivation on anaerobic growth ($P < 0.001$) was stronger than the impact on aerobic growth, suggesting that the impaired growth of the ahpF mutant

FIG. 4. Growth (A) and acidification kinetics (B) of L. lactis TIL46 (WT) and the derived noxE, ahpF, menC, and noxAB mutants in nitrogen-saturated (anaerobic conditions) UHT skim milk and evolution of the redox potential ($E_{h7}$) of milk during growth (C). The data are means ± standard deviations ($n = 3$).

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was not due to its NADH oxidase activity. The growth rates ($P < 0.01$) and final biomasses ($P < 0.01$) of the menC and noxAB mutants were also strongly affected (Fig. 4A).

Under anaerobiosis, the $E_{h7}$ of milk before inoculation was essentially the same as that in the presence of $O_2$ ($350 \pm 50$ mV). Disruption of noxE or ahpF did not affect the ability of TIL46 to decrease the $E_{h7}$ of milk (Fig. 4C). The $E_{h7}$ dropped immediately (before the pH of milk started to decrease) from $350 \pm 50$ mV to a stable value of $-265 \pm 15$ mV. With the menC mutant the $E_{h7}$ dropped 1 h later than it dropped with the WT strain, and it stabilized at a higher value (about $-180 \pm 10$ mV). Finally, the noxAB mutant reduced milk very gradually, and the $E_{h7}$ stabilized at the same level that it stabilized with the menC mutant ($-180 \pm 10$ mV). Thus, our results showed that the NoxA and/or NoxB NADH dehydrogenase and, to a lesser extent, menaquinones are involved in milk reduction by *L. lactis* TIL46 under anaerobic conditions.

**Natural diversity of milk-reducing activity in *L. lactis*. (i) Selection of strains whose ETC activity is affected.** A collection of 50 *L. lactis* strains originating from dairy environments was screened for the ability to reduce tetrazolium violet (TV), which is indicative of ETC activity (38). After inoculation onto MilkAgarTV plates (38), only seven strains were not capable of reducing TV as much as reference strain TIL46. However, the control plate containing a pH indicator showed that six of these strains did not acidify milk either. Only *L. lactis* strain CSK1019, which grew and acidified milk, reduced TV much less than TIL46, in the same way as the noxAB mutant (not shown).

(ii) Milk reduction by natural *L. lactis* strains. The abilities of eight natural strains (including seven strains randomly chosen from the CSK collection and strain CSK1019 that reduced TV poorly) to reduce milk under aerobic and anaerobic conditions were examined.

Under aerobic conditions, all of the strains grew in milk and reached the stationary phase at about 8 and 14 h; CSK1788 grew the fastest, and CSK1019 grew the slowest (see Fig. S4 in the supplemental material). Cultures of all strains reached an OD$_{480}$ close to 3, except for the CSK1019 culture, which stabilized at a higher value (about $0.5$ to $0.7$). In addition, strain CSK2036, which exhibited very low NADH oxidase activity ($0.06 \pm 0.02$ U mg$^{-1}$), consumed $O_2$ very rapidly (the $pO_2$ reached zero when the culture OD$_{480}$ reached 1.8) than the other two strains (the $pO_2$ reached zero when the culture OD$_{480}$ reached 0.5 to 0.7). In addition, strain CSK2036, which exhibited very low NADH oxidase activity ($0.06 \pm 0.02$ U mg$^{-1}$), consumed $O_2$ very rapidly (the $pO_2$ reached zero when the culture OD$_{480}$ reached 0.7). These results suggest that in some *L. lactis* strains, enzymes other than NoxE are capable of consuming $O_2$ in the medium. Under anaerobiosis, all of the strains reduced milk to similar extents and like TIL46, except for CSK1019, which reduced milk much more slowly, like the noxAB mutant (Fig. 6). Also, the growth and acidification kinetics of CSK1019 in nitrogen-saturated UHT skim milk were similar to those of the noxAB mutant (not shown).

**DISCUSSION**

Despite the demonstrated effects of the $E_n$ on dairy product quality, starter lactic acid bacteria are still essentially characterized by their acidification activities and not by their abilities to decrease the $E_n$ of milk (reduction activities). A few studies have reported that the reducing activity of lactic acid bacteria is species dependent and that *L. lactis* is one of the most reducing lactic acid bacteria (5, 8). Also, Jeanson et al. (21) showed that the $E_n$ of milk drops only after all of the dissolved oxygen is consumed by *L. lactis*. However, the molecular basis for the reducing activity of *L. lactis* has not been investigated previously. Our study confirmed that elim-
elimination of $O_2$ is actually a prerequisite for milk reduction and revealed that another mechanism is necessary for the $E_n$ to fall to $-220$ mV. Indeed, we identified and characterized two major mechanisms involved in milk reduction by *L. lactis* (Fig. 7). The first mechanism relied on the elimination of dissolved oxygen. We showed that NoxE activity was essential for early $O_2$ consumption during growth because noxE inactivation hampered a decrease in the $pO_2$ level to zero and consequently prevented a drop in the $E_n$ value. AhpF and the ETC did not appear to contribute notably to $O_2$ consumption during the early growth phase. Indeed, the observed delay in the disappearance of $O_2$ with the *ahpF* mutant was mainly due to the delay in growth, and inactivation of the ETC did not affect $O_2$ consumption significantly. However, AhpF and the ETC may be responsible for the slight consumption of $O_2$ observed with the noxE mutant (Fig. 2). The minor role played by AhpF in oxygen elimination was consistent with the low specific activity of the pure enzyme (15 U mg$^{-1}$) compared to that of NoxE (95 U mg$^{-1}$) (22) and with the fact that its disruption had no impact on the NADH oxidase activity of cell extracts. During the stationary growth phase, NoxE appeared to be much less active, probably because it is unstable when it is subjected to overoxidation and is inhibited by an acidic pH (22).

In this phase, the ETC contributed more markedly to $O_2$ consumption than the milk-reducing activity of the WT. However, the final $E_n$ value was more than the milk-reducing activity of the wild type (5), suggesting that they do not produce menaquinones either. Moreover, we did not find any menaquinone biosynthesis genes in the sequenced genomes of *L. lactis*, *Enterococcus faecalis*, *Streptococcus thermophilus*, or *Lactobacillus plantarum*, which reduce milk in the same way as *S. thermophilus* (5), suggesting that they do not produce menaquinones either. However, several *Lactobacillus plantarum* strains reduce the $E_n$ of milk to a negative value ($-154$ mV) (5), while the sequenced strain (WCF1) appears to lack several genes involved in menaquinone biosynthesis. Other mechanisms may be involved in the reducing activity of *L. plantarum*, or menaquinone production may be strain dependent.

Within *L. lactis* subsp. *cremoris*, we observed quite a broad
diversity of milk-reducing activities. This diversity appeared to be related in part to NADH oxidase activity because the two strains without detectable NADH oxidase activity did not reduce milk. However, some strains with low NADH oxidase activity exhibited O_2 quite rapidly. This might have been due to the possibility that the NADH oxidase activities of these strains in milk were higher than the activities determined in M17 medium, even though we found that the NADH oxidase activities of TIL46 in cells grown in these two media were similar. In fact, two other genes are predicted to encode NADH oxidases in the genomes of _L. lactis_ subsp. _cremoris_ MG1363 and SK11 (30, 40). The first gene, _noxC_, encodes a 547-residue protein in both genomes, while the second gene, _noxD_, is a pseudogene in MG1363 (which was derived from TIL46) but encodes a 443-residue protein in SK11. The latter gene may be present in some natural strains and may be expressed differently in M17 and milk. Moreover, certain natural strains may also exhibit other oxidase activities, such as pyruvate oxidase activity, the genes for which are present in both sequenced genomes, or a strong ETC activity, which enable them to consume all of the oxygen present.

The diversity of the amounts of menaquinone produced by _L. lactis_ strains in milk cultures has been examined previously (31). The amount produced ranged from 50 to 600 nmol per g of lyophilized culture. This great variation could have an impact on the reducing activities of different strains. However, in the present study, the TV reduction test revealed that the ETC activity of only one strain was affected, probably at the level of the NADH dehydrogenases. This result indicates that all of the strains tested produced enough menaquinone to reduce TV. The NoxE activity of the selected strain CSK1019 was also affected. However, the other natural strain without NoxE activity (CSK1382) and the _noxE_ mutant reduced TV as well as reference strain TIL46, indicating that the TV test does not allow selection of strains on the basis of their oxygen consumption. To conclude, great variation in reducing activity was found in natural _L. lactis_ strains originating from the dairy environment. The broad diversity allows selection of specific environments that can be used to modulate the redox potential of fermented dairy products.

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