Generation of a Membrane Potential by *Lactococcus lactis* through Aerobic Electron Transport

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*Lactococcus lactis*, a facultative anaerobic lactic acid bacterium, is known to have an increased growth yield when grown aerobically in the presence of heme. We have now established the presence of a functional, proton motive force-generating electron transfer chain (ETC) in *L. lactis* under these conditions. Proton motive force generation in whole cells was measured using a fluorescent probe (3';3'-dipropylthiadicarbocyanine), which is sensitive to changes in membrane potential (ΔΨ). Wild-type cells, grown aerobically in the presence of heme, generated a ΔΨ even in the presence of the F_{1}-F_{0} ATPase inhibitor N,N'-dicyclohexylcarbodiimide, while a cytochrome bd-negative mutant strain (CydΔA) did not. We also observed high oxygen consumption rates by membrane vesicles prepared from heme-grown cells, compared to CydΔA cells, upon the addition of NADH. This demonstrates that NADH is an electron donor for the *L. lactis* ETC and demonstrates the presence of a membrane-bound NADH-dehydrogenase. Furthermore, we show that the functional respiratory chain is present throughout the exponential and late phases of growth.

*Lactococcus lactis* has a long history of use in the production of fermented dairy products, such as cheese and buttermilk, under mainly anaerobic conditions. Studies on the aerobic growth of *L. lactis* have therefore been focused mainly on the effect of oxygen on fermentation patterns (25) or cell damage due to the formation of reactive oxygen species (3, 8, 32).

These damaging effects of oxygen on *L. lactis* cells are not observed when cells are grown in the presence of both oxygen and a heme source (9, 30, 45). Aerated, heme-grown *L. lactis* cells display new characteristics such as increased growth yield, resistance to oxidative and acid stress, and improved long-term survival when stored at low temperatures (40). These traits are important for industrial applications, and the use of heme to increase the efficiency of biomass production of starter cultures has been described previously (10, 13, 37). The increased growth efficiency of aerated heme-grown *L. lactis* cells is due to a shift from homolactic to mixed-acid fermentation, more complete glucose utilization in non-ph-controlled batch cultures, and possibly energy generation by NADH oxidation via the electron transfer chain (ETC) (9). The ability to generate metabolic energy via NADH oxidation by the ETC will be the subject of this work. Increased growth efficiency will make *L. lactis* more useful as a cell factory for the production of biomass-related compounds such as proteins and vitamins.

Heme is an essential cofactor of cytochrome complexes in the electron transport chains of respiring cells (14, 52). Furthermore, the genomes of several *L. lactis* strains contain genes which, when expressed, could form a simple ETC if supplied with heme (13). Genes encoding menaquinone biosynthesis enzymes and a bd-type cytochrome (mena)quinoloxidase have, for example, been identified in the genomes of strains IL-1403 and SK11 (http://genome.ornl.gov/microbial/lcre/) (6). The (mena)quinoloxidase is a membrane-bound enzyme consisting of two subunits, which are encoded by cydA and cydB. The cydC and cydD genes encode an ABC transporter, which is required for the assembly of the oxidase (7). This type of cytochrome-containing enzyme is found in a variety of facultative aerobic bacteria (16), where it functions as an alternative terminal electron acceptor capable of working under low-oxygen conditions (17, 43).

The higher growth yield in the presence of heme and the presence of ETC-related genes in the genome suggest active respiration in aerated heme-grown cells of *L. lactis* (5). To prove that actual respiration occurs, the formation of a proton motive force (PMF) as a result of ETC activity still needs to be demonstrated. In this paper, we present genetic and physiological evidence for cytochrome bd-associated PMF formation and thus the presence of a functional ETC in *L. lactis*.

**MATERIALS AND METHODS**

**Cultures and growth conditions.** The strains used in these studies were *Lactococcus lactis* MG1363 (11) or derivatives of this strain: a cytochrome-negative mutant (CydΔA Cm') and a cytochrome negative mutant complemented with plasmid pLL253CydABC. Plasmid pLL253CydABC is a pLL253 derivative (46) carrying the cydABC genes (Cm' Ery'). Cells were grown on M17 medium (Difco, Detroit, MI) supplemented with glucose (GM17) to a final concentration of 1% (wt/vol). When indicated, cells were grown in OM17 medium supplemented with heme (hemin) (stock solution, 0.5 mg/ml in 0.05 M NaOH; 8μg/ml) to a final concentration of 2 μg/ml or with the equivalent volume of 0.05 M NaOH as a control. When indicated, chloramphenicol and/or erythromycin was added to a final concentration of 10 μg/ml. Cultures were grown aerobically in 100-ml flasks with shaking at 250 rpm or anaerobically in tubes/glass bottles at 30°C.

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### TABLE 1. Identification by homology searches of NADH-dehydrogenase, (mena)quinoloxidase, and menaquione biosynthesis genes in the genome of *L. lactis* MG1363 compared to *S. aureus* 168

<table>
<thead>
<tr>
<th><strong>L. lactis</strong> MG1363 ORF</th>
<th>Homology to <em>S. aureus</em> 168</th>
<th>% DNA identity/ % aa identity</th>
<th><strong>Identity</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>lllg_1864</td>
<td>cydA</td>
<td>48/68</td>
<td>Cytochrome d ubiquinol oxidase subunit I (EC 1.10.3.–)</td>
</tr>
<tr>
<td>lllg_1883</td>
<td>cydB</td>
<td>43/62</td>
<td>Cytochrome d ubiquinol oxidase subunit II (EC 1.10.3.–)</td>
</tr>
<tr>
<td>lllg_1862</td>
<td>cydC</td>
<td>47/65</td>
<td>ABC transporter component CydC</td>
</tr>
<tr>
<td>lllg_1861</td>
<td>cydD</td>
<td>44/66</td>
<td>ABC transporter component CydD</td>
</tr>
</tbody>
</table>

*a* Predicted identity on the level of amino acid (aa) composition.

### Annotation of *L. lactis* MG1363 genes

Sequence data for *L. lactis* MG1363 were obtained from the *L. lactis* MG1363 sequencing consortium. DNA sequences of open reading frames (ORFs) were translated into amino acid sequences and annotated by homology using the BLAST algorithm as described previously (1). Prediction of membrane-spanning helices was performed as described previously (53).

**Mutant construction.** Molecular cloning techniques were carried out in accordance with standard laboratory procedures (44). For the construction of the knockout plasmid, primers were designed on the basis of MG1363 genome sequence data. A 1-kb fragment upstream of cydA (forward primer p39 [GATC GTCACACTCATACAT] and reverse primer p40 [GGTTACGTTTATCTCC]) and a 1-kb fragment downstream of cydA (forward primer p41 [GTC GTGATGAAATGACTGTA] and reverse primer p42 [GAGCATGACCAATCAACTCT]) were amplified using PCR. The flanking fragments were cloned blunt ended into vector pNZ5317 (23) digested with Swa1 (upstream fragment) and Eco31I (downstream fragment) to produce the knockout vector pRB6671_CydA-KO. The knockout plasmid was transformed into *L. lactis* MG1363, and a chloramphenicol resistance of the cydA gene was obtained by a double-crossover event by homologous recombination as described previously (22), which resulted in mutant strain CydΔΔ. For complementation studies of the cytC-negative mutant (CydΔA), a vector carrying the cyd operon (cydABC) was constructed. The operon was amplified using PCR techniques (forward primer p43 [TGACCGATGGAGGAGTCAGAAGCCTT] and reverse primer p44 [TGACGGCTGCTGATAGCGTACCTGATC]) using the genome of MG1363 as a template. The primer tails (underlined) carried recognition sequences for restriction enzymes SphI and SacI for easy cloning. The PCR product and vector pLJ253 were digested with SphI and SacI, purified from gel, and cloned sticky blunt to construct pLJ253_CydABC. Finally, to complement the cytc-negative mutant, this plasmid was transformed into the CydΔA strain.

### Isolation of membrane vesicles

Cells from a 2-liter culture were grown aerobically to late exponential phase (optical density [OD] of about 2.5 to 3.0), washed twice in 50 mM potassium phosphate (pH 5.0), and resuspended in 20 ml of membrane suspension. The cell suspension was incubated with 10 mg/ml egg lysozyme (Merck, Darmstadt, Germany) for 30 min at 30°C. Cell lysis was achieved by passage two times through a French pressure cell (American Instrument Corp., Silver Spring, MD) at an operating pressure of 20,000 lb/in². The orientation of bacterial membrane vesicles prepared by French press is predominately inside-out (2, 27). The suspension was supplemented with 10 mM MgSO₄ and 100 μg/ml DNase and incubated for 15 min at 30°C, followed by the addition of 15 mM K-EDTA. A low spin at 12,000 rpm was performed to remove cell debris and whole cells. The vesicle-containing supernatant was centrifuged at 150,000 × g to harvest the membranes, which were resuspended in 50 mM potassium phosphate (pH 7.0) containing 10% glycerol to a final concentration of 10 to 20 μg/ml, divided into 500-μl aliquots, and stored at −80°C.

### Extrusion of membrane vesicles

To obtain single unilamellar vesicles suitable for combining enzyme activities, 500 μl of membrane suspension was thawed and diluted with 500 μl of 50 mM potassium phosphate (pH 5.5). The 1-ml mixture was extruded using a Minietruder (Avanti Polar Lipids Inc., Alabaster, United Kingdom) with a 0.4-μm-sized polycarbonate track etch membrane (Whatman International Ltd., Kent, United Kingdom) to generate inside-out, single lamellar vesicles with an average size of 0.4 μm (50).

### Measurements of membrane potential

The fluorescent probe 3,3’-dipropylthiadicarbocyanine [DiSC₃(5)] was used to monitor the membrane potential (ΔΨ) in intact cells (51). The distribution of the probe over the cytoplasmic membrane and the soluble phase is sensitive to changes in the ΔΨ. More probe molecules from the soluble phase will dissolve in the membrane with increasing ΔΨ, causing the quenching of the fluorescence signal by aggregation (20, 48). Nigericin (K⁺/H⁺ exchange) was added to convert ΔΨ to ΔΨ⁺, making it possible to estimate the contribution of the pH gradient to the PMF. Valinomycin (K⁺ ionophore) was added, in combination with nigericin, to cause a total dissipation of the PMF. The fluorescence was measured with a Cary Eclipse fluorescence spectrophotometer combined with a Cary Single Cell Peltier accessory (Varian, Palo Alto, CA) or an SPF-500C spectrofluorometer (SLM Aminco). The fluorescence was measured at an emission wavelength of 660 nm with an excitation wavelength of 643 nm (both with a 5-nm band pass).

Wild-type and CydΔΔ cells were supplemented with heme and grown aerobi-

### RESULTS

In silico evidence for an ETC in *L. lactis* MG1363. An in silico analysis was performed on the genome of *L. lactis* MG1363 to identify possible components of an electron transfer chain. Two type II NADH dehydrogenases, encoded by noxA and noxB (Tables 1 and 2), were predicted on the basis of the *L. lactis* MG1363 genome sequence (13). Both genes are characterized as having flavin adenine dinucleotide binding motifs but differ in the numbers of predicted membrane-spanning segments (four in NoxB and one in NoxA). Although the operon-like structure of noxA and noxB on the genome sug-

### Other analytical procedures

Protein concentrations of membrane preparations were determined using the bicinchoninic acid protein assay reagent (Omnimab Int., Breda, The Netherlands) (49).

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<table>
<thead>
<tr>
<th>\textit{L. lactis} MG1363 ORF</th>
<th>Homology to \textit{L. lactis} IL-1403</th>
<th>% DNA identity/ % aa identity (%)</th>
<th>Identity</th>
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<tbody>
<tr>
<td>lllmg_0196</td>
<td>preA</td>
<td>95/98</td>
<td>Farnesy1 pyrophosphate synthetase (EC 2.5.1.1)</td>
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<td>lllmg_0197</td>
<td>ybg/menA</td>
<td>88/95</td>
<td>1,4-Dihydroxy-2-naphthoate polyprenyltransferase (EC 2.5.1.–)</td>
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<td>lllmg_1828</td>
<td>menF</td>
<td>80/89</td>
<td>Isocitrate synthase (EC 5.4.4.2)</td>
</tr>
<tr>
<td>lllmg_1829</td>
<td>menD</td>
<td>85/91</td>
<td>2-Succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase (EC 2.5.1.64)</td>
</tr>
<tr>
<td>lllmg_1830</td>
<td>menX</td>
<td>77/85</td>
<td>YtxM-like protein/menaquinone biosynthesis-related protein</td>
</tr>
<tr>
<td>lllmg_1831</td>
<td>menB</td>
<td>98/100</td>
<td>Naphthoate synthase (EC 4.1.3.36)</td>
</tr>
<tr>
<td>lllmg_1832</td>
<td>menE</td>
<td>85/93</td>
<td>O-Succinylbenzoic acid coenzyme A ligase (EC 6.2.1.26)</td>
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<tr>
<td>lllmg_1833</td>
<td>yhdB/menC</td>
<td>91/97</td>
<td>O-Succinylbenzoate synthase (EC 4.2.1.–)</td>
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<td>lllmg_1735</td>
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<td>99/99</td>
<td>NADH dehydrogenase (EC 1.6.99.3)</td>
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<tr>
<td>lllmg_1734</td>
<td>noxB</td>
<td>99/99</td>
<td>NADH dehydrogenase (EC 1.6.99.3)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Predicted identity on the level of amino acid (aa) composition.

heme-grown wild-type cells. Subsequent complementation of the Cyd\textDelta\textDelta mutant with a vector carrying the (mena)quinoloxidase coding operon (pIL253CydABCD) restored the wild-type-like phenotype when grown aerobically with heme.

Measurement of \( \Delta \Psi \) in whole cells of \textit{L. lactis}. The formation of a membrane potential by \textit{L. lactis}, as a result of electron transport, was determined with the fluorescent probe DiSC\textsubscript{3}(5). The intensity of fluorescence of the probe is sensitive to changes in the \( \Delta \Psi \), and it decreases with increasing \( \Delta \Psi \) and versa. The PMF is composed of \( \Delta \psi \) and \( \Delta \xi \). In order to estimate the contribution of a pH gradient to the PMF, we added nigericin (a \( K^+ / H^+ \) exchanger) to convert the \( \Delta \psi \) into a \( \Delta \xi \). Furthermore, the addition of valinomycin (\( K^+ \) ionophore) plus nigericin collapsed the \( \Delta \xi \) completely. The main proton pump in \textit{L. lactis} responsible for PMF generation is the \( F_1-F_0 \) ATPase, by pumping protons at the expense of metabolic ATP (26). To discriminate between PMF generation by the ETC and that by the \( F_1-F_0 \) ATPase, the \( F_1-F_0 \) ATPase-specific inhibitor DCCD was used (47). To further validate PMF formation via the ETC, we used a cytochrome \( bd \)-negative mutant (Cyd\textDelta) as a control.

The changes in membrane potential, DiSC\textsubscript{3}(5) fluorescence, were recorded as a function of time (Fig. 2). In wild-type cells and Cyd\textDelta cells with no DCCD treatment, the addition of glucose led to an increase in \( \Delta \Psi \). However, for Cyd\textDelta cells incubated with DCCD, this increase in \( \Delta \Psi \) was negligible. The increase in \( \Delta \Psi \) after the addition of glucose was transient, since the membrane potential is subsequently converted into a pH gradient (see also Discussion). Accordingly, the addition of

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Membrane preparation} & \textbf{OD\textsubscript{600}} & \textbf{pH} \\
\hline
Wild type + heme & 5.25 & 5.04 \\
Wild type & 2.60 & 4.34 \\
Cyd\textDelta + heme & 2.57 & 4.33 \\
Cyd\textDelta & 2.66 & 4.27 \\
Cyd\textDelta + pIL253CydABCD + heme & 5.07 & 5.06 \\
Cyd\textDelta + pIL253CydABCD & 2.62 & 4.30 \\
\hline
\end{tabular}
\caption{Aerobic growth (OD\textsubscript{600}) and acidification of \textit{L. lactis} with and without heme\textsuperscript{a}}
\end{table}

\textsuperscript{a} \textit{L. lactis} wild-type, Cyd\textDelta, and Cyd\textDelta cells, complemented with plasmid pIL253CydABCD (carrying the cydABCD operon), were grown aerobically overnight in M17G medium at 30°C in the presence or absence of heme.
nigericin resulted in an increase in ΔΨ. The gradual decrease of the ΔΨ, that is, upon the addition of nigericin to wild-type cells inhibited with DCCD and of CydΔΔ cells (Fig. 2B and C), was caused by the excess of nigericin and could be prevented by using lower nigericin concentrations (data not shown). The subsequent addition of valinomycin dissipated the ΔΨ completely. The fluorescence measurements clearly show that the cytochrome-negative CydΔΔ strain is unable to generate a ΔΨ when the F$_r$F$_o$ ATPase is inhibited by DCCD. In contrast, wild-type heme-grown cells are able to build up ΔΨ even in the presence of DCCD. Taken together, these findings indicate the presence of a cytochrome bd-dependent mechanism of PMF generation in wild-type L. lactis cells.

It has been suggested that respiration is induced in the late exponential/early stationary phases of growth when glucose becomes limiting or the pH drops below a certain threshold (9, 12, 54). To investigate this hypothesis, ETC activity measurements were performed by using cells harvested at different phases of growth, corresponding to early, mid-, and late exponential/stationary phases of growth when glucose is an electron acceptor (Fig. 1). To prove that NADH can indeed serve as an electron donor, oxygen consumption by membrane vesicles of aerobically grown wild-type cells (with and without heme) and CydΔΔ cells (with heme) was measured (Table 5). Membrane vesicles prepared from heme-grown wild-type cells showed a greater-than-6.5-fold increase in oxygen consumption rates for buffer with heme were not detected). These results confirm that respiration in L. lactis is not a growth-phase-dependent event but is present throughout aerated heme-supplemented growth.

**NADH-dependent oxygen consumption by membrane vesicles.** The results with whole cells of L. lactis led to a model of a simple ETC, which uses NADH as an electron donor and oxygen as an electron acceptor (Fig. 1). To prove that NADH can indeed serve as an electron donor, oxygen consumption by membrane vesicles of aerobically grown wild-type cells (with and without heme) and CydΔΔ cells (with heme) was measured (Table 5). Membrane vesicles prepared from heme-grown wild-type cells showed a greater-than-6.5-fold increase

**Rate of oxygen uptake by whole cells.** The terminal electron acceptor of aerobic ETC in L. lactis is oxygen through the activity of the oxygen-requiring cytochrome bd complex. This complex most likely oxidizes menaquinol and reduces oxygen to water. ETC activity should thus lead to increased oxygen consumption. For the measurements of oxygen uptake, aerobically grown, early-exponential-phase cells (OD$_{600}$ of 0.5 to 0.58) were used, rather than stationary-phase cells, to eliminate possible differences in cell viability. Upon the addition of glucose, heme-supplemented wild-type cells showed a higher oxygen consumption rate (25.72 ± 2.76 nmol O$_2$ depletion/min/mg [dry weight] at an OD of 0.50 to 0.58) than heme-supplemented CydΔΔ cells (13.51 ± 0.02 nmol O$_2$ depletion/min/mg [dry weight] at an OD of 0.50 to 0.58) or wild-type cells grown in the absence of heme (13.02 ± 0.01 nmol O$_2$ depletion/min/mg [dry weight] at an OD of 0.50 to 0.58) (oxygen consumption rates for buffer with heme were not detected). These results confirm that respiration in L. lactis is not a growth-phase-dependent event but is present throughout aerated heme-supplemented growth.

**TABLE 4. Relative drop in fluorescence of early-exponential-phase, mid-exponential-phase, and overnight (or late-stationary-phase) cultures of DCCD-treated and untreated wild-type and CydΔΔ cells after glucose addition**

<table>
<thead>
<tr>
<th>Strain</th>
<th>OD$_{600}$ (phase)</th>
<th>% Fluorescence drop</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>−DCCD</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.48 (early)</td>
<td>60.7</td>
</tr>
<tr>
<td>CydΔΔ</td>
<td>0.4 (early)</td>
<td>40.0</td>
</tr>
<tr>
<td>Wild type</td>
<td>1.08 (early/mid)</td>
<td>71.8</td>
</tr>
<tr>
<td>CydΔΔ</td>
<td>1.04 (early/mid)</td>
<td>58.6</td>
</tr>
<tr>
<td>Wild type</td>
<td>1.53 (mid)</td>
<td>38.0</td>
</tr>
<tr>
<td>CydΔΔ</td>
<td>1.49 (mid)</td>
<td>46.9</td>
</tr>
<tr>
<td>Wild type</td>
<td>4.49 (O/N)</td>
<td>46.9</td>
</tr>
<tr>
<td>CydΔΔ</td>
<td>2.6 (O/N)</td>
<td>45.7</td>
</tr>
</tbody>
</table>

*The percent fluorescence drop compares two fluorescence values in one trace at one time point with a maximal difference in value: the low trace value is the measured level of fluorescence after the addition of glucose; the high trace value was extrapolated from the fluorescence recording before the addition of glucose. Experimental conditions are described in Materials and Methods.

*These fluorescence traces are described in detail in the legend of Fig. 2.

*Cultures grown overnight (O/N) were grown for more than 20 h and considered (late) stationary-phase cells.

FIG. 2. Fluorescence traces of DiSC$_3$(5) in whole cells of L. lactis showing the generation of a membrane potential. Cells were prepared as described in Materials and Methods. A decrease in fluorescence signifies an increase in membrane potential. At 1 min, glucose (15 mM) was added; at 2 min, nigericin (0.1 μM) was added; and at 3 min, valinomycin (2 μM) was added. (A) Wild-type cells. (B) Wild-type cell treated with DCCD. (C) CydΔΔ cells. (D) CydΔΔ cells treated with DCCD.
in oxygen consumption upon the addition of NADH compared to membrane vesicles from wild-type cells grown without heme or to membrane vesicles from heme-grown CydAΔ cells. The oxygen consumptions by the latter two were comparable. No oxygen consumption was observed when membrane vesicles of heme-grown wild-type cells were supplied with NAD⁺, demonstrating the need for the reduced form of NAD. Furthermore, NADH added to buffer without membrane vesicles led to only a limited amount of oxygen consumption (data not shown).

**DISCUSSION**

In this report, we have shown, for the first time, that aerobic electron transport in *L. lactis* MG1363 actually leads to the generation of a PMF. A cytochrome bd-negative mutant was constructed (CydAΔ) as a negative control lacking the respiratory phenotype. We used DCCD to inhibit the F₁-Fₒ ATPase, the primary PMF-generating system in fermentative lactic acid bacteria (LAB) using ATP as the energy source (19). DCCD-treated heme-supplemented wild-type cells were still capable of generating a PMF, while in contrast, DCCD-treated heme-supplemented CydAΔ cells were not. These two observations demonstrate that heme-supplemented wild-type cells have an additional PMF-generating system besides the F₁-Fₒ ATPase. This PMF-generating system requires a functional cytochrome bd complex and implies the presence of a functional ETC.

The slight decrease in fluorescence upon the addition of glucose to DCCD-treated CydAΔ cells is explained by the incomplete inhibition of the F₁-Fₒ ATPase by DCCD (23). In addition to the F₁-Fₒ ATPase and the ETC, the contribution of alternative mechanisms of PMF generation, if present at all, to the overall energy conservation in *L. lactis* seems minimal (24, 29, 34, 35). What is clearly seen in the fluorescence recordings of the heme-supplemented wild-type cells is that the addition of glucose leads to an initial rapid increase in ΔΨ and a subsequent conversion into a ΔpH. This conversion in ΔpH is deduced from the increase in ΔΨ upon the addition of nigericin. Although the membrane of a cell acts as a capacitor, its capacitance is low. Consequently, the extrusion of a few protons already leads to a large ΔΨ. To generate a ΔpH of a similar size, the cell needs to pump out far more protons, and this would lead to a very high ΔΨ. Therefore, mechanisms are present to increase the ΔpH at the expense of ΔΨ, i.e., through the electrogenic uptake of K⁺ ions, which allows more protons to be pumped out (4, 21, 36).

**TABLE 5. Oxygen consumption by membrane vesicles at 25°C after the addition of NADH or NAD⁺**

<table>
<thead>
<tr>
<th>Membrane preparation</th>
<th>Addition</th>
<th>mean μmol O₂ consumption/min/mg protein ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type + heme</td>
<td>NADH</td>
<td>42.18 ± 0.12</td>
</tr>
<tr>
<td>Wild type</td>
<td>NADH</td>
<td>6.62 ± 0.35</td>
</tr>
<tr>
<td>CydAΔ + heme</td>
<td>NADH</td>
<td>8.41 ± 1.32</td>
</tr>
<tr>
<td>Wild type + heme</td>
<td>NAD</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Membrane vesicles were prepared from the different cells as described in Materials and Methods. ND, no consumption of oxygen detected after the addition of NAD.

Respiring cells (aerated and heme supplemented) from an exponentially growing culture showed an increased oxygen consumption rate compared to similarly grown cells containing a disruption in the cytochrome genes or compared to non-heme-grown wild-type cells. These results confirm that the *L. lactis* ETC leads to the reduction of oxygen. An indication of the fact that respiration is not growth phase dependent is the observation that in heme-supplemented early-exponential-phase wild-type cells, respiration is already maximal. Additionally, we have observed that heme-grown wild-type cells incubated with DCCD can still form a clear PMF, irrespective of the growth phase from which they were harvested. Therefore, we can conclude that a fully functional ETC is present in heme-grown wild-type cells throughout growth and is not limited to the late exponential or stationary phase.

In this work and that of others (13), it has been proposed that NADH is an important electron donor for the ETC, which explains the observation of mixed acid fermentation under respiratory conditions. Membrane vesicles prepared from wild-type cells grown with heme showed a greater-than-6.5-fold increase in oxygen consumption compared to wild-type cells grown without heme and CydAΔ cells grown with heme. Furthermore, this oxygen consumption was dependent on the reduced form of NAD (NADH). We have thus clearly demonstrated that NADH is a likely electron donor for the ETC in *L. lactis* and that a membrane-bound NADH dehydrogenase is present.

When NADH is added to membrane vesicles of heme-grown CydAΔ or non-heme-grown wild-type cells, there is still some oxygen consumed. Roughly 10% of this oxygen consumption can be attributed to a direct chemical reaction of NADH with oxygen. The rest of the observed NADH-dependent oxygen consumption in the control experiments can be attributed to the NADH oxidases that are known to be present in *L. lactis*. Although these NADH oxidases do not contain any membrane-spanning helices, they can be (loosely) associated with the membrane fraction (http://genome.ornl.gov/microbial/lcre/). This could explain the NADH-dependent, membrane-associated oxygen consumption seen in the membrane fractions from non-heme-grown wild-type cells or heme-grown CydAΔ cells.

Since in the dairy environment, little or no heme or oxygen is present, respiration is not expected to contribute significantly to growth and metabolic conversion. Heme-dependent respiration is therefore most likely a trait that confers a significant selective advantage in the original habitat of *L. lactis*: the plant surface or phyllosphere. An intriguing question, then, is the origin of the heme source in the phyllosphere. This and other questions concerning the respiratory capacities of LAB remain and promise increased scientific insight and novel industrial applications.

The definition of *L. lactis* as a facultative anaerobe seems not to be true in all situations (e.g., when heme and oxygen are present). In light of this study, a better definition of *L. lactis* would be a facultative aerobe. It is still largely unknown how many other LAB with a similar facultative aerobic metabolism exist. An extensive screening among the different species (pediococci, lactococci, and lactobacilli) is required to better define and characterize LAB as a group. Some information on the respiratory capabilities of a limited number of LAB, mostly streptococci and enterococci, can already be found in the lit-
erule (41, 42, 45, 56, 59, 60). Interestingly, analysis of the large 3.3-Mb genome of Lactobacillus plantarum WCF5 revealed the presence of genes coding for a fumarate reductase and heme-dependent nitrate reductase complex, creating a branched ETC capable of oxygen and nitrogen respiration (18, 38). This would point to more possibilities for electron transfer and energy conservation in Lactobacillus plantarum than in L. lactis. The future exploitation of the respiratory capacities of LAB could result in improved industrially important traits (higher biomass/gram carbon source, increased resistance to acid stress and oxygen stress, and increased survival rate when stored at low temperatures), making the organisms even more attractive as cell factories.

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REFERENCES


