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The electron transport chains of *Lactobacillus plantarum* WCFS1

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Abstract

Lactobacillus plantarum WCFS1 requires both heme and menaquinone to induce a respiration-like behavior under aerobic conditions. 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 (1-2% v/v 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 include 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 non-redundant branched electron transport chain in *L. plantarum* WCFS1 is proposed that is capable of using oxygen or nitrate as terminal electron acceptor.

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Introduction

Lactic acid bacteria (LAB) are extensively used for 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, production of cytochromes, typical constituents of respiratory chains, has been observed in several LAB species, when grown in the presence of heme. These include *Lactococcus lactis* (*Streptococcus lactis*), *Leuconostoc mesenteroides* and *Enterococcus faecalis* (37, 43).

Recently in *Lactococcus 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 *Lactococcus lactis*. *Lactococcus lactis* cells grown in these respiration-permissive conditions have enhanced biomass yields and are more robust (more resistant to oxygen-, acid- and cold-storage stress) (16, 19, 32). Respiratory-like behavior has also been reported for *Streptococcus agalactiae* and *Oenococcus oeni* (A. Gruss, unpublished results) (43, 44). However, no publications exist, to date, of heme-induced respiration-like behavior in any member of the genus *Lactobacillus*. This genus contains many species that are used extensively in food fermentations, such as *Lactobacillus plantarum*.

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L. plantarum has been isolated from the human gastrointestinal tract and plant surfaces. *L. plantarum* is an economically important starter-culture bacterium, to initiate food fermentations, and (certain strains) is even merchandised as a probiotic (2, 3, 13, 41). Improvements in the efficiency of biomass formation and robustness, which are associated with respiration in *Lactococcus lactis*, are desirable traits for starter cultures as well as probiotics.

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

Materials and Methods

Cultures and growth conditions

The four strains used in this study are *Lactobacillus plantarum* WCFS1, an isolate from NCIMB8826, and the three isogenic strains NarGΔ, Ndh1Δ and CydAΔ. These *L. plantarum* strains were cultivated on MRS broth (Difco) or chemically defined media (CDM) (39). When mentioned citrate, 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 conc. of 2.5 µg/ml (stock 0.5 mg/ml in 0.05 M NaOH Sigma-Aldrich), vitamin K_2 (or menaquinone 4)

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to a final concentration of 1 µg/ml (stock 2mg/ml in ethanol Sigma-Aldrich) and $NaNO_3$ (Sigma-Aldrich) to various concentrations. For nitrite-reduction assays, $NaNO_2$ (Sigma-Aldrich) was added to a final concentration of 500 mg/L. Cultures were grown anaerobic under N_2-atmosphere at 37˚C.

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

pH-controlled batch fermentation

pH-controlled (pH 5.5, 37˚C) batch fermentations were performed with modified MRS-broth: 10mM glucose and no sodium acetate or ammonium citrate. Heme, vitamin K_2 or ethanol, were added when mentioned. 1L fermentors were filled with 500ml medium and the headspace flushed with 20ml/min nitrogen gas.

Mutant construction

Molecular cloning techniques were carried out in accordance with standard laboratory procedures (35). For 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 (24). The plasmid pNZ5319_NarG_KO contains two ~1kb regions that are identical to DNA sequences that flank *narG*,

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on the chromosome. These flanking regions, 1 upstream and 1 downstream of *narG*, were amplified with PCR-techniques and using genomic *L. plantarum* WCFS1 DNA as template (for primers used see table 1). The amplified DNA fragments were cloned, using *Escherichia coli* as host strain, blunt-ended in vector pNZ5319 digested with SwaI (upstream fragment) and Ecl36II (downstream fragment) to produce the knock-out vector pNZ5319_NarG_KO. The knock-out plasmid was subsequently transformed in *L. plantarum* WCFS1. A chloramphenicol replacement of *narG* by a double crossover event was isolated: NarGΔ (Cm^r, Ery^s). This procedure for construction of the *narG* mutant follows the general procedures as described (31). 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 NO_3^-" kits (Roche Diagnostics GmbH, Mannheim Germany) as described (4, 6, 23). 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 of 1 ml/min at room temperature. The anions were detected with suppressed conductivity. Ammonia

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was quantified using the standard glutamate dehydrogenase-based method. (Roche Diagnostics GmbH, Mannheim, Germany).

HPLC analyses

Organic acids were also measured by HPLC techniques (38). 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 (in a proportion of 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 cation exhange column (Rezex ROA-Organic Acid H+ (8%) 300*7.8mm, Phenomenex) the samples were eluted with an isocratic pump system (Shimadzu Corporation, Kyoto, Japan) using 0.005M H2SO4 as the mobile phase. Detection was carried out using a refractive index detector, ERC-7512 (Erma), and spectrophotometrically measuring UV-light absorbance at 210nm and 290nm (Spectra Physics UV1000).

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Oxygen uptake measurements

A Biological Oxygen Monitor (YSI model 5300, YSI Scientific, Ohio, USA) with a Clark type polarographic oxygen probe and a 15 ml sample chamber were used to measure dissolved oxygen (DO) at room temperature (20 °C). Cells
165 were washed thrice in 50 mM potassium phosphate (pH 5.0) prior to each measurement. The DO of air-saturated buffer was calibrated using air-saturated water. The electrode was allowed to equilibrate, in air-saturated buffer before the start of each experiment for 5 min. After this equilibration, cells were added to a final conc. of OD_{600} of 1.0. After approximately 5 min, glucose was added to
170 a final conc. of 13mM and the decrease in oxygen-levels followed in time for 10 min. At the end of this analysis 10ml of the cell-suspension from the chamber was used for dry-weight determination.

Results

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The *L. plantarum* WCFS1 aerobic electron transport chain

A *cydABCD* operon that encodes a *bd*-type cytochrome was found in the genome of *Lactobacillus plantarum* WCFS1. However, supplementation with heme alone did not result in a respiratory-like phenotype (increase in biomass),
180 in aerobic non-pH controlled batch cultures. This supplementation with heme did

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induce catalase (peroxidase) activity (data not shown), as reported for other LAB (1).

Apparently, heme-cultivated *L. plantarum* still lacks essential components for restoration of the aerobic electron transport chain. Indeed the genome of *L. plantarum* lacks a complete (mena)quinones biosynthesis gene-set. Menaquinones are common membrane-integral components of bacterial electron transport chains (40).

When both heme and a menaquinone source, in the form of vitamin K_2, were supplemented to aerobic cultures of *L. plantarum*, incubated for 48 hours, a higher biomass and higher final pH was observed (Table 2). The higher final pH, induced by the combined addition of heme and menaquinone, coincides with a pronounced shift from lactic- to acetic acid production (data not shown). This metabolic shift clearly indicates that a more oxidative metabolism has occurred.

Loss of heme and menaquinone stimulation in CydAΔ and Ndh1Δ

The aerobic branch of the *Lactococcus lactis* electron transport chain terminates in a, *cydABCD* encoded, *bd* –type cytochrome (9, 16). A mutant of *L. plantarum* WCFS1, of 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 are annotated (*ndh1* and *ndh2*). As aerobic electron transport chains canonically use NADH as electron donor, these genes are likely

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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 (d), with which it may be associated (Fig 1). The acetate kinase gene (*ack2*) is positioned up-stream of *ndh1*. Activation of the aerobic electron transport chain induced a higher metabolite flux toward acetate. We have 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 equals that of non-supplemented wild-type cells, while their final pH is 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.

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 (22). The homologues genes in *Bacillus subtilis* and *Escherichia 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,

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molybdopterin cofactor biosynthesis genes (*moaABEDA*,*mobAB*,*moeAB*) were found in close proximity of the *narGHJI* operon (Fig 2). In addition genes coding for an iron transport complex (*fecBED*), nitrite extrusion protein (*narK*) and a kinase/response regulator system (*rrp4*, *hpk4*) were found in its vicinity (8, 36). 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, the *narGHJI* operon is predicted to encode for a nitrate reductase A-type that, in other microorganisms, is associated with electron transport (7, 18). Reconstitution of the *L. plantarum* electron transport chain by addition of heme and menaquinone (vitamin K_2), did lead to an observable, but small, level of nitrite production. When the glucose levels (normally present in 20 g/L = 110 mM in MRS-broth) were reduced, however, nitrite production was stimulated (Fig 3).

The requirement for both heme and menaquinone (vitamin K_2), to induce nitrite production, was verified in chemically defined medium, with 10 mM glucose. When heme or menaquinone alone are added to the culture medium only minimal amounts of nitrite were formed (26 µM, stdev 1.3). Addition of both

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these compounds increased nitrite formation by 25-fold to 728 µM (stdev 77.3). Next, to demonstrate the requirement of the *narGHJI* operon to reduce nitrate, a *narG* knockout (NarGΔ) was constructed. In this case, no nitrite production could be measured by the NarGΔ when grown in the presence of heme and menaquinone. As mentioned, traces of nitrite were still produced by the wild-type cells when either heme or menaquinone were ommited. Clearly the nitrate reductase complex has a much reduced, but still retains some residual activity, even without these co-factors.

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 redox sink. Using anaerobic batch conditions that stimulate nitrite formation in the wild-type, we observed that ndh1Δ still produced high levels of nitrite (at about 67% of wild-type levels).

Effect of mannitol on nitrate-reductase activity

As shown, the presence of high glucose levels resulted in a clear 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 main carbon source requires a proportional concentration of an electron acceptor (28). 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

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

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

Discussion

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L. plantarum, as a typical lactic acid bacterium, has traditionally been considered an obligate fermentor. Here we propose that *L. plantarum* in fact contains a branched electron transport chain, capable of using oxygen or nitrate as 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 (22). The electron transport chain requires activation by addition of the heme-cofactor and a menaquinone-pool, in the form of vitamin K_2.

The proposed aerobic electron transport chain is simple, non-redundant and consists of a NADH-dehydrogenase (Ndh1), menaquinone pool (vitamin K_2) 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, menaquinol pool and the nitrate reductase complex. As the ndh1Δ was still able to reduce nitrate, another dehydrogenase must be involved in menaquinone reduction in the anaerobic branch. Anaerobic growth on mannitol as main carbon source requires a proportional presence of an extracellular electron acceptor (28). 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 lactic acid bacterium that is associated with an electron transport chain.

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The *bd*-type cytochrome consists of 2 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 (20), where it functions as, a proton motive force generating, (alternative) terminal electron acceptor. Its ability to generate a proton motive force, and permit respiration, has also been shown in *Lactococcus lactis* MG1363 (9). This cytochrome is capable of working (and activate) particularly under low-oxygen conditions (21, 34). 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 contain a molybdenum-pterin cofactor and 4 iron-sulfur clusters (5, 27). The *narI* subunit contains two heme moieties, provides binding to the membrane and interacts with the quinone pool (33, 45). *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 (25, 40, 42, 46). The nitrate-reductase complex and the *bd*-type cytochrome of *Escherichia coli* are assumed not to be actual proton pumps themselves, since scalar chemistry alone could account for the $2H^+/ NO_3$ and $2H^+/ O_2$ translocation efficiencies (18, 30).

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

The use of either oxygen or nitrate as extracellular electron acceptor by *L. plantarum* only in the presence of heme and menaquinone may seem an artificially induced phenotype. However, these compounds are also present in more "natural" environments, such as plant and meat -based food fermentations and in the human GI-tract. Firstly, nitrate is a natural compound found in green plants and drinking water and is also used as a curing salt in meat fermentations (17, 26, 29). Secondly, live *L. plantarum* cells, present in many fermented food products, are consumed by humans. Thirdly, heme, as a component of cytochromes, is present in all consumed meat and plant products. Lastly, menaquinones are produced in the GI-tract by the indigenous micro-flora (11).

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The combination of nitrate (or maybe also oxygen), live *Lactobacillus* sp., 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) effect of nitrate reduction on the (probiotic) effect of *Lactobacillus* strains on human health. Furthermore, as in the case of *Lactococcus lactis*, exploitation of the aerobic respiration-like phenotype for commercial purposes such as the starter-culture industry appears likely (15, 31).

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Table 1. Primers used in this study

PCR-amplify	Forward primer	Reverse primer
1kb upstream *ndh1*	P93: GGCAAATCAGCATAGTGTTCCTG	P92: GGCTCGCATCCCTGCGTAAC
1kb downstream *ndh1*	P94: GGAACGGTCTTCAGTAAAGG	P95: CGCATCAATGAATCAGTCCATG
1kb upstream *narG*	P101: CCAGTCAGTAATAGCTGCTAA	P100: CGATAAGACCTCCTTTATCAC
1kb downstream *nar*G	P102: CGGAAGTTAAAGAAGGTGAAC	P103: CGAATTCTGAGCAGCTTCCA
1kb upstream *cydA*	P111: GCTGAATCGGTCGTTGATTTAG	P110: CGCCAAGGATAAAATACTTAATC
1kb downstream *cydA*	P112: GCGTTCAGTCATGAGTAATCT	P113: CGCACCAGTGATACTGTCGT

530 **Table 2.** Aerobic phenotype of *L. plantarum* WCFS1 wild-type and CydAΔ and Ndh1Δ isogenic mutant cells

Cells	addition		OD_{600}	pH	O_2-consumption (mg O_2/gDW/min)
	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	n.d.[a]
Wild-type	-	+	7.55 ±0.11	3.96 ±0.01	n.d.
Wild-type	-	-	7.04 ±0.08	3.96 ±0.01	12.2 ± 0.7
CydAΔ	+	+	5.56 ±0.17	3.94 ±0.02	8.9 ± 1.2
CydAΔ	-	-	5.69 ±0.13	3.96 ±0.01	n.d.
Ndh1Δ	+	+	7.22 ±0.2	3.96 ±0.01	15.2 ± 0.4
Ndh1Δ	-	-	6.79 ±0.1	3.95 ±<0.01	n.d.

[a] The values represent averages ± the standard deviation, n.d. indicates not 535 done.

540 **Table 3.** Residual nitrate concentration and biomass of *L. plantarum* WCFS1 wild-type and NarGΔ, grown anaerobically with mannitol as carbon source.

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Cells	additions		Residual NO_3[b] (mM)		Biomass (OD600)	
wild-type	heme	K_2 NO_3	10.12	±0.82	3.88	±0.51
wild-type	heme	- NO_3	16.69	±1.42	0.88	±0.02
wild-type	-	K_2 NO_3	18.94	±1.59	0.85	±0.03
wild-type	heme	K_2 -	0.46	±0.46	0.95	±0.02
NarGΔ	heme	K_2 NO_3	19.34	±0.53	0.91	±0.02

545 [a] The values represent averages ± the standard deviation.

[b] 20mM of nitrate was used as starting concentration

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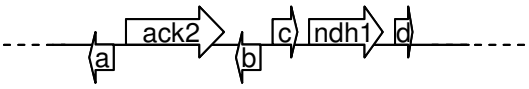

555 **Figure 1.** The genomic region of *ndh1* and *ack2*. *ndh1* (NADH-dehydrogenase) forms part of an operon-like structure with a transcription factor (c) and an integral membrane protein (d) that neighbors the acetate kinase gene (*ack2*). *ack2* itself is flanked by another integral membrane protein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

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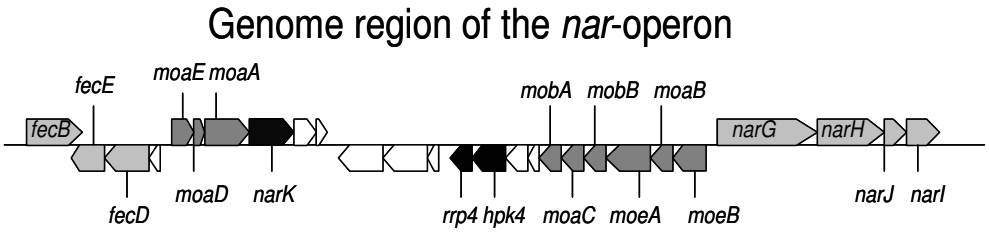

565 **Figure 2.** The genomic region upstream of the *nar*-operon (*narGHJI*) in *Lactobacillus plantarum* WCFS1. Genes coding for molybdo-pterin cofactor biosynthesis are (dark-grey; *moaABEDA*,*mobAB*,*moeAB*), genes for iron transport (*fecBED*) and the (*narGHJI*) in light grey, and for nitrite extrusion (*nark*) and a kinase/response regulator system (*rrp4*, *hpk4*) in black, are found in this region.

570 Putative genes are indicated with white arrows.

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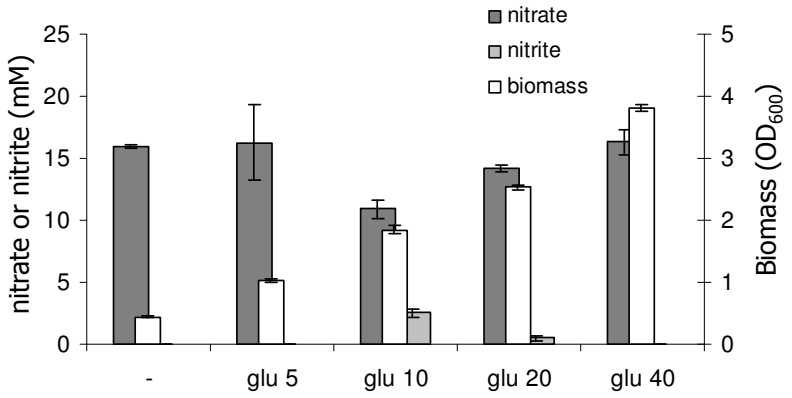

Figure 3. Nitrate reduction and nitrite formation by *Lactobacillus plantarum* WCFS1 in the presence of glucose. The Y-error bars indicate the average values ± the standard deviation. Wild-type cells were grown overnight in anaerobic
580 batch cultures in modified MRS broth. Acetate and citrate were omitted from the standard MRS-broth recipe. This medium was supplemented with heme, menaquinone, 16mM nitrate and a variable concentration of glucose (glu 0, 5, 10, 20 and 40 mM).

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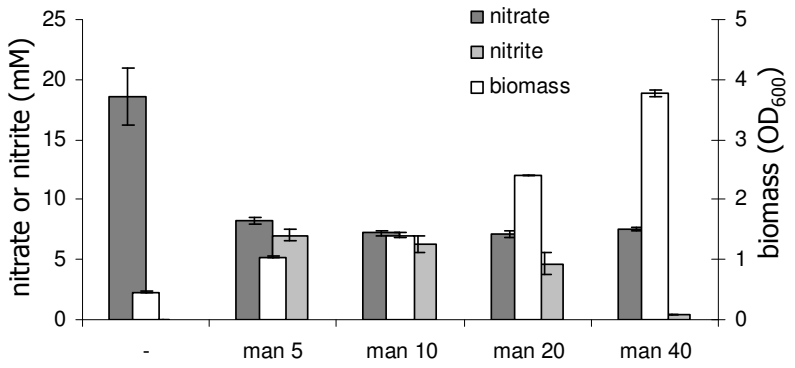

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Figure 4. Nitrate reduction and nitrite formation by *Lactobacillus plantarum* WCFS1 in the presence of mannitol. The Y-error bars indicate the average values ± the standard deviation. Wild-type cells were grown overnight in anaerobic batch cultures in MRS broth, from which acetate, citrate and glucose were
590 omitted (modified MRS-broth). This medium was further supplemented with heme, menaquinone, nitrate and variable amounts of mannitol (mM). Nitrate-reduction is not repressed at high mannitol concentrations.

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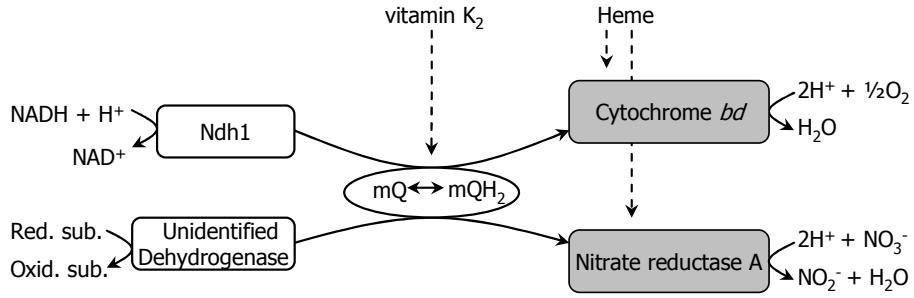

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Figure 5. Proposed branched electron transport chain of *Lactobacillus plantarum*
600 WCFS1 that terminates in either a nitrate reductase A or a *bd*-type cytcochrome complex. Dashed (- - -) lines represent the extra cellular origin of menaquinone (vitamin K_2) and heme. Abbreviations: reduced substrate (Red. sub.), oxidized substrate (Oxid. sub.), menaquinone (mQ) and menaquinol (mQH_2).