Lactobacillus plantarum WCFS1 Electron Transport Chains[▽]

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Lactobacillus plantarum WCFS1 requires both heme and menaquinone to induce respiration-like behavior under aerobic conditions. The addition of these compounds enhanced both biomass production, without progressive acidification, and the oxygen consumption rate. When both heme and menaquinone were present, *L. plantarum* WCFS1 was also able to reduce nitrate. The ability to reduce nitrate was severely inhibited by the glucose levels that are typically found in *L. plantarum* growth media (1 to 2% [vol/vol] glucose). In contrast, comparable mannitol levels did not inhibit the reduction of nitrate. *L. plantarum* reduced nitrate with concomitant formation of nitrite and ammonia. Genes that encode a *bd*-type cytochrome (*cydABCD*) and a nitrate reductase (*narGHJI*) were identified in the genome of *L. plantarum*. The *narGHJI* operon is part of a cluster of genes that includes the molybdopterin cofactor biosynthesis genes and *narK*. Besides a menaquinone source, isogenic mutants revealed that *cydA* and *ndh1* are required for the aerobic-respiration-like response and *narG* for nitrate reduction. The *ndh1* mutant was still able to reduce nitrate. The existence of a nonredundant branched electron transport chain in *L. plantarum* WCFS1 that is capable of using oxygen or nitrate as a terminal electron acceptor is proposed.

Lactic acid bacteria (LAB) are extensively used for the production of fermented foods from dairy, meat, fruit, and vegetable sources. These fermented foods are valued for their enhanced shelf life, flavor, and structural properties. LAB have been exploited for this purpose for millennia and generally behave as facultative anaerobic, obligate fermentative bacteria.

However, the production of cytochromes, typical constituents of respiratory chains, has been observed in several LAB species when they are grown in the presence of heme. These include *Lactococcus lactis* (*Streptococcus lactis*), *Leuconostoc mesenteroides*, and *Enterococcus faecalis* (36, 42).

Recently, in *L. lactis*, generation of a proton motive force by a heme-dependent aerobic electron transport chain was demonstrated (9). In other words, heme induces respiration in *L. lactis*. *L. lactis* cells grown under these respiration-permissive conditions have enhanced biomass yields and are more robust (more resistant to oxygen, acid, and cold-storage stress) (15, 18, 31). Respiration-like behavior has also been reported for *Streptococcus agalactiae* and *Oenococcus oeni* (43; A. Gruss, unpublished results). However, there have been no published reports of heme-induced respiration-like behavior in any member of the genus *Lactobacillus*. This genus contains many species that are used extensively in food fermentation, such as *Lactobacillus plantarum*.

L. plantarum has been isolated from the human gastrointestinal tract and plant surfaces. It is an economically important starter culture bacterium, to initiate food fermentation, and certain strains are even sold as probiotics (2, 3, 13, 40). Improvements in the efficiency of biomass formation and robustness, which are associated with respiration in *L. lactis*, are desirable traits for starter cultures, as well as probiotics.

In this work, we investigated whether functional electron transport chains are present in *L. plantarum*. We analyzed the genome for components of electron transport chains and investigated the ability of *L. plantarum* to exploit extracellular electron acceptors.

MATERIALS AND METHODS

Cultures and growth conditions. The four strains used in this study were *L. plantarum* WCFS1 (an isolate from NCIMB8826) and the three *NarGΔ*, *Ndh1Δ*, and *CydAΔ* isogenic strains. These *L. plantarum* strains were cultivated on MRS broth (Difco) or chemically defined media (38). When indicated, citrate and acetate were omitted, glucose titrated, or replaced by mannitol. The isogenic mutants were grown in the presence of 5 μg/ml chloramphenicol. For the induction of nitrate reductase activity, heme (heme or hemin) was added to a final concentration of 2.5 μg/ml (0.5-mg/ml stock in 0.05 M NaOH; Sigma-Aldrich), vitamin K₂ (or menaquinone 4) to a final concentration of 1 μg/ml (2-mg/ml stock in ethanol; Sigma-Aldrich), and NaNO₃ (Sigma-Aldrich) to various concentrations. For nitrite reduction assays, NaNO₂ (Sigma-Aldrich) was added to a final concentration of 500 mg/liter. Cultures were grown anaerobically under an N₂ atmosphere at 37°C.

Escherichia coli (strain E10) was used as the host for constructing plasmids and was cultivated aerobically at 37°C on TYB medium (Difco) with 10 μg/ml chloramphenicol and/or 10 μg/ml erythromycin, as indicated.

pH-controlled batch fermentation. pH-controlled (pH 5.5; 37°C) batch fermentations were performed with modified MRS broth: 10 mM glucose and no sodium acetate or ammonium citrate. Heme, vitamin K₂, or ethanol was added as indicated. One-liter fermentors were filled with 500 ml medium, and the headspaces were flushed with 20 ml/min nitrogen gas.

Mutant construction. Molecular cloning techniques were carried out in accordance with standard laboratory procedures (34). For the construction of a mutant lacking a functional *narG* (*NarGΔ*), the plasmid pNZ5319_*NarG_KO* was constructed, derived from plasmid pNZ5319. Plasmid pNZ5319 was specifically developed for the creation of double-crossover replacement mutants (23). The plasmid pNZ5319_*NarG_KO* contains two ~1-kb regions that are identical to DNA sequences that flank *narG* on the chromosome. These flanking regions, one upstream and one downstream of *narG*, were amplified with PCR techniques using genomic *L. plantarum* WCFS1 DNA as a template (Table 1 lists the primers used). The amplified DNA fragments were cloned, using *Escherichia coli* as the host strain, and blunt ended in vector pNZ5319 digested with *Swa*I (upstream fragment) and *Ecl*36II (downstream fragment) to produce the knockout vector pNZ5319_*NarG_KO*. The knockout plasmid was subsequently trans-

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TABLE 1. Primers used in this study

PCR amplification 1 kb:	Forward primer		Reverse primer	
	Name	Sequence	Name	Sequence
Upstream of <i>ndh1</i>	P93	GGCAAATCAGCATAGTGTTCCTG	P92	GGCTCGCATCCCTGCGTAAC
Downstream of <i>ndh1</i>	P94	GGAACGGTCTTCAGTAAAGG	P95	CGCATCAATGAATCAGTCCATG
Upstream of <i>narG</i>	P101	CCAGTCAGTAATAGCTGCTAA	P100	CGATAAGACCTCCTTTATCAC
Downstream of <i>narG</i>	P102	CGGAAGTTAAAGAAGGTGAAC	P103	CGAATTCTGAGCAGCTTCCA
Upstream of <i>cydA</i>	P111	GCTGAATCGGTCGTTGATTAG	P110	CGCCAAGGATAAAATACTTAATC
Downstream of <i>cydA</i>	P112	GCGTTCAGTCATGAGTAATCT	P113	CGCACCAGTGATACTGTCGT

formed in *L. plantarum* WCFS1. A chloramphenicol replacement of *narG* by a double-crossover event was isolated: NarGΔ (Cm^r Ery^r). This procedure for construction of the *narG* mutant follows the general procedures described previously (23). The isogenic mutants lacking a functional *ndh1* or *cydA* were constructed in a similar process using primers described in Table 1.

Analytical determinations. Nitrate and nitrite were determined by photometric endpoint determination, using the nitrite/nitrate colorimetric method and Nitrate NO₃⁻ kits (Roche Diagnostics GmbH, Mannheim, Germany), as described previously (4, 6, 22).

Nitrate reduction was further confirmed via high-pressure liquid chromatography. Ions were separated on a Dionex column (Ionpac AS9-SC) with an eluent consisting of 1.8 mM Na₂CO₃ and 1.7 mM NaHCO₃ at a flow rate of 1 ml/min at room temperature. The anions were detected with suppressed conductivity. Ammonia was quantified using the standard glutamate dehydrogenase-based method (Roche Diagnostics GmbH, Mannheim, Germany).

HPLC analyses. Organic acids were also measured by high-performance liquid chromatography (HPLC) techniques (37). Sugars were analyzed by HPLC using a chromatographic system consisting of a precolumn packed with a cation-exchange resin, AG50W-X4, 400 mesh (Bio-Rad, Hercules, CA), and AG3-X4A, 200/400 mesh (35:65; Bio-Rad), and a cation exchanger in a prepacked column (RT 300-7.8 Polyspher CHPb; 300 by 7.8 mm; Merck, Darmstadt, Germany). The samples were eluted with an isocratic pump system (Shimadzu Corporation, Kyoto, Japan) using water as the mobile phase. Detection was carried out using a refractive index detector, ERC-7512 (Erma). Organic acids were analyzed by HPLC using a chromatographic system consisting of a cation-exchange column (Rezex ROA-Organic Acid H+ [8%]; 300 by 7.8 mm; Phenomenex), and the samples were eluted with an isocratic pump system (Shimadzu Corporation, Kyoto, Japan) using 0.005 M H₂SO₄ as the mobile phase. Detection was carried out using a refractive index detector, ERC-7512 (Erma), and by spectrophotometrically measuring UV light absorbance at 210 nm and 290 nm (Spectra Physics UV1000).

Oxygen uptake measurements. A biological oxygen monitor (YSI model 5300; YSI Scientific, OH) with a Clark-type polarographic oxygen probe and a 15-ml sample chamber were used to measure dissolved oxygen at room temperature (20°C). Cells were washed three times in 50 mM potassium phosphate (pH 5.0) prior to each measurement. The dissolved oxygen of air-saturated buffer was calibrated using air-saturated water. The electrode was allowed to equilibrate in air-saturated buffer for 5 min before the start of each experiment. After this equilibration, cells were added to a final optical density at 600 nm (OD₆₀₀) of 1.0. After approximately 5 min, glucose was added to a final concentration of 13 mM, and the decrease in oxygen levels was followed for 10 min. At the end of this analysis, 10 ml of the cell suspension from the chamber was used for dry-weight determination.

RESULTS

The *L. plantarum* WCFS1 aerobic electron transport chain. A *cydABCD* operon that encodes a *bd*-type cytochrome was found in the genome of *L. plantarum* WCFS1. However, supplementation with heme alone did not result in a respiration-like phenotype (increase in biomass) in aerobic non-pH-controlled batch cultures. This supplementation with heme did induce catalase (peroxidase) activity (data not shown), as reported for other LAB (1).

Apparently, heme-cultivated *L. plantarum* still lacks components essential for restoration of the aerobic electron transport

chain. Indeed, the genome of *L. plantarum* lacks a complete (mena)quinone biosynthesis gene set. Menaquinones are common membrane-integral components of bacterial electron transport chains (39).

When aerobic cultures of *L. plantarum* were supplemented with both heme and a menaquinone source, in the form of vitamin K₂, and incubated for 48 h, a greater biomass and higher final pH were observed (Table 2). The higher final pH, induced by the combined addition of heme and menaquinone, coincided with a pronounced shift from lactic to acetic acid production (data not shown). This metabolic shift clearly indicated that a more oxidative metabolism had occurred.

Loss of heme and menaquinone stimulation in *CydAΔ* and *Ndh1Δ* strains. The aerobic branch of the *L. lactis* electron transport chain terminates in a *cydABCD*-encoded *bd*-type cytochrome (9, 15). A mutant of *L. plantarum* WCFS1 in which the *cydA* gene was replaced by an antibiotic resistance marker lost the heme- and menaquinone-induced phenotype (Table 2). Furthermore, in the genome of *L. plantarum*, two putative NADH dehydrogenase genes (*ndh1* and *ndh2*) have been annotated. As aerobic electron transport chains canonically use NADH as an electron donor, these genes are likely candidates to encode this activity. The predicted *ndh1* gene product has no membrane-spanning helices, although it lies in an operon-like structure upstream of a gene encoding an integral membrane protein, with which it may be associated (Fig. 1). The acetate kinase gene (*ack2*) is positioned upstream of *ndh1*. Activation of the aerobic electron transport chain induced a higher metabolite flux toward acetate. We constructed a mutant lacking a complete *ndh1* gene, which also failed to be stimulated by heme and menaquinone (Table 2).

The final biomass of the two isogenic mutants (*CydAΔ* and *Ndh1Δ*) at most equaled that of unsupplemented wild-type

TABLE 2. Aerobic phenotypes of *L. plantarum* WCFS1 wild-type and *CydAΔ* and *Ndh1Δ* isogenic mutant cells

Cell	Addition ^a		OD ₆₀₀ ^b	pH ^b	O ₂ consumption (mg O ₂ /g [dry wt]/min) ^b
	Heme	K ₂			
Wild type	+	+	9.88 ± 0.25	4.51 ± <0.01	36.4 ± 3.1
Wild type	+	–	6.75 ± 0.22	3.96 ± 0.01	ND ^c
Wild type	–	+	7.55 ± 0.11	3.96 ± 0.01	ND
Wild type	–	–	7.04 ± 0.08	3.96 ± 0.01	12.2 ± 0.7
<i>CydAΔ</i>	+	+	5.56 ± 0.17	3.94 ± 0.02	8.9 ± 1.2
<i>CydAΔ</i>	–	–	5.69 ± 0.13	3.96 ± 0.01	ND
<i>Ndh1Δ</i>	+	+	7.22 ± 0.2	3.96 ± 0.01	15.2 ± 0.4
<i>Ndh1Δ</i>	–	–	6.79 ± 0.1	3.95 ± <0.01	ND

^a +, added; –, not added.

^b The values are averages ± standard deviations.

^c ND, not done.

FIG. 1. Genomic region of *ndh1* and *ack2.ndh1* (NADH dehydrogenase) forms part of an operon-like structure with a transcription factor (c) and an integral membrane protein (d) that neighbors the acetate kinase gene (*ack2*). *ack2* itself is flanked by another integral membrane protein (a) and a putative acetyl transferase (b).

cells, while their final pH was unaffected by supplementation. Furthermore, heme and menaquinone addition approximately tripled the oxygen consumption rate of wild-type cells (Table 2). Again, heme and menaquinone addition did not induce a comparable change in phenotype of the *CydAΔ* and *Ndh1Δ* isogenic mutants.

The *L. plantarum* WCFS1 genome codes for nitrate reductase genes. Genes that encode a putative nitrate-reductase complex (*narGHJI*) were identified in the genome of *L. plantarum* WCFS1 (21). The homologous genes in *Bacillus subtilis* and *E. coli* encode a membrane-bound nitrate reductase that permits anaerobic nitrate respiration. This active nitrate reductase A contains several cofactors: a heme moiety, a molybdenum-pterin cofactor, and iron-sulfur clusters. Although *L. plantarum* WCFS1 does not produce heme, molybdopterin cofactor biosynthesis genes (*moaABEDA*, *mobAB*, and *moeAB*) were found in close proximity to the *narGHJI* operon (Fig. 2). In addition, genes coding for an iron transport complex (*fecBED*), a nitrite extrusion protein (*narK*), and a kinase/response regulator system (*rrp4* and *hpk4*) were found in its vicinity (8, 35). The gene product of *narK* may also promote nitrate import into the cytoplasm, in addition to its role in nitrite excretion (10).

Growth conditions that allow nitrate reduction. Anaerobic overnight cultivation of *L. plantarum* WCFS1 in standard MRS broth supplemented with nitrate did not lead to production of detectable amounts of nitrite. Indeed, as mentioned above, the *narGHJI* operon is predicted to encode a nitrate reductase A type that in other microorganisms is associated with electron transport (7, 17). Reconstitution of the *L. plantarum* electron transport chain by the addition of heme and menaquinone (vitamin K_2) did lead to an observable, but low, level of nitrite production. When the glucose levels (normally present at 20 g/liter [110 mM] in MRS broth) were reduced, however, nitrite production was stimulated (Fig. 3).

The requirement for both heme and menaquinone (vitamin K_2) in order to induce nitrite production was verified in chemically defined medium with 10 mM glucose. When heme or menaquinone alone was added to the culture medium, only minimal amounts of nitrite were formed ($26 \pm 1.3 \mu\text{M}$). Addition of both compounds increased nitrite formation by 25-

FIG. 3. Nitrate reduction and nitrite formation by *L. plantarum* WCFS1 in the presence of glucose. The bars indicate the average values $\pm$ the standard deviation. Wild-type cells were grown overnight in anaerobic batch cultures in modified MRS broth. Acetate and citrate were omitted from the standard MRS broth recipe. This medium was supplemented with heme, menaquinone, 16 mM nitrate, and various concentrations of glucose (glu): 0, 5, 10, 20, and 40 mM.

fold to $728 \pm 77.3 \mu\text{M}$. Next, to demonstrate the requirement for the *narGHJI* operon in order to reduce nitrate, a *narG* knockout mutant (*NarGΔ*) was constructed. In this case, no nitrite production by the *NarGΔ* strain could be measured when it was grown in the presence of heme and menaquinone. As mentioned above, traces of nitrite were still produced by the wild-type cells when either heme or menaquinone was omitted. Clearly, the nitrate reductase complex has a much reduced activity, but it still retains some residual activity even without these cofactors.

Finally, we tested whether the *ndh1* gene product, required for the activity of the aerobic electron transport chain, is also involved in the ability of cells to use nitrate as a redox sink. Using anaerobic batch conditions that stimulated nitrite formation in the wild type, we observed that the *Ndh1Δ* mutant still produced high levels of nitrite (at about 67% of wild-type levels).

Effect of mannitol on nitrate reductase activity. As shown above, the presence of high glucose levels resulted in a clearly decreased level of (anaerobic) nitrate reduction (Fig. 3). In contrast, high concentrations of the reduced sugar mannitol did not seem to repress nitrate reduction (Fig. 4). Anaerobic growth with the reduced sugar mannitol as the main carbon source requires a proportional concentration of an electron acceptor (27). Standard MRS broth contains citrate, which can be used for this purpose. Extensive anaerobic growth of *L. plantarum* in MRS broth (in which glucose was replaced with mannitol) required the presence of citrate or a combination of heme, vitamin K_2 , nitrate, and a functional *narG* gene (Table 3). In addition, these results indicate that the residual nitrate reductase activity observed in the presence of only heme or menaquinone does not sustain anaerobic growth on mannitol.

FIG. 2. The genomic region upstream of the *nar* operon (*narGHJI*) in *L. plantarum* WCFS1. Genes coding for molybdopterin cofactor biosynthesis (*moaABEDA*, *mobAB*, and *moeAB*) (dark grey), for iron transport (*fecBED* and *narGHJI*) (light grey), and for nitrite extrusion (*narK*) and a kinase/response regulator system (*rrp4* and *hpk4*) (black) are found in this region. Putative genes are indicated by white arrows.

FIG. 4. Nitrate reduction and nitrite formation by *L. plantarum* WCFS1 in the presence of mannitol. The bars indicate the average values $\pm$ standard deviations. Wild-type cells were grown overnight in anaerobic batch cultures in MRS broth from which acetate, citrate, and glucose were omitted (modified MRS broth). This medium was further supplemented with heme, menaquinone, nitrate, and various concentrations of mannitol (man) (in mM). Nitrate reduction is not repressed at high mannitol concentrations.

Thus, only the fully reconstituted anaerobic electron transport chain of *L. plantarum* is able to use nitrate as an electron sink.

At higher mannitol concentrations (10 mM to 40 mM), progressively less nitrite was formed, while nitrate consumption remained relatively stable. As growth on mannitol requires an electron sink, it appears that nitrite can be actively consumed as an electron sink, as well (Fig. 4). We have experimentally verified that *L. plantarum* cells actively remove nitrite and that the contribution of chemical degradation to this removal is minimal. Furthermore, during pH-controlled anaerobic growth on 40 mM mannitol with nitrate as a redox sink, nitrite was mostly transient, and high levels of ammonia were formed (10.7 ± 1.03 mM). In comparison, only 1.35 ± 0.63 mM of ammonia was produced when citrate was supplied as a redox sink. The reduction of nitrite appears not to be negatively affected by a high glucose concentration and, in contrast to the reduction of nitrate, does not require heme, menaquinone, or a functional *narG* (data not shown).

DISCUSSION

L. plantarum, as a typical LAB, has traditionally been considered an obligate fermenter. Here, we propose that *L. plantarum* in fact contains a branched electron transport chain capable of using oxygen or nitrate as an extracellular electron acceptor. The genome of *L. plantarum* WCFS1 indeed contains the *cydABCD* genes, encoding a *bd*-type cytochrome, and the *narGHJI* genes, encoding a nitrate reductase A (21). The electron transport chain requires acti-

FIG. 5. Proposed branched electron transport chain of *L. plantarum* WCFS1 that terminates in either a nitrate reductase A or a *bd*-type cytochrome complex. The dashed arrows represent the extracellular origin of menaquinone (vitamin K₂) and heme. Red. sub., reduced substrate; Oxid. sub., oxidized substrate; mQ, menaquinone; mQH₂, menaquinol.

vation by the addition of the heme cofactor and a menaquinone pool in the form of vitamin K₂.

The proposed aerobic electron transport chain is simple and nonredundant and consists of an NADH dehydrogenase (Ndh1), a menaquinone pool (vitamin K₂), and a *bd*-type cytochrome (Fig. 5). We also propose a simple structure of the nitrate-reducing chain, consisting of at least three parts, a dehydrogenase, a menaquinol pool, and the nitrate reductase complex. As the Ndh1Δ mutant was still able to reduce nitrate, another dehydrogenase must be involved in menaquinone reduction in the anaerobic branch. Anaerobic growth on mannitol as the main carbon source requires the presence of a proportional amount of an extracellular electron acceptor (27). The putative candidate for the dehydrogenase of the anaerobic electron transport chain is therefore likely to oxidize (indirectly) one of the intermediates of primary metabolism. In any case, to our knowledge, this is the first report of nitrate reduction by a LAB that is associated with an electron transport chain.

The *bd*-type cytochrome consists of two subunits (CydA and CydB). CydC and CydD are required for assembly of the oxidase (12). This *bd*-type cytochrome is found in various (facultative) aerobic bacteria (19), where it functions as a proton motive force generating an (alternative) terminal electron acceptor. Its ability to generate a proton motive force and to permit respiration has also been shown in *L. lactis* MG1363 (9). This cytochrome is capable of working (and being activated) particularly under low-oxygen conditions (20, 33). Activation of this aerobic electron transport chain leads to a significant increase in biomass formation in non-pH-controlled batch cultures of *L. plantarum* WCFS1.

The second branch of the electron transport chain is active under anaerobic conditions and terminates in a nitrate reductase A complex. This type of nitrate reductase A has been extensively studied in *E. coli*. It consists of three subunits (α , β , and γ) encoded by *narG*, *narH*, and *narI*, respectively. The α and β subunits form a complex that contains a molybdenum-pterin cofactor and four iron-sulfur clusters (5, 26). The *narI* subunit contains two heme moieties, provides binding to the membrane, and interacts with the quinone pool (32, 44). *narJ* is required for full activity of the membrane-bound nitrate reductase (14). It has an established function in an anaerobic electron transport chain and generates a proton motive force (24, 39, 41, 45). The nitrate reductase complex and the *bd*-type cytochrome of *E. coli* are assumed not to be actual proton

TABLE 3. Residual nitrate concentration and biomass of *L. plantarum* WCFS1 wild type and NarGΔ grown anaerobically with mannitol as a carbon source

Cell	Addition(s)	Residual NO ₃ (mM) ^{a,b}	Biomass ^a (OD ₆₀₀)
Wild type	Heme, K ₂ , NO ₃	10.12 $\pm$ 0.82	3.88 $\pm$ 0.51
Wild type	Heme, NO ₃	16.69 $\pm$ 1.42	0.88 $\pm$ 0.02
Wild type	K ₂ , NO ₃	18.94 $\pm$ 1.59	0.85 $\pm$ 0.03
Wild type	Heme, K ₂	0.46 $\pm$ 0.46	0.95 $\pm$ 0.02
NarGΔ	Heme, K ₂ , NO ₃	19.34 $\pm$ 0.53	0.91 $\pm$ 0.02

^a The values are averages $\pm$ standard deviations.

^b 20 mM of nitrate was used as the starting concentration.

pumps themselves, since scalar chemistry alone could account for the $2\text{H}^+/\text{NO}_3^-$ and $2\text{H}^+/\text{O}_2$ translocation efficiencies (17, 29).

We have shown conclusively that heme and menaquinone can reconstitute an active aerobic and anaerobic electron transport chain in *L. plantarum*. Whether a proton motive force can be formed via these, and possibly other, electron transport chains and thus enable a respiratory metabolism will form the subject of further research.

As shown here, high mannitol levels do not inhibit nitrate reductase activity, in contrast to high glucose levels. This specific sensitivity to glucose suggests an active glucose-induced repression. The location of the response regulator and the sensor protein (*rrp4* and *hpk4*) that are found on the genome in close proximity to the *nar* genes makes them promising candidates to be involved in transcriptional regulation of the nitrate reductase genes.

The use of either oxygen or nitrate as an extracellular electron acceptor by *L. plantarum* only in the presence of heme and menaquinone may seem to be an artificially induced phenotype. However, these compounds are also present in more "natural" environments, such as plant- and meat-based food fermentation and the human gastrointestinal (GI) tract. First, nitrate is a natural compound found in green plants and drinking water and is also used as a curing salt in meat fermentation (16, 25, 28). Second, live *L. plantarum* cells, present in many fermented food products, are consumed by humans. Third, heme, as a component of cytochromes, is present in all meat and plant products that are consumed. Finally, menaquinones are produced in the GI tract by the indigenous microflora (11). The combination of nitrate (and perhaps also oxygen), live *Lactobacillus* spp., heme, and menaquinones in the human GI tract, for example, may be quite common.

It would be extremely interesting, and relevant, to study the positive or negative influence of nitrate reduction on the (probiotic) effect of *Lactobacillus* strains on human health. Furthermore, as in the case of *L. lactis*, exploitation of the aerobic-respiration-like phenotype for commercial purposes, such as the starter culture industry, appears likely (30; P. Duwat, A. Gruss, Y. Le Loir, and P. Gaudu, 1999, French patent application FR2798670).

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