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$_{3}$-dicyclohexylcarbodiimide, while a cytochrome bd-negative mutant strain (CydA$^{-}$/H9004) did not. We also observed high oxygen consumption rates by membrane vesicles prepared from heme-grown cells, compared to CydA$^{+}$ 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 (faculative) 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 (CydA$^{-}$/H9004) and a cytochrome negative mutant complemented with plasmid pIL253CydABC. Plasmid pIL253CydABC is a pIL253 derivative (46) carrying the cydABC genes (Cyd$^{-}$/H9262). 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 GM17 medium supplemented with heme (hemin) (stock solution, 0.5 mg/ml in 0.05 M NaOH; Sigma) 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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thiadicarbocyanine [DiSC 3(5)] was used to monitor the membrane potential in single laminar vesicles with an average size of 0.4 μm (50).

### 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 [GATG GTAACCACATCAACCAT] and reverse primer p40 [GGTTGATGTTTAT CTCG]) and a 1-kb fragment downstream of cydA (forward primer p41 [GTT GATGAAATGACTGOGA] and reverse primer p42 [CCACGATGACAA TAAACCTG]) were amplified using PCR. The flanking fragments were cloned blunt-ended into vector pNZ5317 (23) digested with Swal (upstream fragment) and Ecl3I (downstream fragment) to produce the knockout vector pRB6761_CydA_KO. The knockout plasmid was transformed into L. lactis MG1363, and a chloramphenicol replacement of the cydA gene was obtained by a double-crossover event by homologous recombination as described previously (53).

### 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), resuspended in the same buffer to an OD 600 of 5.0, and placed on ice. Prior to each measurement, the buffer was heated to 30°C, and the electrode was allowed to equilibrate for 10 min. At time zero, cells were added to a final concentration of an OD 600 of 0.4 to 0.48, early/mid-exponential phase (OD 600 of 1.04 to 1.08) and mid-exponential phase (OD 600 of 1.49 to 1.53) and after overnight growth (OD 600 of 4.49 for the wild type and 2.6 for CydAΔ). Cell samples were washed twice in 50 mM potassium phosphate (pH 5.0), and resuspended in the same buffer to an OD 600 of 5.0. Subsequently, samples were diluted to an OD 600 of 0.3, and N,N′-dicyclohexylcarbodiimide (DCCD; Sigma-Aldrich) (stock, 1 mM in ethanol) was added to a final concentration of 1 mM when indicated, or, as a control, the equivalent volume of ethanol was added. DCCD is a well-known inhibitor of the F1Fo ATPase and prevents PMF formation by the hydrolysis of ATP (15, 47). The samples were incubated for 45 min on ice in the presence of DCCD or ethanol. After incubation, 2 ml of fresh buffer was added to the 1-ml samples to optimize the cell density for measuring fluorescence, after which the samples were transferred into a 3-ml cuvette. Finally, a final concentration of 133 nM Cuvettes containing this mixture were warmed at 30°C, and the dissolved oxygen was measured at room temperature for 3 min prior to measuring. 15 mM glucose, 0.1 μM nigericin, or 2 μM valinomycin was added when indicated.

### Oxygen uptake measurements.

A biological oxygen monitor (model 5300; YSI Scientific, OH) with a Clark-type polarographic oxygen probe and a 15-ml sample chamber was used to measure dissolved oxygen. To measure oxygen consumption, cells were washed twice in 50 mM potassium phosphate (pH 5.0), resuspended in the same buffer to an OD 600 of 5.0, and placed on ice. Prior to each measurement, the buffer was heated to 30°C, and the electrode was allowed to equilibrate for 10 min. At time zero, cells were added to a final concentration of an OD 600 of 0.2. After 5 min, 13 mM glucose was added. The dissolved oxygen of air-saturated buffer was calibrated using air-saturated water. To measure oxygen consumption by membrane vesicles, 1 ml of membrane vesicle mixture was added to 10 ml 50 mM potassium phosphate (pH 5.0). After 5 min, either 5 mM NADH or NADH− was added (both obtained from Sigma-Aldrich).

### 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 suggests coregulation (28), these type II NADH dehydrogenases are always observed to function as separate, individual

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

<table>
<thead>
<tr>
<th>L. lactis MG1363 ORF</th>
<th>Homology to B. subtilis 168</th>
<th>% DNA identity/ % aa identity</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>llmg_1864 cydA</td>
<td></td>
<td>48/68</td>
<td>Cytochrome d ubiquinol oxidase subunit I (EC 1.10.3.–)</td>
</tr>
<tr>
<td>llmg_1883 cydB</td>
<td></td>
<td>43/62</td>
<td>Cytochrome d ubiquinol oxidase subunit II (EC 1.10.3.–)</td>
</tr>
<tr>
<td>llmg_1862 cydC</td>
<td></td>
<td>47/65</td>
<td>ABC transporter component CydC</td>
</tr>
<tr>
<td>llmg_1861 cydD</td>
<td></td>
<td>44/64</td>
<td>ABC transporter component CydD</td>
</tr>
</tbody>
</table>

* 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).

Wild-type and CydΔΔ cells were supplemented with heme and grown aerobically. During growth, samples of cells were harvested at early exponential phase (OD at 600 nm [OD600] of 0.4 to 0.48), early/mid-exponential phase (OD600 of 1.04 to 1.08) and mid-exponential phase (OD600 of 1.49 to 1.53) and after overnight growth (OD600 of 4.49 for the wild type and 2.6 for CydAΔ). Cell samples were washed twice in 50 mM KPi (pH 5.0) and resuspended in the same buffer to an OD600 of 5.0. Subsequently, samples were diluted to an OD600 of 0.3, and N,N′-dicyclohexylcarbodiimide (DCCD; Sigma-Aldrich) (stock, 1 mM in ethanol) was added to a final concentration of 1 mM when indicated, or, as a control, the equivalent volume of ethanol was added. DCCD is a well-known inhibitor of the F1Fo ATPase and prevents PMF formation by the hydrolysis of ATP (15, 47). The samples were incubated for 45 min on ice in the presence of DCCD or ethanol. After incubation, 2 ml of fresh buffer was added to the 1-ml samples to optimize the cell density for measuring fluorescence, after which the samples were transferred into a 3-ml cuvette. Finally, a final concentration of 133 nM Cuvettes containing this mixture were warmed at 30°C, and the dissolved oxygen was measured at room temperature for 3 min prior to measuring. 15 mM glucose, 0.1 μM nigericin, or 2 μM valinomycin was added when indicated.

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### Other analytical procedures.

Protein concentrations of membrane preparations were determined using the bicinchoninic acid protein assay reagent (Omninlabo Int., Breda, The Netherlands) (49).
polypeptides. The genes for the biosynthesis of riboflavin, the precursor of flavin adenine dinucleotide, can also be found, and the biosynthesis of this vitamin by the biosynthesis of riboflavin, the dehydrogenase complex, the menaquinol/menaquinone couple, and the lysis of ATP via F1-Fo ATPase.

L. lactis MG1363 also contains the gene for the biosynthesis of riboflavin. The genes for the biosynthesis of riboflavin have also been identified in Bacillus subtilis. The genes for the biosynthesis of riboflavin, the dehydrogenase complex, the menaquinol/menaquinone couple, and the lysis of ATP via F1-Fo ATPase.

L. lactis MG1363 experimentally verified in L. lactis MG1363 (6). Menaquinone production has been observed in many L. lactis strains (33), and menaquinone biosynthesis genes (menFDXBEC and preA-menA) were identified. Genes encoding a (mena)quinoloxidase have also been identified in the L. lactis MG1363 genome (cydABCD), which show a high degree of similarity to the well-characterized Bacillus subtilis strain 168 cyd genes (55, 57, 58). This type of cytochrome (bd type) is not considered to be an actual proton pump, as scalar chemistry alone could account for the stoichiometry of 1H+/e−, during the reduction of oxygen to water (16, 31, 39). Thus, all the genetic elements needed to form a functional ETC are present in the genome of MG1363, with the exception of a complete heme biosynthesis pathway (Fig. 1).

Growth improvement by heme supplementation. Wild-type L. lactis cells, when grown aerobically and supplemented with heme, showed increased biomass and less acidification of their medium (Table 3), suggesting the presence of a functional heme-grown wild-type cells. Subsequent complementation of the CydΔA mutant with a vector carrying the (mena)quinoloxidase coding operon (pIL253CydABCD) restored the wild-type-like phenotype when grown aerobically with heme.

Measurement of ΔΨ in whole cells of L. lactis. The formation of a membrane potential by L. lactis, as a result of electron transport, was determined with the fluorescent probe DiSC3(5). The intensity of fluorescence of the probe is sensitive to changes in the ΔΨ and its decrease with increasing ΔΨ and vice versa. The PMF is composed of ΔpH and ΔΨ. In order to estimate the contribution of a pH gradient to the PMF, we added nigericin (a K+/H+ exchanger) to convert the ΔpH into a ΔΨ. Furthermore, the addition of valinomycin (K+ ionophore) plus nigericin collapsed the ΔΨ completely. The main proton pump in L. lactis responsible for PMF generation is the F1-Fo ATPase, by pumping protons at the expense of metabolic ATP (26). To discriminate between PMF generation by the ETC and that by the F1-Fo ATPase, the F1-Fo ATPase-specific inhibitor DCCD was used (47). To further validate PMF formation via the ETC, we used a cytochrome bd-negative mutant (CydΔA) as a control.

The changes in membrane potential, DiSC3(5) fluorescence, were recorded as a function of time (Fig. 2). In wild-type cells and CydΔA cells with no DCCD treatment, the addition of glucose led to an increase in ΔΨ. However, for CydΔA cells incubated with DCCD, this increase in ΔΨ was negligible. The increase in ΔΨ 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...
nigericin resulted in an increase in $\Delta \Psi$. The gradual decrease of the $\Delta \Psi$, that is, upon the addition of nigericin to wild-type cells inhibited with DCCD and of Cyd$\Delta\Delta$ 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 $\Delta \Psi$ completely. The fluorescence measurements clearly show that the cytochrome-negative Cyd$\Delta\Delta$ strain is unable to generate a $\Delta \Psi$ when the $F_{1}-F_{0}$ ATPase is inhibited by DCCD. In contrast, wild-type heme-grown cells are able to build up $\Delta \Psi$ 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 phase (OD$_{600}$ of ~0.5, ~1.0, and ~1.5, respectively) and the late stationary phase (cultures grown overnight). A clear buildup of $\Delta \Psi$ was observed upon the addition of glucose to DCCD-treated and untreated wild-type cells and untreated Cyd$\Delta\Delta$ cells but never in DCCD-treated Cyd$\Delta\Delta$ cells, irrespective of the growth phase (Table 4). These results show that a functional ETC in wild-type cells is formed in all stages and not restricted to late exponential and stationary growth phases.

**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 Cyd$\Delta\Delta$ 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.

**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 Cyd$\Delta\Delta$ cells (with heme) was measured (Table 5). Membrane vesicles prepared from heme-grown wild-type cells showed a greater-than-6.5-fold increase

**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 Cyd$\Delta\Delta$ 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>-DCCD</td>
<td>+DCCD</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.48 (early)</td>
<td></td>
</tr>
<tr>
<td>Cyd$\Delta$</td>
<td>0.4 (early)</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>1.08 (early/mid)$^c$</td>
<td></td>
</tr>
<tr>
<td>Cyd$\Delta$</td>
<td>1.04 (early/mid)$^c$</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>1.53 (mid)</td>
<td></td>
</tr>
<tr>
<td>Cyd$\Delta$</td>
<td>1.49 (mid)</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>4.49 (O/N)$^c$</td>
<td></td>
</tr>
<tr>
<td>Cyd$\Delta$</td>
<td>2.6 (O/N)$^c$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$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.

$^b$These fluorescence traces are described in detail in the legend of Fig. 2.

$^c$Cultures grown overnight (O/N) were grown for more than 20 h and considered (late) stationary-phase cells.
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 NADH, 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-F0 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 observations demonstrate that heme-supplemented wild-type cells have an additional PMF-generating system besides the F1-F0 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-F0 ATPase by DCCD (23). In addition to the F1-F0 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).

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-

<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.
erature (41, 42, 45, 56, 59, 60). Interestingly, analysis of the large 3.3-Mb genome of Lactobacillus plantarum WCSS revealed the presence of genes coding for a furamate reductase and heme-dependent nitrate reductase complex, creating a branched ETC capable of oxygen and nitrate 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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