Electron Transport Chains of Lactic Acid Bacteria

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Contents

Abstract

Lactic acid bacteria are generally considered facultative anaerobic obligate fermentative bacteria. They are unable to synthesize heme. Some lactic acid bacteria are unable to form menaquinone as well. Both these components are cofactors of respiratory (electron transport) chains of prokaryotic bacteria.

Lactococcus lactis, and several other lactic acid bacteria, however respond to the addition of heme in aerobic growth conditions. This response includes increased biomass and robustness. In this study we demonstrate that heme-grown *Lactococcus lactis* in fact do have a functional electron transport chain that is capable of generating a proton motive force in the presence of oxygen. In other words, heme addition induces respiration in *Lactococcus lactis*. This aerobic electron transport chain contains a NADH-dehydrogenase, a menaquinone-pool and a *bd*-type cytochrome.

A phenotypic and genotypic screening revealed a similar response, induced by heme (and menaquinone) supplementation, in other lactic acid bacteria.

The genome of *Lactobacillus plantarum* WCFS1 was predicted to encode a nitrate reductase A complex. We have found that *Lactobacillus plantarum* is capable of using nitrate as terminal electron acceptor, when heme and menaquinone are provided. Nitrate can be used by *Lactobacillus plantarum* as effective electron sink and allows growth on a extended range of substrates. The impact of both the aerobic and anaerobic electron transport chain, on the metabolism and global transcriptome of *Lactobacillus plantarum* were studied in detail.

This work has resulted in the discovery of novel electron transport chains and respiratory capabilities of lactic acid bacteria. The potential respiratory capabilities of other, previously considered (strictly) anaerobic prokaryotic bacteria, were reviewed.

Chapter 1

Introduction and outline of thesis

Introduction

This chapter will introduce the reader to lactic acid bacteria (LAB), general concepts of respiration and electron transport chains. Firstly, the general features, applications, fermentative metabolism and the main current research topics will be described. The second part will focus on bacterial respiration with a particular emphasis on *Escherichia coli* as model organism. Then the scientific observations are presented that have suggested heme-induced aerobic respiration in several LAB, such as *Lactooccus lactis* and *Enterococcus faecalis*. The introduction concludes with the outline of this thesis.

The Lactic acid bacteria

The LAB are defined here as belonging to the order *Lactobacillales*, a related group of prokaryotic bacteria that are descended from a common ancestor (Fig. 1)

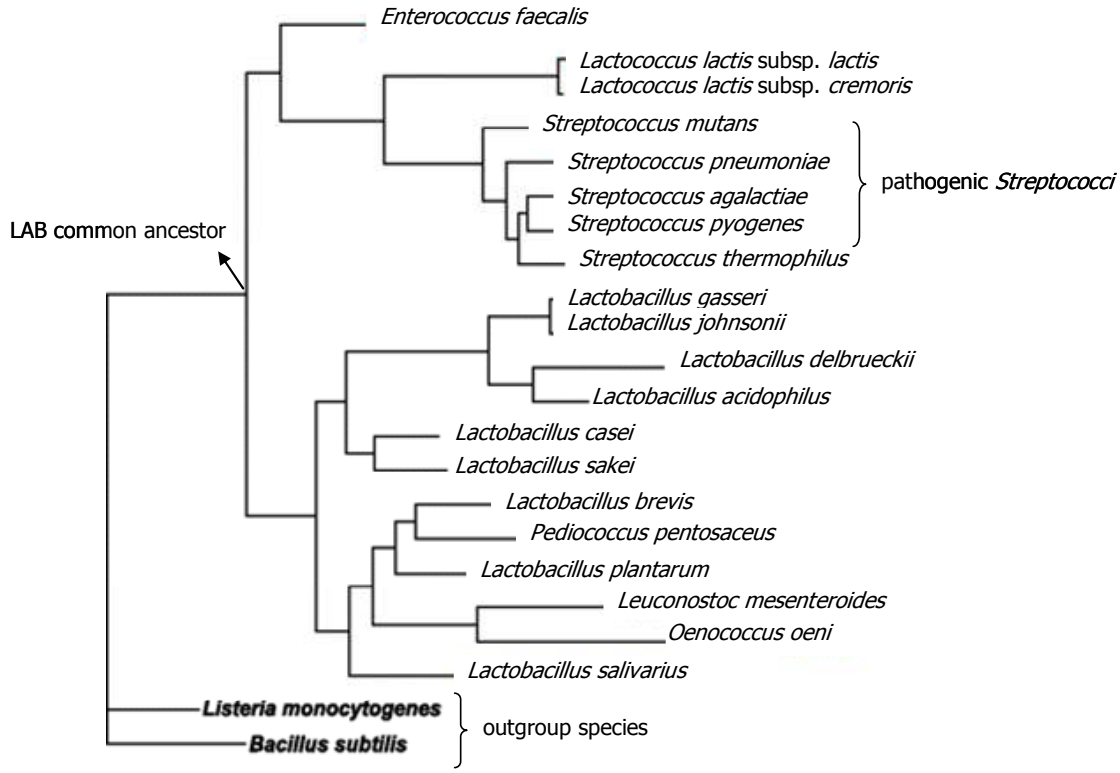


Figure 1. Evolutionary relationship of LAB. The phylogenetic tree of Lactobacillales constructed on the basis of concatenated alignments of four subunits (α, ß, ß', and δ) of the DNA-dependent RNA polymerase (based on Makarova et. al.) (37). The length of the branches represents evolutionary distance.

The members of this group share several physiological features and include Gram-positive, acid tolerant, non-spore forming, rod- or cocci-shaped bacteria that are able to ferment carbohydrates to (lactic) acids (59). LAB have been used for the fermentation of foods already since prehistoric times, and do so without the production of toxins, so that it can still be consumed, safely (Table 1).

Product	**Microorganisms**	**Substrate**
Wine, beer	*Saccharomyces cerevisiae*, LAB	grapes, grain, hops
Bread	*Saccharomyces cerevisiae*, LAB	wheat, rye, grains
Cheddar cheese	*Lactococcus* (*cremoris, lactis*), *Leuconostoc*	milk
Swiss type cheese	*Lactobacillus* (*delbrueckii*, *bulgaricus*, *helveticus*)	milk
Mould- and smear ripened cheeses	*Carnobacterium piscicola*, *Brevibacterium linens*	milk
Yoghurts	*Streptococcus thermophilus*, *Lactobacillus bulgaricus*	milk
Kefir	*Lactococci*, yeast, *Lactobacillus kefir* (and others)	milk
Fermented meats	*Pediococci*, *Staphylococci*, various LAB	pork, beef
Sauerkraut	*Lactococcus lactis*, *Leuconostoc mesenteroides*, *Lactobacillus* (*brevis*, *plantarum*, *curvatus*, *sake*)	cabbage
Soy sauce	*Aspergillus* (*oryzae*, *soyae*), *Lactobacilli*, *Zygosaccharomyces rouxii*	soy beans, wheat
Vegetables	*Enterococcus* (*mundtii*, *faecium*), *Lactococcus* (*cremoris*, *lactis*), *Lactobacillus* (*casei*, *plantarum*)	vegetables
Fish	*Carnobacterium* (*piscicola*, *divergens*)	fish

Table 1. Examples of foods that use LAB for their production, taken from Ross et. al. (52).

Several species are also able to produce anti-microbial compounds that in conjunction with the process of acidification inhibits growth of other spoilage, and possibly toxin-producing, bacteria or fungi (43). Fermentation thus increases the shelf-life of foods and can reduce the chance of spoilage. These attributes are especially of great importance in many third-world countries with lower hygiene standards.

Historically, mankind has used LAB to ferment their foods, without knowledge of the existence of (these) bacteria. Fermented foods have increased levels of nutrients, such as vitamins, are sometimes easier to digest then the raw food-product, and have altered and enhanced flavor profiles (29, 33).

Typical examples of centuries-old food fermentations that involve LAB are the production of many types of yoghurts and cheeses from milk. The discovery that these fermentation processes could be initiated by active inoculation of (fresh) milk, with old

batches of yoghurt and cheese, has inevitably led to a symbiosis between LAB and man that is still evolving.

For example, nutrient rich food-environments contain many complex organic compounds, such as peptides, vitamins and other co-factors. This “free” availability of these compounds relieves the selective pressure on the bacteria to maintain the metabolic capacity to produces them. Many species of LAB have been completely sequenced and the evolutionary (adaptive) trends in gene loss and gain can be deduced from this information. The LAB in general show extensive adaptation to the nutrient rich food-environments with significant gene loss (Fig. 2).

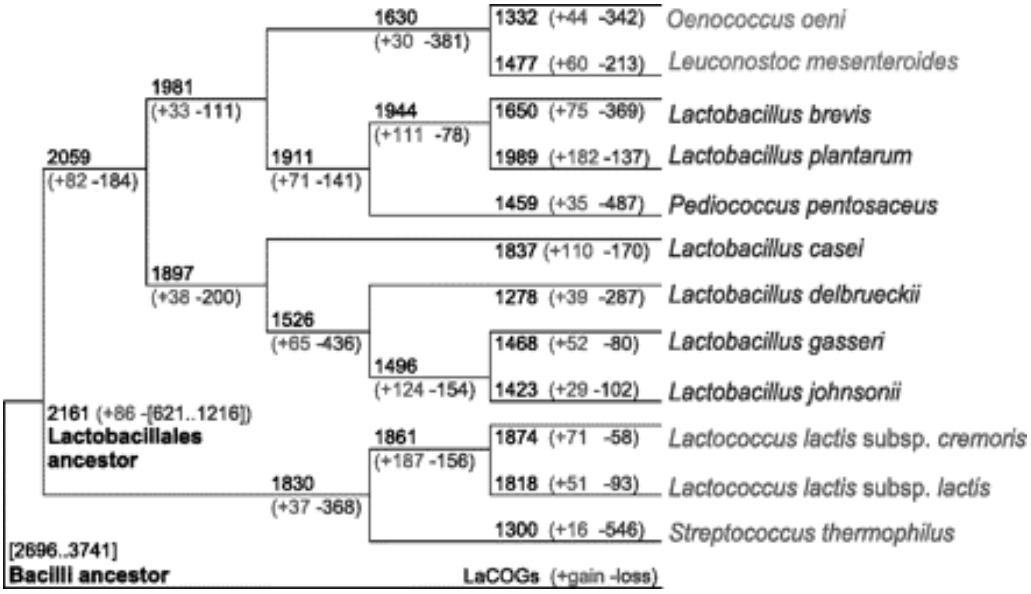


Figure 2. Progressive loss of genes as seen in the genomes of LAB indicate extensive specialization, taken from Makarova et.al. (37). The numbers of the LAB specific clusters of orthologous protein encoding genes, found in the species and inferred at the various nodes of speciation, are given, as well as the concomitant gain (+) and loss (-) events.

Most striking is that they have lost many biosynthetic capacities (8). A clear example of this is that cultivation of LAB on chemically defined medium requires supplementation with a variety of amino acids (26, 68).

Main research topics of lactic acid bacteria

LAB are also found in a wide variety of natural environments, such as plants. The research interest of LAB has however been rather anthropocentric. It has predominantly focused on isolates from (industrial) food-fermentations and the (human) gastrointestinal tract and on human and animal pathogens (1, 59). In fact the human body can be considered a natural habitat for some LABl species as well. The research focus of LAB falls into three broad categories: the relationship between their metabolic activity and the characteristics of the fermented food products, and the positive (probiotics) or negative (pathogens) effects of their interaction with the human body.

By studying bacteria derived from fermentations, a more controlled fermentation process may be achieved and a precise manipulation of nutrient and flavor production is expected.

The study of LAB that are human pathogens aims to improve the knowledge on bacterial virulence. Indeed, although many LAB have the GRAS-status (generally regarded as safe), certain species in particular from the genus *Streptococci* form the exception and have been associated with human infections. *Streptococcus pneumonia* for example, is a causative agent of community-acquired pneumonia, typically affecting children and the elderly, and *Streptococcus agalactiae* as causative agent of pneumonia, sepsis and meningitis in neonates (2, 60).

The consumption of specific strains of (live) LAB however, exerts beneficial effects of on human health (beyond that of the consumption of fermented foods). Such strains are currently marketed as probiotics and their consumption is associated with lower incidence of some types of allergies and diseases. There is clinical evidence that some probiotic strains can reduce the duration of diarrhea, ameliorate certain inflammatory bowel diseases and may even reduce levels of cancer-promoting enzymes and putrefactive metabolites in the gut (12-14, 47) . Furthermore administration of probiotics may decrease incidence of necrotizing enterocolitis (and thereby mortality rate) of neonates, prevent the emergence of allergies and atopic disease (5, 6, 44). There is considerable interest to elucidate the mechanisms by which these probiotic strains exert their beneficial effects. Hence the specific interaction of these probiotic bacteria with the human gastro-intestinal tract is the subject of many current research studies (54).

LAB with the GRAS status, probiotics and pathogens can be studied in comparison to distill the mechanisms of their (positive, neutral or negative) interactions with humans (30).

Fermentation and respiration

Respiration is defined as the oxidation of oxidize organic substrates using extracellular electron acceptors, via an electron transport chain (ETC) that can generate a proton motive force (PMF). This is in contrast to fermentation, defined as the oxidation of organic compounds using endogenous electron acceptors that are usually catabolic intermediates of the same organic compounds.

LAB as a group are generally considered non-respiring and obligate fermentors. Fermenting bacteria generate most of their energy (ATP) by substrate level phosphorylation. Characteristic for all LAB is the absence of a complete citric acid cycle which is indicative of their specialization to a fermentative life-style. Not surprisingly, they are not known to completely degrade sugars (or other carbon sources) to CO_2 (and H_2O). LAB convert sugars mainly to acids, principally lactate and acetate, which is rather inefficient since the excreted acids are still rich in high-energy bonds. Lactate production, however, requires relatively few enzymes, compared to the enzymes of the citric acid cycle needed for complete breakdown to CO_2. Current thoughts are that the production of lactate, in nutrient rich environments, allows for higher growth rates. At the same time lactate production acidifies the environment and inhibits growth of competitors. Indeed metabolic models of *Lactobacillus plantarum* demonstrated optimisation for growth rate rather than efficiency (61).

The anaerobic fermentative conversion of glucose to L-lactate via glycolysis must be redox-balanced (Fig. 3). The electrons that are released in the oxidation of the catabolic intermediates, such as glyceraldehyde-3-phosphate, are sequestered by a pool of redox-carriers. The principal redox carrier of glycolysis, NAD^+ is itself reduced to NADH in the process. Since the redox-carrier pools are finite, for glycolysis to proceed, NADH must be eventually be re-oxidised by other intermediates in glycolysis (e.g. fermentation). This occurs further “down-stream” when pyruvate is reduced by the lactate-dehydrogenase, to lactate. Under some conditions pyruvate is converted to acetate, which yields additional ATP. Anaerobic conversion of requires additional mechanisms of re-oxidizing excess NADH. For example, the partial conversion of the pyruvate pool to ethanol or the conversion

of extra-cellular citrate to succinate can act as effective redox sinks (Fig 3). As mentioned most LAB, such as *Lactococcus lactis* and *Lactobacillus plantarum,* produce almost exclusively lactate when grown on glucose in batch culture.

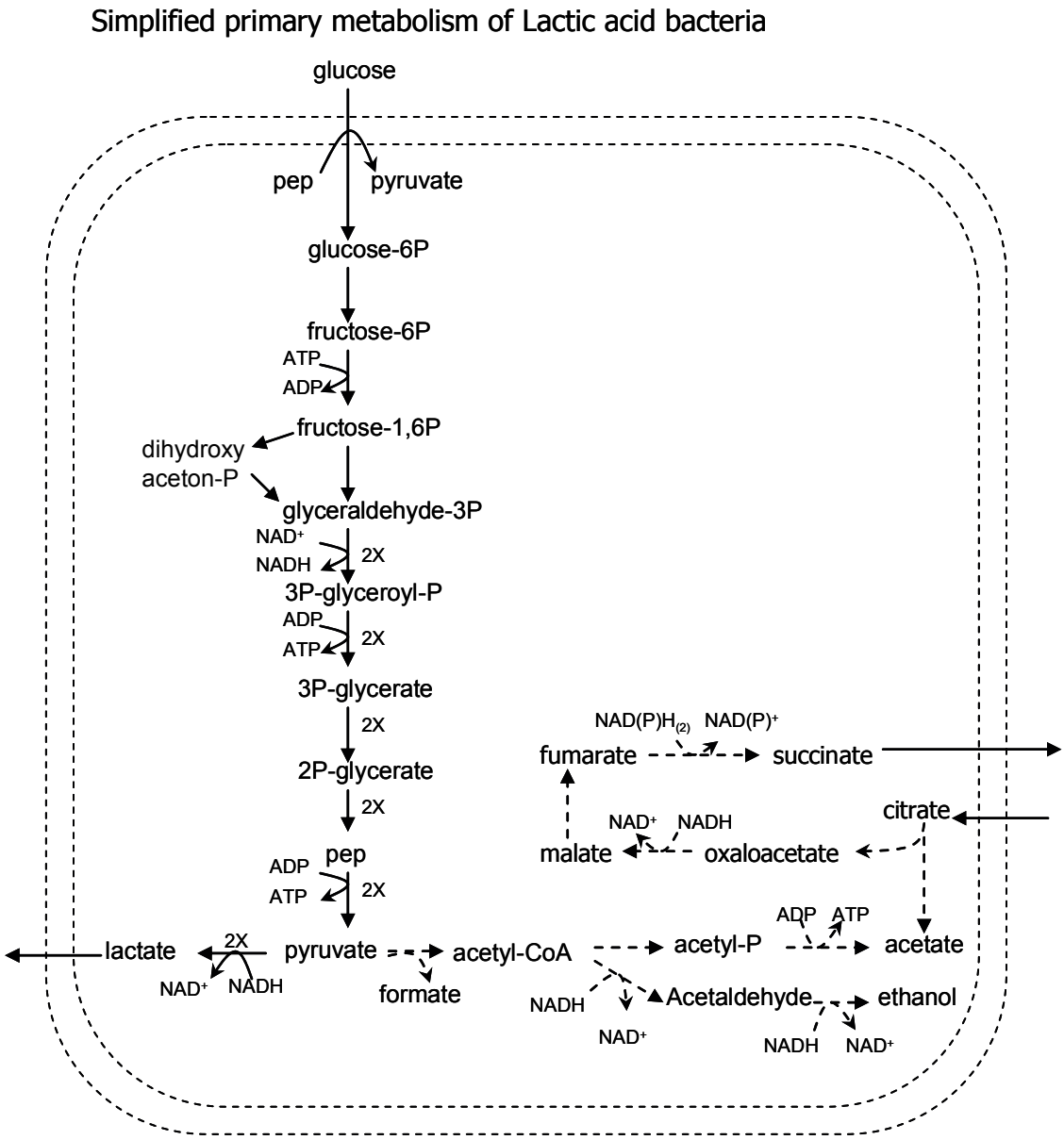


Figure 3. Simplified primary metabolism of LAB, modified from Chapter VI of this thesis. Glucose-6-phosphate can be used for biomass formation or energy production. 2x indicates a molar duplication of metabolites at the point of glyceraldehyde-3P. Single dotted lines represent the alternative conversion of pyruvate to acetate and the use extracellular citrate-consumption and ethanol-production as redox sinks. Abbreviations: phosphate (-P), phosphoenolpyruvate (pep). Dotted double lines symbolise the cell membrane.

There are observations that several LAB, when grown in the presence of certain cofactors (heme and menaquinone) display a respiratory-like behavior (16, 69, 70). Bacterial respiration has been well studied the model-organism *Escherichia coli* (3, 51). This bacterium has a complete citric acid cycle and is able to fully catabolize glucose to CO_2 and H_2O, when a suitable electron acceptor is available. The citric acid cycle generates high levels of reduced electron carriers, typically NADH. For the complete oxidation of glucose to continue, extracellular electron acceptors are needed to regenerate the finite pool of NAD^+. The ETC can couple the oxidation of substrates (in this case NADH) and the reduction of electron acceptors (in this case oxygen) to the formation of a PMF (Fig 4).

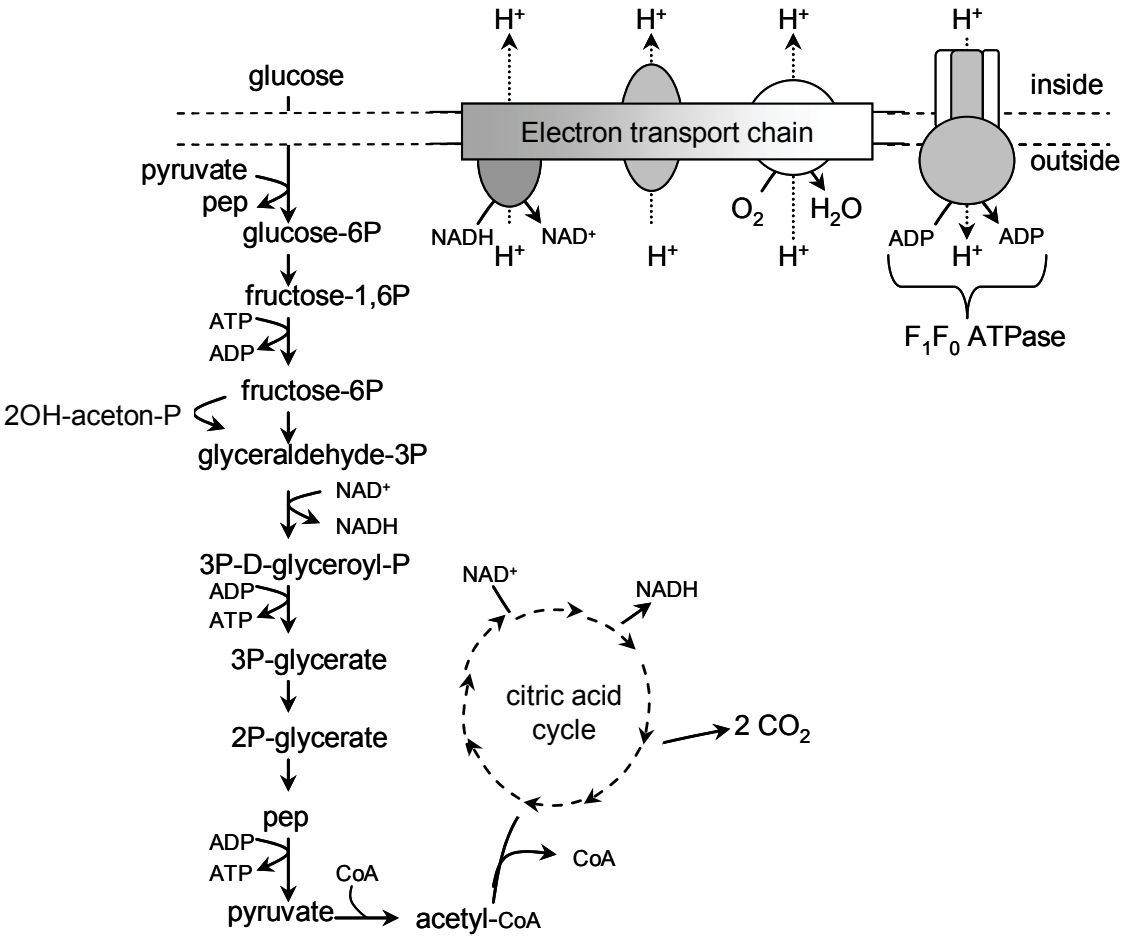


Figure 4. The complete catabolism of glucose to CO_2 and H_2O by the combined activity of glycolysis, the citric acid cycle and an ETC. The double dotted lines represent the cell membrane (lipid bi-layer). Abbreviations: pep: phosphoenolpyruvate, -P (phosphate).

In prokaryotic bacteria, the ETC typically consists of dehydrogenases, a quinone or menaquinone pool, and reductases. These components work in unison to conserve the energy that is released in the passage of the electrons by the generation of a PMF.

The proton-dependent (F_1F_0) ATPase

The lipid bi-layer of the cell membrane forms a barrier to charged protons. The charge difference on both sides of the membrane (typically negative on the cytosolic side) is used, for example to transport solutes (against their gradient) and even to phosphorylate ADP (PMF-driven ATP generation), using the F_1F_0 ATPase (Fig. 5). The efficiency of ATP formation is assumed to be approximately $3H^+$/ATP (56, 64).

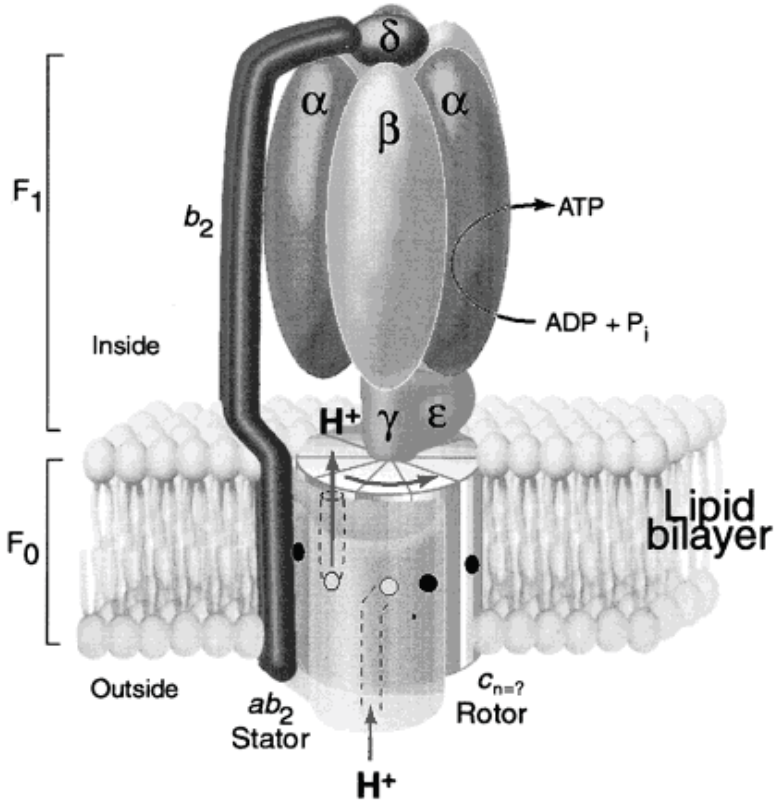


Figure 5. Structural model of the bacterial (*Escherichia coli*) F_1F_0 ATPase (taken from Jiang et. al., 2001) (27). The F_1F_0 ATPase is a membrane-bound multi-protein complex that consists of many distinct subunits (α, β, δ, γ, ε). An ATP-driven (F_1) and proton driven (F_0) part rotate in opposite directions and allow interchange of the energy stored in proton motive force and ATP.

Most bacteria have a proton driven F_1F_0 ATPase complex to generate ATP, although some bacteria (and Archaea), such as *Acetobacterium woodii*, have sodium driven ATPase systems (23, 41). LAB also require the F_1F_0 ATPase for growth, although they, when fermenting, do not use this protein complex to generate ATP (7, 17). During fermentation, the ATPase is assumed to work "in reverse" and generate PMF that is essential for transport processes, at the cost of ATP. In particular the ability of the ATPase to efficiently export protons is an important system to maintain intracellular pH-homeostasis (32).

Branched ETC of *Escherichia coli*

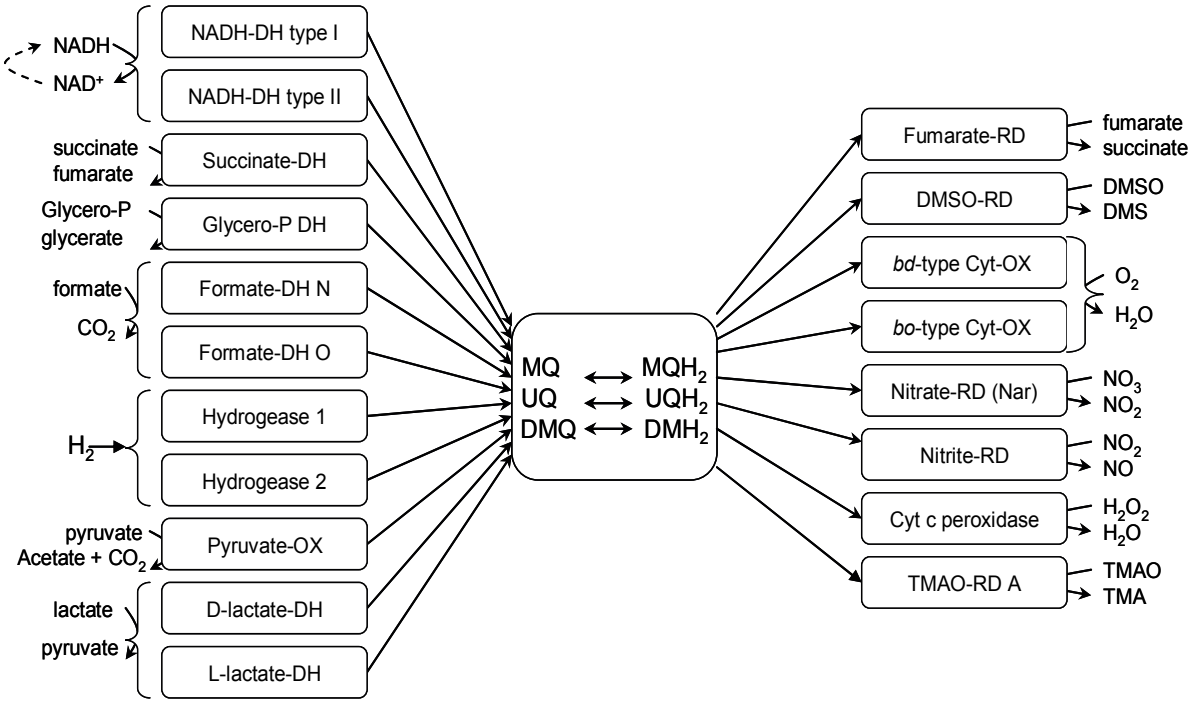
Aerobic respiration is a complex process in which a great number of electron donors and electron acceptors can participate. This can be observed In *Escherichia coli* where several membrane-associated dehydrogenases are able to directly reduce the (mena)quinone pool, using electrons donors, such as NADH, formate, succinate and lactate (Fig. 6).

Indirectly, many other substrates can act as electron donor. For example any substrate that is oxidized in an enzymatic reaction which concomitantly produces NADH, is in essence able to (indirectly) donate electrons to the (mena)quinone pool. *E. coli* also has an array of reductases that can in turn accept electrons from menaquinol and reduce specific electron acceptors (oxygen, nitrate, fumarate etc…). Specific combinations of dehydrogenases and reductases usually operate together. Redundancies in the activity of dehydrogenases and reductase may exist, as in the case of the *bd*- and *bo*-type cytchrome that both reduce oxygen.

Extra cellular electron acceptors

The list of electron acceptors that can be used by *E. coli* is not exclusive by far for bacteria and Archaea as a whole. For example sulfate reducing bacteria have a set of reductases that can reduce inorganic sulfate completely to H_2S, while acetogens and methanogens are able to reduce CO_2 (with hydrogen) (Chapter 6). Various types of metal ions are used by many bacteria, of which Fe^{3+} is a widespread anaerobic terminal electron acceptor. Fe^{3+} is one of the most abundant metals and, as it has low solubility, it is usually present in the form of ferric oxides in the soil. *Shewanella putrefaciens* and *Geobacter metallireducens* are model organisms to study the reduction of ferric iron and have specific ferric iron reductases (36).

Indeed many types of metal-ions or their respective oxides can be used as electron acceptor by the microbial community, such as oxides of manganese, selenium, chromium, vanadium, copper, molybdenum, or even the more exotic metal-oxides of uranium, mercury, techneticum, silver and gold.



* NO_2 can be fully reduced to ammonia.

Figure 6. Dehydrogenases and reductases as found in *Escherichia coli* K-12 that respectively donate or accept electrons from the quinone-pool (modified from Richardson, 2000) (51). The quinone pool can vary in composition and (in *E. coli*) can consist of menaquinones (MQ), ubiquinones (UQ), demethylmenaquinone (DMQ). Abbreviations: -DH (dehydrogenase), -RD (reductase), Cyt (cytochrome).

A recent paper showed that in fact the lactic acid bacterium *Lactococcus lactis* was also capable of reducing Fe^{3+} and Cu^{2+} (50). An exhaustive review of metal reduction by bacteria is given by Lovley, 1993 (35). The reduction of metals by microbes has been of interest for bioremediation of heavy metals and radionuclides. In the light of bioremediation, the use of toxic hydrocarbons, such as organic solvents, chlorinated solvent as electron donor

or acceptors should be mentioned (34). The list of compounds that bacteria can use either as extracellular electron donor or acceptor (in an ETC) to generate PMF is virtually endless.

cytochromes

In many processes, where the oxidation of extracellular compounds is linked to cytosolic reduction processes, cytochromes are involved. Cytochromes can be broadly defined as membrane-bound hemo-proteins that can carry out electron transport. We will use the term cytochrome and cytochrome-containing complexes interchangeably. Aerobic cytochromes are usually defined based on their heme-cofactors. *Escherichia coli*, for example, contains the *bo*-type cytcochrome, with a b-heme, an o-heme and copper as cofactor, while the *bd*-type cytochome has two b-hemes and a single d-heme (24, 63). There is an apparent redundancy of having two cytochromes that carry out the reduction of oxygen. However, the *bo*-type cytochrome is active during oxygen-rich conditions, while the bd-type cytochrome is produced during oxygen limiting conditions. In accordance to this differential expression, the *bo*-cytochrome has a low affinity for oxygen but a high K_m, relative to the *bd*-type. The *bd*-type cytochrome with a higher oxygen affinity is able to scavenge oxygen to such an extent that it provides resistance against oxidative stress (11, 21). Not all cytochromes are so clearly named. The nitrate reductase A for example, encoded by the *narGHJI* genes, is also a membrane-bound hemo-protein that carries out electron transport, and therefore is a cytochrome (53).

Membrane-integral electron carriers

We have defined respiration as a system that employs an electron transport chain, with membrane-integral electron carriers, to generate a proton motive force. In this sense electron carriers, such as quinones, are essential for respiration. These compounds are usually membrane-soluble that shuttle “electrons” from electron donors, as typically catalyzed by membrane-associated dehydrogenases, to electron acceptors, as typically catalyzed by membrane-associated reductases. Bacterial quinones are classified based on their structure and can be divided in two main types: the naphthoquinones (menaquinone, phylloquinone) and the benzoquinones (plastoquinones, ubiquinones) (Fig. 7). These quinone types can have tails of various lengths. For example, menaquinones have been

detected with C-3 isoprenyl side-chains that vary from 1 to 14 (10). As mentioned *Escherichia coli* produces three types of quinones: ubiquinones, menaquinones and demethylmenaquinones (Fig 7.).

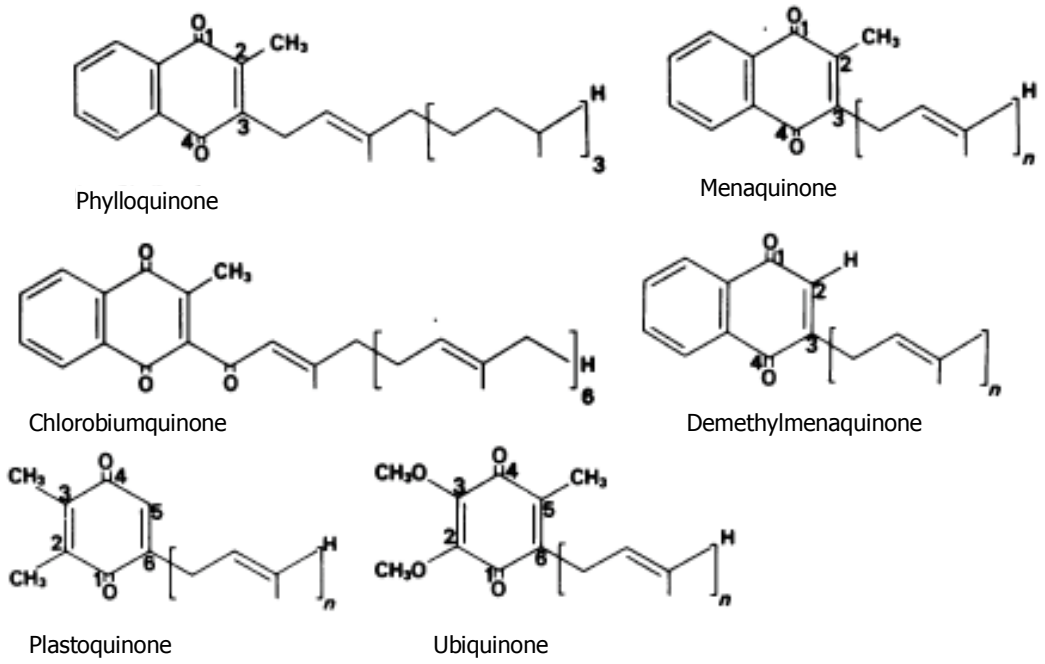


Figure 7. Structures of quinone-types found in bacteria (modified from Collines *et. al.*, 1981) (10). The number (or n) next to the brackets indicate the number of tail-structure repeats. Vitamin K_2 is a menaquinone with 4 side chains (n = 4).

In Gram-negatives, ubiquinones are the major quinones species when oxygen is used as terminal electron acceptor, menaquinones when fumarate or DMSO are used and demethylmenaquinones during nitrate respiration, while in Gram-positives (demethyl)menaquinones are used for both aerobic and anaerobic respiration (50, 64). Phylloquinones are more rarely found in bacteria. Plastoquinones are typical for cyanobacteria and chlorobium quinones were isolated from green photosynthetic bacteria (10).

Besides their role in ETC's, quinones have been associated with diverse functions such as disulfide bond formation of extracellular proteins, reduction of oxidative stress, redox-sensing and more (4, 20, 38, 50, 58, 67). Menaquinones are also produced by several

species (but not all) of LAB, such as by *Lactococcus lactis*, *Leuconostoc mesenteroides* and more rarely by *Lactobacillus* sp (39).

Not all prokaryotes use quinones as electron transfer components in respiratory chains as Archaea are known to use polyferredoxins, flavoproteins or phenazine derivatives as membrane-integral electron carriers (55). Respiration is associated with an ETC with membrane-integral electron carriers. In this regard for example, the oxidation of lactate with the reduction of sulfate to H_2S in sulfate reducing bacteria does not constitute respiration, since no membrane-integral electron carrier partakes. In these systems the passive diffusion of hydrogen itself over the cell membrane functions as "electron carrier" (22).

General principals of electron transport – redox-couples.

There are certain energetic principles that determine the efficiency of the conversion of proton motive force to generation of ATP. For example, for microbial ETC's to generate ATP, generally with a stoichiometry of $3H^+$/ATP, the electron transfer from the electron donors to the electron acceptor must release an amount of energy that is at least one-third of the energy required to phosphorylate ADP (62):

$$ATP + H_2O \longrightarrow ADP + Pi \qquad (\Delta G^\circ = -31.8 kJ/mol)$$

The energy release of -31.8 kJ/mol is an approximation, since it is dependent on temperature and the intracellular concentration of ATP, ADP and Pi. However, as a general principle, the Gibbs free energy released in the oxidation of substrates by an (extracellular) electron acceptor must be sufficient to drive ATP synthesis.

For redox-reaction actually to occur, the electron donor must in principle have a lower redox-potential (*E*) than the electron acceptor (Table. 2). The Gibbs free energy that is released in these redox reactions can be calculated from the difference in redox potential. ($\Delta G' = n.F. \Delta E'$, in which 'n' is the number of electrons transferred and F the Faraday constant) Thauer et. al. calculated, based on a electron transfer reaction which involves 2 electrons, that a $\Delta E'$ of 250 mV would release 48 kJ/mol.

Redox compound	E_0' (mV)	Redox compound	E_0' (mV)
CO_2 / formate	-432	Menaquinone / menaquinol	-74
H^+ / H_2	-414	glycine / $acetate^-$ + NH_4^+	-10
NAD^+ / NADH	-320	fumarate / succinate	+33
CO_2 / acetate	-290	ubiquinone / ubiquinol	+113
CO_2 / CH_4	-244	NO_2 / NO	+350
FAD / $FADH_2$	-220	NO_3 / NO_2	+433
Pyruvate / lactate	-190	Fe^{3+} / Fe^{2+}	+772
Oxaloacetate / malate	-172	O_2 / H_2O	+818

Table 2. standard redox potentials of a few selected electron donors and acceptors (modified from Thauer et. al., 1977) (62).

These types of calculations are valuable to understand which redox-reactions could theoretically be used by microbes to generate ATP by oxidative phosphorylation. As an example, it shows that hydrogen and formate can be used as electron donor to reduce CO_2 to acetate, while lactate cannot. Furthermore, it shows why pyruvate reduction with $FADH_2$ as electron donor is unlikely to generate ATP by oxidative phosphorylation. As noted before, since the Gibbs free energy change is affected by various parameters, including concentration of reactants and temperature, these factors should be considered.

Evidence for respiration in Lactic acid bacteria

LAB as a group are classified as facultative anaerobic fermentative bacteria. There are several reasons why these bacteria are unlikely respirators. Firstly, LAB do not produce heme, which are essential cofactors of cytochromes. Secondly, many species of LAB do not even produce quinones, such as *Latobacillus* sp. and *Streptococcus* sp. There are several species however that are known to produce menaquinones, such as *Lactococcus* sp. *Leuconostoc* sp (39). Quinones are the membrane-integral electron shuttles commonly found in bacterial respiratory chains.

However, in 1970 observations were published that heme stimulated growth of several LAB under aerobic conditions. Sijpesteijn et. al. observed that addition of hemin, to

cultures of *Lactococcus lactis* (then called *Streptococcus lactis*) and *Leuconostoc mesenteroides*, stimulated oxygen uptake with a corresponding shift from homo-lactic to mixed acid fermentation. Furthermore cyanide reduced the effect of hemin on oxygen uptake (57). Cyanide is a known inhibitor of cellular respiration. Similar heme-induced responses were observed in 1977 for *Enterococcus faecalis* (then called *Streptococcus faecalis*), where also cytochrome-peaks of a b-hemes (558nm and 562nm) and a d-heme (627nm) were observed in the reduced-oxidized difference spectra of the membrane (48). Only at the start of the new millennium did research on the heme-induced responses in LAB revive. It was shown that the cyd-operon (*cydABCD*) of *Enterococcus faecalis* V583, could be transferred, functionally expressed and compensate a *bd* cytochrome deficient mutant of *Bacillus subtilis* (69). These *cydABCD* genes encode the structural subunits (and maturation enzymes) of the *bd*-type cytochrome. This aerobic cytochrome is found in a wide variety of bacteria where it functions in an electron transport chain context (oxidizes menaquinol) and generates a proton motive force via scalar chemistry (28).

In 2001, the involvement of *cydA* in the heme-induced response of *Lactococcus lactis* was also shown (16). Furthermore, in this study a more detailed characterization of the response was performed that revealed several interesting traits. These included an increase in biomass production with a clear shift from homo-lactic to mixed-acid production, resistance to oxygen induced stress and a markedly improvement in long-term survival in storage conditions (4 ˚C). Evidence that heme addition allow growth of a *L. lactis* H^+-ATPase mutant to grow on air-exposed agar plates was published in the same year (7). Finally, in 2002 an aerobic electron transport chain was proposed to exist in *Lactococus lactis*, consisting of a NADH-dehydrogenase a menaquinone pool and a *bd*-type cytochrome as menaquinol oxidase (19). Since then, the idea that *Lactococcus lactis* actually respires has been largely accepted. More recently, studies have shown that the activity of the *bd* cytochrome results in low-intracellular oxygen-levels, even in aerated conditions, which forms a plausible mechanism for its protective role against oxidative stress (49). Even the effect of heme (and the assumed respiratory metabolism) on cellular protein content and genome wide transcription levels have been studied, using proteomics and microarray studies (45, 66).

The term "assumed respiratory metabolism" in *Lactococcus lactis* is used, since direct evidence linking the presence of *cyd*-genes to PMF generation was still lacking. The heme-induced improvement in biomass formation for example can also be explained by the observed shift toward mixed-acid production, since acetate yields more (substrate level) ATP. Furthermore the oxygen scavenging activity of the *bd*-cytochrome does not automatically imply that it generates a PMF.

Nitrate-respiration and the *narGHJI* genes

Escherichia coli can use many different types of terminal electron acceptors, besides oxygen, among which nitrate (25). The *narGHJI* genes encode for a nitrate-reductase A complex in *E. coli* that functions as quinol oxidase in the ETC and generates PMF (18). This *nar*-operon was found in the published genomes of *Lactobacillus plantarum*, *Lactobacillus reuteri* and *Lactobacillus fermentum* (31, 40) (http://genome.jgi-psf.org/draft_microbes/lacro/lacro.home.html). This is surprising since *Lactobacillus plantarum* is not only unable to produce heme, that is an essential co-factor of this nitrate-reductase, but also unable to produce menaquinones. In the *Lactobacillus plantarum* genome, however, there are genes present that encode for the biosynthesis of the molybdenum cofactor. This co-factor is also required for activity of the nitrate reductase A complex (65). In fact they are found in close proximity to the *nar* genes with which they form a gene-cluster (31). There are reports however that a combination of heme and menaquinone supplementation induced a respiratory-like response in *Streptococcus agalactiae*, which is also unable to produce menaquinone (71). The supplied menaquinone is menaquinone-4 that is more commonly known as vitamin K_2. Since the genome of *Lactobacillus plantarum* also harbors the *cydABCD* genes, there is a possibility that this lactic acid bacterium may have a heme- and menaquinone-dependent branched-ETC.

Impact of assumed respiration in LAB on the main research topics

The heme (and menaquinone) induced phenotype of LAB has wide implications for three main fields of LABl research (food fermentation, virulence and probiotic activity), for several reasons.

For example, when *Lactococcus lactis* MG1363 is grown aerobically in the presence of heme, a drastically altered phenotype is observed (16, 19). This includes many industrially important traits, such as increased biomass-yield, growth-efficiency, stress resistances (oxygen, acid) and prolonged survival at low temperatures. Growth of *Lactococcus lactis* in the presence of heme has already found its way into industrial applications patents (15, 46). Although the main commercial interest in LAB focuses on their ability to ferment foods anaerobically, there are extensive industrial activities that provide (defined) starter cultures for these fermentations. This starter-culture industry has used the aerobic heme-stimulation as a more economic (and efficient) method to produce biomass.

A combination of heme and menaquinone induces enhanced aerobic growth of pathogens, such as *Streptococcus agalactiae*. Interestingly, mutation of a gene that is involved in the respiration phenotype (*cydA*) led to a decrease of virulence, in a rat-model (70, 71). The study of how respiration and/or respiration controlled functions may contribute to dissemination of these pathogens in the host may help in furthering the understanding of virulence and develop targets for medical treatment.

For probiotic activities that rely on live LAB in the gut, heme- (and menaquinone-) increased robustness could contribute to enhanced survival and thus probiotic effectiveness. Furthermore, in the gut and some types of food-matrices (such as meat) there are alternative electron acceptors, besides oxygen, such as nitrate and nitrites. Many bacteria, such as *Escherichia coli* and the, previously considered obligate aerobe, *Bacillus subtilis*, are able to exploit these inorganic substrates as redox-sinks (9, 42). In fact, nitrate-reductase genes (*narGHJI*) that are involved in the dissimilatory nitrate reductase are also found in *Lactobacillus plantarum*, *Lactobacillus reuteri* and *Lactobacillus fermentum* (31).

Outline of thesis

This study examines the presence and functionality of electron transport chains in LAB. Firstly, we determined whether *Lactococcus lactis* is capable of aerobic respiration. In order to do so we have to provide evidence that its ETC is capable of generating PMF. Secondly, we will explore the distribution of the *cyd*-genes and the heme- (and menaquinone-) induced aerobic response in LAB as a group. This is facilitated by the fact that many genomes of LAB have been completely sequenced, and that the respiration-like

phenotypes can be easily detected. Thirdly, we aim to study the nitrate reducing ability of *Lactobacillus plantarum* that involves an ETC. Nitrate-respiration does not require aeration and thus is potentially more easily applicable in fermentation processes, and of importance for probiotic gut-activity.

In **Chapter 2** the PMF-generating capability of the *Lactcoccus lactis* ETC is studied. A fluorescent probe is used to measure changes in proton motive force, called $DiSC_3(5)$ or 3',3'-dipropylthiadicarbocyanine. Aerated cultures of heme-grown wild-type cells, and cells that have their *cydA* replaced by a chloramphenicol-resistance gene, are compared. Although growing cells maintain a PMF to transport, for example amino acids, washing of cells in buffer together with an incubation period on ice is expected to dissipate the PMF to a measurable extent. Subsequent addition of glucose will provide the ATP (and NADH) needed to (re)generate PMF levels. During fermentation, the F_1F_0 ATPase generates PMF by expending ATP. Therefore, in order to attribute formation of PMF to activity of the electron transport chain the ATPase activity must be inhibited. DCCD (*N,N*'-dicyclohexylcarbodiimide), is a well known inhibitor of H^+ ATPase activity and will be used for this purpose. Thus, by observing differences in the PMF generating capabilities of wild-type and cydAΔ in the presence of DCCD, we attempt to visualize PMF generation by the ETC.

In **Chapter 3** the distribution of the *cydABCD* genes in LAB, that code for the aerobic *bd*-type cytochrome with menaquinol oxidase activity, is studied. In the NCBI database there are about 40 genomes of LAB that have been completely sequenced, and 18 of which are assayed using whole genome shotgun approaches. Among these are 24 different species, so that for several species multiple strains have been sequenced. In any case, there is a wealth of genomic sequence data available, which provides an opportunity to examine the distribution of the *cyd*-genes in these bacteria.

The heme-induced aerobic phenotype of *Lactococcus lactis* is readily observed by the increased biomass production that is not associated with a lower final pH. We set-up a standard aerobic cultivation procedure to screen a number of LAB for a heme (and menaquinone) induced response. In addition, since the respiratory-like phenotype of *L. lactis*

includes several industrial interesting traits, attempts are made to develop high-throughput screening methods.

Chapters 4, 5 and 6 specifically focus on the combined heme- and menaquinone-induced phenotype of *Lactobacillus plantarum* WCFS1. *Lactobacillus plantarum* WCFS1 contains genes that encode a *bd*-type (aerobic) cytochrome (*cydABCD*) and a nitrate-reductase A complex (*narGHJI*). Homologues of these protein complexes participate in aerobic and nitrate respiration in *Escherichia coli* and *Bacillus subtilis*.

In **Chapter 4** cultivation conditions are examined that induce heme- and menaquinone-dependent oxygen and nitrate dissimilation and the involvement of the *nar*- and *cyd*-operons. **Chapter 5** deals with the detailed characterization of the aerobic phenotype that is induced by these cofactors. *Lb. plantarum* cells are grown in complex medium with and without these cofactors and the organic acid profiles are followed in time. The differences between these two conditions are further compared with micro-array techniques that allow the global genome expression to be analyzed. This chapter also describes other genes that are involved in the aerobically cofactor-induced phenotype by creating selected mutants. **Chapter 6** deals with the detailed characterization of the anaerobic phenotype in a similar way. Growth and organic acid composition of wild-type cells is followed in time under conditions that allow for nitrate reduction, as described in chapter 4. In particular, the ability of nitrate to function as effective redox sink is compared to citrate. Micro-arrays are used to investigate differences in global genome expression profiles. Furthermore, the genomes of several (~20) strains of *Lactobacillus plantarum* were compared in a different study by genotyping techniques, using the *Lactobacillus plantarum* WCFS1 genome sequence as template. This allows us to investigate the presence of the *nar*-genes these strains and whether other genes are co-conserved

Chapter 7 is a review of the electron transport chains in LAB and other (strict) anaerobic prokaryotic bacteria. LAB, propionibacteria, sulfate-reducing bacteria and acetogens are best known for their anaerobic-life styles. In fact, various species of LAB and propionibacteria grow poorly on agar plates that are exposed to air, while sulfate-reducing bacteria and acetogens are considered strict anaerobes. Surprisingly, in all these groups of

bacteria the *cyd*-genes were found that, in other bacteria, function in aerobic respiratory chains. This warrants a review of the electron transport chains of these bacteria, and the presence of alternative (aerobic) ETC's and how these may impact their primary metabolism.

In **Chapter 8** the main results are summarized and discussed with specific attention to whether the evidence obtained in this study supports the concept of active ETC's and respiration in LAB. Suggestions are made for further scientific study to answer several remaining questions. Finally, future perspectives are given of the impact that respiratory-like behavior of LAB may have on (industrial) applications and scientific endeavors.

1. **Aguirre, M., and M. D. Collins.** 1993. Lactic acid bacteria and human clinical infection. J Appl Bacteriol **75:**95-107.
2. **Alonso De Velasco, E., A. F. M. Verheul, J. Verhoef, and H. Snippe.** 1995. *Streptococcus pneumoniae*: Virulence Factors, Pathogenesis, adn Vaccines. Microbiol Rev**:**591-603.
3. **Anraku, Y.** 1988. Bacterial electron transport chains. Annu Rev Biochem **57:**101-32.
4. **Bader, M., W. Muse, D. P. Ballou, C. Gassner, and J. C. Bardwell.** 1999. Oxidative protein folding is driven by the electron transport system. Cell **98:**217-27.
5. **Bin-Nun, A., R. Bromiker, M. Wilschanski, M. Kaplan, B. Rudensky, M. Caplan, and C. Hammerman.** 2005. Oral probiotics prevent necrotizing enterocolitis in very low birth weight neonates. J Pediatr **147:**192-6.
6. **Bjorksten, B.** 2005. Evidence of probiotics in prevention of allergy and asthma. Curr Drug Targets Inflamm Allergy **4:**599-604.
7. **Blank, L. M., B. J. Koebmann, O. Michelsen, L. K. Nielsen, and P. R. Jensen.** 2001. Hemin reconstitutes proton extrusion in an H(+)-ATPase-negative mutant of *Lactococcus lactis*. J Bacteriol **183:**6707-9.
8. **Bolotin, A., B. Quinquis, P. Renault, A. Sorokin, S. D. Ehrlich, S. Kulakauskas, A. Lapidus, E. Goltsman, M. Mazur, G. D. Pusch, M. Fonstein, R. Overbeek, N. Kyprides, B. Purnelle, D. Prozzi, K. Ngui, D. Masuy, F. Hancy, S. Burteau, M. Boutry, J. Delcour, A. Goffeau, and P. Hols.** 2004. Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. Nat Biotechnol **22:**1554-8.
9. **Bonnefoy, V., and J. A. Demoss.** 1994. Nitrate reductases in *Escherichia coli*. Antonie Van Leeuwenhoek **66:**47-56.
10. **Collins, M. D., and D. Jones.** 1981. Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implication. Microbiol Rev **45:**316-54.
11. **D'Mello, R., S. Hill, and R. K. Poole.** 1995. The oxygen affinity of cytochrome *bo'* in *Escherichia coli* determined by the deoxygenation of oxyleghemoglobin and oxymyoglobin: Km values for oxygen are in the submicromolar range. J Bacteriol **177:**867-70.

12. **de Roos, N. M., and M. B. Katan.** 2000. Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998. Am J Clin Nutr **71:**405-11.
13. **de Vrese, M., and P. R. Marteau.** 2007. Probiotics and prebiotics: effects on diarrhea. J Nutr **137:**803S-11S.
14. **de Vrese, M., and J. Schrezenmeir.** 2008. Probiotics, Prebiotics, and Synbiotics. Adv Biochem Eng Biotechnol.
15. **Duwat P, G. A., LeLoir Y, Gaudu P.** 1999. Bactéries lactiques transformées pour leur conférer un métabolisme respiratoire, et levains comprenant lesdites bactéries. French patent application FR2798670.
16. **Duwat, P., S. Sourice, B. Cesselin, G. Lamberet, K. Vido, P. Gaudu, Y. Le Loir, F. Violet, P. Loubiere, and A. Gruss.** 2001. Respiration capacity of the fermenting bacterium *Lactococcus lactis* and its positive effects on growth and survival. J Bacteriol **183:**4509-16.
17. **Ferrandiz, M. J., and A. G. de la Campa.** 2002. The membrane-associated F(0)F(1) ATPase is essential for the viability of *Streptococcus pneumoniae*. FEMS Microbiol Lett **212:**133-8.
18. **Garland, P. B., J. A. Downie, and B. A. Haddock.** 1975. Proton translocation and the respiratory nitrate reductase of *Escherichia coli*. Biochem J **152:**547-59.
19. **Gaudu, P., K. Vido, B. Cesselin, S. Kulakauskas, J. Tremblay, L. Rezaiki, G. Lamberret, S. Sourice, P. Duwat, and A. Gruss.** 2002. Respiration capacity and consequences in *Lactococcus lactis*. Antonie Van Leeuwenhoek **82:**263-9.
20. **Georgellis, D., O. Kwon, and E. C. Lin.** 2001. Quinones as the redox signal for the arc two-component system of bacteria. Science **292:**2314-6.
21. **Govantes, F., A. V. Orjalo, and R. P. Gunsalus.** 2000. Interplay between three global regulatory proteins mediates oxygen regulation of the *Escherichia coli* cytochrome *d* oxidase (cydAB) operon. Mol Microbiol **38:**1061-73.
22. **Heidelberg, J. F., R. Seshadri, S. A. Haveman, C. L. Hemme, I. T. Paulsen, J. F. Kolonay, J. A. Eisen, N. Ward, B. Methe, L. M. Brinkac, S. C. Daugherty, R. T. Deboy, R. J. Dodson, A. S. Durkin, R. Madupu, W. C. Nelson, S. A. Sullivan, D. Fouts, D. H. Haft, J. Selengut, J. D. Peterson, T. M. Davidsen, N. Zafar, L. Zhou,**

D. Radune, G. Dimitrov, M. Hance, K. Tran, H. Khouri, J. Gill, T. R. Utterback, T. V. Feldblyum, J. D. Wall, G. Voordouw, and C. M. Fraser. 2004. The genome sequence of the anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris Hildenborough*. Nat Biotechnol **22:**554-9.

23. **Heise, R., J. Reidlinger, V. Muller, and G. Gottschalk.** 1991. A sodium-stimulated ATP synthase in the acetogenic bacterium *Acetobacterium woodii*. FEBS Lett **295:**119-22.
24. **Hill, J. J., J. O. Alben, and R. B. Gennis.** 1993. Spectroscopic evidence for a heme-heme binuclear center in the cytochrome bd ubiquinol oxidase from *Escherichia coli*. Proc Natl Acad Sci U S A **90:**5863-7.
25. **Ingledew, W. J., and R. K. Poole.** 1984. The respiratory chains of *Escherichia coli*. Microbiol Rev **48:**222-71.
26. **Jensen, P. R., and K. Hammer.** 1993. Minimal requirements for exponential growth of *Lactococcus lactis*. Appl Environ Microbiol **59:**4363-4366.
27. **Jiang, W., J. Hermolin, and R. H. Fillingame.** 2001. The preferred stoichiometry of c subunits in the rotary motor sector of *Escherichia coli* ATP synthase is 10. Proc Natl Acad Sci U S A **98:**4966-71.
28. **Junemann, S.** 1997. Cytochrome bd terminal oxidase. Biochim Biophys Acta **1321:**107-27.
29. **Kalantzopoulos, G.** 1997. Fermented products with probiotic qualities. Anaerobe **3:**185-90.
30. **Klaenhammer, T., E. Altermann, F. Arigoni, A. Bolotin, F. Breidt, J. Broadbent, R. Cano, S. Chaillou, J. Deutscher, M. Gasson, M. van de Guchte, J. Guzzo, A. Hartke, T. Hawkins, P. Hols, R. Hutkins, M. Kleerebezem, J. Kok, O. Kuipers, M. Lubbers, E. Maguin, L. McKay, D. Mills, A. Nauta, R. Overbeek, H. Pel, D. Pridmore, M. Saier, D. van Sinderen, A. Sorokin, J. Steele, D. O'Sullivan, W. de Vos, B. Weimer, M. Zagorec, and R. Siezen.** 2002. Discovering lactic acid bacteria by genomics. Antonie Van Leeuwenhoek **82:**29-58.
31. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R.**

Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen. 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc Natl Acad Sci U S A **100:**1990-5.

32. **Konings, W. N.** 2006. Microbial transport: adaptations to natural environments. Antonie Van Leeuwenhoek **90:**325-42.
33. **Leroy, F., and L. De Vuyst.** 2004. Lactic acid bacteria as functional starters for the fermentation industry. Trends Food Sci Technol **15:**67-78.
34. **Lovley, D. R.** 2003. Cleaning up with genomics: applying molecular biology to bioremediation. Nat Rev Microbiol **1:**35-44.
35. **Lovley, D. R.** 1993. Dissimilatory metal reduction. Annu Rev Microbiol **47:**263-90.
36. **Luu, Y., and J. A. Ramsay.** 2003. Review: microbial mechanisms of accessing insoluble Fe(III) as an energy source. World J Microb Biot **19:**215-225.
37. **Makarova, K. S., and E. V. Koonin.** 2007. Evolutionary genomics of lactic acid bacteria. J Bacteriol **189:**1199-208.
38. **Maruyama, A., Y. Kumagai, K. Morikawa, K. Taguchi, H. Hayashi, and T. Ohta.** 2003. Oxidative-stress-inducible qorA encodes an NADPH-dependent quinone oxidoreductase catalysing a one-electron reduction in *Staphylococcus aureus*. Microbiology **149:**389-98.
39. **Morishita, T., N. Tamura, T. Makino, and S. Kudo.** 1999. Production of menaquinones by lactic acid bacteria. J Dairy Sci **82:**1897-903.
40. **Morita, H., H. Toh, S. Fukuda, H. Horikawa, K. Oshima, T. Suzuki, M. Murakami, S. Hisamatsu, Y. Kato, T. Takizawa, H. Fukuoka, T. Yoshimura, K. Itoh, D. J. O'Sullivan, L. L. McKay, H. Ohno, J. Kikuchi, T. Masaoka, and M. Hattori.** 2008. Comparative Genome Analysis of Lactobacillus reuteri and Lactobacillus fermentum Reveal a Genomic Island for Reuterin and Cobalamin Production. DNA Res **15:**151-61.
41. **Mulkidjanian, A. Y., P. Dibrov, and M. Y. Galperin.** 2008. The past and present of sodium energetics: May the sodium-motive force be with you. Biochim Biophys Acta **1777:**985-992.
42. **Nakano, M. M., and P. Zuber.** 1998. Anaerobic growth of a "strict aerobe" (*Bacillus subtilis*). Annu Rev Microbiol **52:**165-90.

43. **Nes, I. F., D. B. Diep, L. S. Havarstein, M. B. Brurberg, V. Eijsink, and H. Holo.** 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie Van Leeuwenhoek **70:**113-28.

44. **Ogden, N. S., and L. Bielory.** 2005. Probiotics: a complementary approach in the treatment and prevention of pediatric atopic disease. Curr Opin Allergy Clin Immunol **5:**179-84.

45. **Pedersen, M. B., C. Garrigues, K. Tuphile, C. Brun, K. Vido, M. Bennedsen, H. Mollgaard, P. Gaudu, and A. Gruss.** 2008. Impact of aeration and heme-activated respiration on *Lactococcus lactis* gene expression: identification of a heme-responsive operon. J Bacteriol **190:**4903-11.

46. **Pedersen, M. B., S. L. Iversen, K. I. Sorensen, and E. Johansen.** 2005. The long and winding road from the research laboratory to industrial applications of lactic acid bacteria. FEMS Microbiol Rev **29:**611-24.

47. **Pham, M., D. A. Lemberg, and A. S. Day.** 2008. Probiotics: sorting the evidence from the myths. Med J Aust **188:**304-8.

48. **Pritchard, G. G., and J. W. Wimpenny.** 1978. Cytochrome formation, oxygen-induced proton extrusion and respiratory activity in *Streptococcus faecalis* var. *zymogenes* grown in the presence of haematin. J Gen Microbiol **104:**15-22.

49. **Rezaiki, L., B. Cesselin, Y. Yamamoto, K. Vido, E. van West, P. Gaudu, and A. Gruss.** 2004. Respiration metabolism reduces oxidative and acid stress to improve long-term survival of Lactococcus lactis. Mol Microbiol **53:**1331-42.

50. **Rezaiki, L., G. Lamberet, A. Derre, A. Gruss, and P. Gaudu.** 2008. *Lactococcus lactis* produces short-chain quinones that cross-feed Group B *Streptococcus* to activate respiration growth. Mol Microbiol **67:**947-57.

51. **Richardson, D. J.** 2000. Bacterial respiration: a flexible process for a changing environment. Microbiology **146 (Pt 3):**551-71.

52. **Ross, R. P., S. Morgan, and C. Hill.** 2002. Preservation and fermentation: past, present and future. Int J Food Microbiol **79:**3-16.

53. **Rothery, R. A., F. Blasco, A. Magalon, and J. H. Weiner.** 2001. The diheme cytochrome *b* subunit (Narl) of *Escherichia coli* nitrate reductase A (NarGHI):

structure, function, and interaction with quinols. J Mol Microbiol Biotechnol **3:**273-83.

54. **Saxelin, M., S. Tynkkynen, T. Mattila-Sandholm, and W. M. de Vos.** 2005. Probiotic and other functional microbes: from markets to mechanisms. Curr Opin Biotechnol **16:**204-11.
55. **Schafer, G., M. Engelhard, and V. Muller.** 1999. Bioenergetics of the Archaea. Microbiol Mol Biol Rev **63:**570-620.
56. **Senior, A. E.** 1990. The proton-translocating ATPase of *Escherichia coli*. Annu Rev Biophys Biophys Chem **19:**7-41.
57. **Sijpesteijn, A. K.** 1970. Induction of cytochrome formation and stimulation of oxidative dissimilation by hemin in *Streptococcus lactis* and *Leuconostoc mesenteroides*. Antonie Van Leeuwenhoek **36:**335-48.
58. **Soballe, B., and R. K. Poole.** 2000. Ubiquinone limits oxidative stress in *Escherichia coli*. Microbiology **146 (Pt 4):**787-96.
59. **Stiles, M. E., and W. H. Holzapfel.** 1997. Lactic acid bacteria of foods and their current taxonomy. Int J Food Microbiol **36:**1-29.
60. **Tettelin, H., V. Masignani, M. J. Cieslewicz, J. A. Eisen, S. Peterson, M. R. Wessels, I. T. Paulsen, K. E. Nelson, I. Margarit, T. D. Read, L. C. Madoff, A. M. Wolf, M. J. Beanan, L. M. Brinkac, S. C. Daugherty, R. T. DeBoy, A. S. Durkin, J. F. Kolonay, R. Madupu, M. R. Lewis, D. Radune, N. B. Fedorova, D. Scanlan, H. Khouri, S. Mulligan, H. A. Carty, R. T. Cline, S. E. Van Aken, J. Gill, M. Scarselli, M. Mora, E. T. Iacobini, C. Brettoni, G. Galli, M. Mariani, F. Vegni, D. Maione, D. Rinaudo, R. Rappuoli, J. L. Telford, D. L. Kasper, G. Grandi, and C. M. Fraser.** 2002. Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V *Streptococcus agalactiae*. Proc Natl Acad Sci U S A **99:**12391-6.
61. **Teusink, B., A. Wiersma, D. Molenaar, C. Francke, W. M. de Vos, R. J. Siezen, and E. J. Smid.** 2006. Analysis of growth of *Lactobacillus plantarum* WCFS1 on a complex medium using a genome-scale metabolic model. J Biol Chem **281:**40041-8.
62. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev **41:**100-80.

63. **Tsubaki, M., T. Mogi, H. Hori, S. Hirota, T. Ogura, T. Kitagawa, and Y. Anraku.** 1994. Molecular structure of redox metal centers of the cytochrome *bo* complex from *Escherichia coli*. Spectroscopic characterizations of the subunit I histidine mutant oxidases. J Biol Chem **269:**30861-8.

64. **Unden, G., and J. Bongaerts.** 1997. Alternative respiratory pathways of *Escherichia coli*: energetics and transcriptional regulation in response to electron acceptors. Biochim Biophys Acta **1320:**217-34.

65. **Vergnes, A., K. Gouffi-Belhabich, F. Blasco, G. Giordano, and A. Magalon.** 2004. Involvement of the molybdenum cofactor biosynthetic machinery in the maturation of the Escherichia coli nitrate reductase A. J Biol Chem **279:**41398-403.

66. **Vido, K., D. Le Bars, M. Y. Mistou, P. Anglade, A. Gruss, and P. Gaudu.** 2004. Proteome analyses of heme-dependent respiration in *Lactococcus lactis*: involvement of the proteolytic system. J Bacteriol **186:**1648-57.

67. **Wang, G., and R. J. Maier.** 2004. An NADPH quinone reductase of *Helicobacter pylori* plays an important role in oxidative stress resistance and host colonization. Infect Immun **72:**1391-6.

68. **Wegkamp, A.** 2007. Modulation of folate production in lactic acid bacteria. Dissertation. University of Wageningen, Wageningen.

69. **Winstedt, L., L. Frankenberg, L. Hederstedt, and C. von Wachenfeldt.** 2000. *Enterococcus faecalis* V583 contains a cytochrome *bd*-type respiratory oxidase. J Bacteriol **182:**3863-6.

70. **Yamamoto, Y., C. Poyart, P. Trieu-Cuot, G. Lamberet, A. Gruss, and P. Gaudu.** 2005. Respiration metabolism of Group B *Streptococcus* is activated by environmental haem and quinone and contributes to virulence. Mol Microbiol **56:**525-34.

71. **Yamamoto, Y., C. Poyart, P. Trieu-Cuot, G. Lamberet, A. Gruss, and P. Gaudu.** 2006. Roles of environmental heme, and menaquinone, in streptococcus agalactiae. Biometals **19:**205-10.

Chapter 2

Generation of a membrane potential by *Lactococcus lactis* through aerobic electron transport

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Chapter 2 – PMF generation by *Lactococcus lactis* ETC

Abstract

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 in *L. lactis* under these conditions. Proton motive force generation in whole cells was measured using a fluorescent probe ($DiSC_3(5)$), which is sensitive to changes in the membrane potential ($\Delta\psi$). Wild-type cells, grown aerobically in the presence of heme, generated a $\Delta\psi$ even in the presence of the F1-F0 ATPase inhibitor DCCD, while a cytochrome *bd* negative mutant strain (CydAΔ) did not. We also observed high oxygen consumption rates by membrane vesicles prepared from heme-grown cells, when compared to CydAΔ cells, upon addition of NADH. This demonstrates that NADH is an electron donor for the *L. lactis* ETC, and 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.

Introduction

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 aerobic growth of *L. lactis* were therefore mainly focussed on the effect of oxygen on fermentation patterns (26) or cell damage due to the formation of reactive oxygen species (3, 9, 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 (10, 31, 46). 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 (41). 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 before (7, 11, 38). The increased growth efficiency of aerated heme-grown *L. lactis* cells is due to a shift from homo-lactic to mixed-acid fermentation, more complete glucose utilization in non pH-controlled batch cultures and possibly energy-generation by NADH oxidation via the ETC (10). The capacity 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 cell factories 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 (15, 53). Furthermore, the genomes of several *L. lactis* strains contain genes which, when expressed, could form a simple Electron Transfer Chain (ETC) if supplied with heme (14). Genes encoding menaquinone biosynthesis enzymes and the production of a *bd* type cytochrome-containing (mena)quinoloxidase have, for example, been identified in the genomes of IL1403 and SK11 (http://genome.ornl.gov/microbial/lcre/) (6). The (mena)quinoloxidase is a membrane-bound enzyme consisting of 2 subunits, which are encoded by *cydA* and *cydB*. The c*ydC* and *cydD* genes encode an ABC-transporter, required for assembly of the oxidase (8). This type of cytochrome-containing enzyme is found in a variety of (facultative) aerobic bacteria (17), where it functions as an (alternative) terminal electron acceptor capable of working under low-oxygen conditions (18, 44).

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 functionality of the ETC in *L. lactis*.

Materials and Methods.

Cultures and growth conditions.

The strains used in these studies were *Lactococcus lactis* MG1363 (12) or its derivatives: the cytochrome negative mutant (CydAΔ Cm^r) and the cytochrome negative mutant complemented with the plasmid pIL253CydABCD. pIL253CydABCD is a pIL253 derivative (47), carrying the *cydABCD* genes (Cm^r Ery^r). Cells were grown on M17 medium (Difco, Detroit, Mich.), supplemented with glucose (GM17), to a final concentration of 1% (wt/vol). When indicated cells were grown in GM17 supplemented with heme (hemin) (Sigma, stock solution: 0.5 mg/ml in 0.05M NaOH) to a final conc. of 2 μg/ml or with the equivalent volume of 0.05M NaOH as a control. When indicated chloroamphenicol and/or erythromycin were added to a final concentration of 10 μg/ml. Cultures were grown aerobically in 100 ml flasks shaking at 250 rpm or anaerobically in tubes/glass bottles at 30°C.

Annotation of *L. lactis* MG1363 genes

Sequence data of *L. lactis* MG1363 was obtained from the *L. lactis* MG1363 sequencing consortium. DNA sequences of open reading frames (ORF's) were translated into amino acid sequences and annotated by homology using the BLAST algorithm as described (1). Prediction of membrane spanning helices was performed as described (54).

Mutant construction.

Molecular cloning techniques were carried out in accordance with standard laboratory procedures (45). For the construction of the knock-out plasmid, primers were designed on the basis of MG1363 genome sequence data. A 1 kb fragment upstream (forward primer p39 GATCGTCAAC CATCAACCAT, reverse primer p40 GGTTAGCATT

GTTTATCTCC) and downstream of *cydA* (forward primer p41 GTGGATGAAT AATGACTGGA, reverse primer p42 CCAGCGATAG CAATAAACTG) were PCR amplified. The flanking fragments were cloned blunt-ended in vector pNZ5317 (24) digested with SwaI (upstream fragment) and Ecl36II (downstream fragment) to produce the knock-out vector pRB6671_CydA_KO. The knock-out plasmid was transformed into *L. lactis* MG1363 and a chloramphenicol replacement of the *cydA* gene was obtained by a double cross over event by homologous recombination as described (23), which resulted in the mutant strain CydAΔ. For complementation studies of the cytochrome-negative mutant (CydAΔ) a vector was constructed carrying the cyd-operon (*cydABCD*). The operon was amplified using PCR techniques (forward primer P43: TGACGCATGC GAGGCCTCAAGAAAGCACTT, reverse primer P44: TGAC GAGCTC CGTAGACGAGTAACGCATCT), using the genome of MG1363 as template. The primer tails (underlined) carried recognition sequences for restriction enzymes SphI and SacI for easy cloning. The PCR product and the vector pIL253 were digested with SphI and SacI, purified from gel, and cloned sticky-blunt to construct pIL253_CydABCD. Finally to complement the cytochrome-negative mutant this plasmid was transformed into the CydAΔ strain.

Isolation of membrane vesicles

Cells from a 2 liter culture were grown aerobically to late-exponential phase (~OD 2.5-3.0), washed twice in 100mM potassium phosphate (pH7.0) and re-suspended in 20ml of the same buffer. The cell suspension was incubated with 10mg/ml egg-lysozyme (Merck, Darmstadt, Germany) for 30 min at 30°C. Cell lysis was achieved by 2 times passage through a French pressure cell (American Instrument Corp., Silver Spring, Md.) at an operating pressure of 20.000 psi. The orientation of bacterial membrane-vesicles prepared by French press is predominantly inside-out (2, 28). The suspension was supplemented with 10mM $MgSO_4$ and 100 µg/ml DNAse and incubated for 15 min at 30°C, after which 15mM K-EDTA was added. A low-spin at 12.000 rpm was performed to remove cell-debris and whole cells. The vesicle-containing supernatant was centrifuged at 150.000g to harvest the membranes, which were re-suspended in 50 mM potassium phosphate pH 7.0, containing 10% glycerol, to a final conc. of 10-20 µg/ml, divided in 500 µl aliquots and stored at -80°C.

Extrusion of membrane vesicles

To obtain single unilamellar vesicles, suitable for comparing enzyme activities, 500 µl membrane suspension was thawed and diluted with 500 µl 50 mM potassium phosphate (pH5.5). The 1 ml mixture was extruded using a mini-extruder (Avanti polar lipids Inc., Alabaster, UK) with a 0.4 µm size nucleopore polycarbonate track-etch membrane (Whatman International Ltd., Kent, UK) to generate inside-out, single laminar vesicles with an average size of 0.4 µm (51).

Measurements of membrane potential

The fluorescent probe $DiSC_3(5)$ (3',3'-dipropylthiadicarbocyanine) was used to monitor the membrane potential ($\Delta\Psi$) in intact cells (52). The distribution of the probe over the cytoplasmic membrane and the soluble phase is sensitive to changes in the $\Delta\Psi$. More probe molecules, from the soluble phase, will dissolve in the membrane with increasing $\Delta\Psi$, causing quenching of the fluorescence signal by aggregation (21, 49). Nigericin (K^+/H^+ exchange) was added to convert ΔpH into $\Delta\Psi$, 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, USA) or an SPF-500C spectrofluorometer (SLM Aminco). The fluorescence was measured at an emission of 666 nm with an excitation of 643 nm (both with a 5 nm band pass).

Wild-type and CydAΔ cells were supplemented with heme and grown aerobically. During growth, samples of cells were harvested at early- (OD_{600} 0.4-0.48), early-/ mid- (OD_{600} 1.04-1.08), mid- (OD_{600} 1.49-1.53) and overnight (OD_{600} wild-type 4.49, OD_{600} CydAΔ 2.6) growth phases. Cell samples were washed twice in 50 mM KPi (pH 5.0) and resuspended in the same buffer to an optical density at 600 nm (OD_{600}) of 5.0. Subsequently, samples were diluted to an OD_{600} of 0.3 and DCCD (N,N'-dicyclohexylcarbodiimide, Sigma-Aldrich)(stock: 1M in ethanol) was added to a final concentration of 1 mM when indicated or as a control the equivalent volume of ethanol. DCCD is a well-known inhibitor of the F1-F0 ATPase and prevents PMF formation by hydrolysis of ATP (16, 48). The samples were incubated for 45 min on ice in the presence of DCCD or ethanol. After incubation, 2 ml fresh

buffer was added to the 1 ml samples, to optimize the cell density for measuring fluorescence, after which the samples were transferred to 3 ml cuvets. Finally $DiSC_3(5)$ was added to a final conc. of 133 nM. Cuvets containing this mixture were warmed at room temperature for 3 min prior to measuring; 15 mM glucose, 0.1 μM nigericin or 2 μM valinomycin were added when indicated.

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). To measure oxygen-comsumption, cells were washed twice in 50 mM potassium phosphate (pH 5.0) and resuspended in the same buffer to OD_{600} 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 point zero, cells were added to a final conc. of OD_{600} of 0.2. After 5 min 13 mM glucose was added. The DO of air-saturated buffer was calibrated using air-saturated water. To measure oxygen-consumption by membrane vesicles, 1 ml membrane vesicle-mixture was added to 10ml 50 mM potassium phosphate (pH 5.0). After 5 min either 5 mM NADH or NAD^+ was added (both obtained from Sigma-Aldrich).

Other analytical procedures.

Protein concentrations of membrane preparations were determined using the bicinchoninic acid protein assay reagent (Omnilabo Int., Breda, the Netherlands) (50).

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-2 NADH-dehydrogenases, encoded by *noxA* and *noxB* (Table 1), were predicted on the basis of the *L. lactis* MG1363 genome sequence (14). Both genes are characterized as having FAD binding-motifs, but differ in the number of predicted membrane spanning segments (four in NoxB and one in Nox1). Although the operon-like structure of *noxA* and *noxB* on the genome suggests co-regulation (29), these type-2 NADH dehydrogenases are always observed to function as separate, individual, polypeptides. The genes for biosynthesis of riboflavin – the precursor for FAD - can also be found and the biosynthesis of this vitamin by L. lactis has been experimentally verified in L. lactis MG1363 (6). Menaquinone production has been observed in many *L. lactis* strains (34) and menaquinone biosynthesis genes (*menFDXBEC*, *preA-menA*) were identified.

ORF *L. lactis* MG1363	Homology to *Bac. subtilis* 168	DNA-identity / AA-identity[a]	identity
llmg_1864	*cydA*	48% / 68%	Cytochrome d ubiquinol oxidase subunit I (EC 1.10.3.-)
llmg_1883	*cydB*	43% / 62%	Cytochrome d ubiquinol oxidase subunit II (EC 1.10.3.-)
llmg_1862	*cydC*	47% / 65%	ABC transporter component CydC
llmg_1861	*cydD*	44% / 64%	ABC transporter component CydD
	Homology to *L. lactis* IL1403		
llmg_0196	*preA*	95% / 98%	Farnesyl pyrophosphate synthetase (EC 2.5.1.1)
llmg_0197	*ybiG* / *menA*	88%/ 95%	1,4-dihydroxy-2-naphthoate polyprenyltransferase (EC 2.5.1.-)
llmg_1828	*menF*	80% / 89%	Isochorismate synthase (EC 5.4.4.2)
llmg_1829	*menD*	85% / 91%	2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase (EC 2.5.1.64)

ORF *L. lactis* MG1363	Homology to *L. lactis* IL1403	DNA-identity / AA-identity[a]	identity
llmg_1830	*menX*	77% / 85%	YtxM-like prot. / Menaquinone biosynthesis related prot.
llmg_1831	*menB*	98% / 100%	Naphthoate synthase (EC 4.1.3.36)
llmg_1832	*menE*	85% / 93%	O-succinylbenzoic acid-CoA ligase (EC 6.2.1.26)
llmg_1833	*yhdB* / *menC*	91% / 97%	O-succinylbenzoate synthase (EC 4.2.1.-)
llmg_1735	*noxA*	99% / 99%	NADH dehydrogenase (EC 1.6.99.3)
llmg_1734	*noxB*	99% / 99%	NADH dehydrogenase (EC 1.6.99.3)

[a] Predicted identity on the level of amino acid composition

Table 1. Identification by homology searches of NADH-dehydrogenase, (mena)quinoloxidase and menaquinone biosynthesis genes in the genome of *L. lactis* MG1363.

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 characterised *Bacillus subtilis* strain 168 *cyd*-genes (56, 58, 59). 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 1 H^+/e^-, during the reduction of oxygen to water (17, 33, 40). 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)

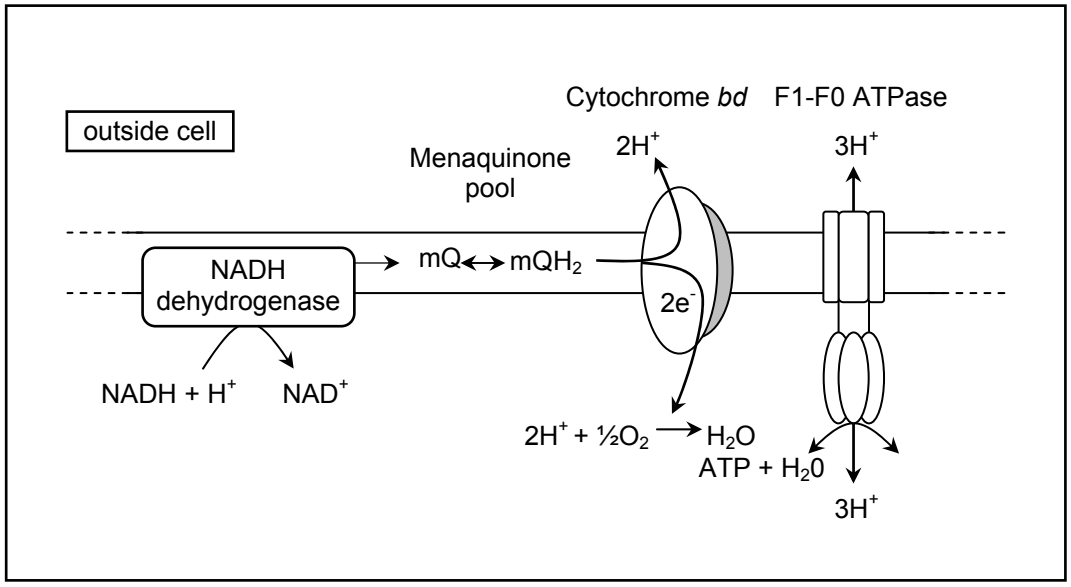

Figure 1. Predicted aerobic ETC of heme-grown *L. lactis* MG1363. The three principal components of the ETC are the type II NADH-dehydrogenase complex, the menaquinol/menaquinone couple and the cytochrome *bd* complex. A PMF can be also formed by the hydrolysis of ATP via F1-F0 ATPase.

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 2), suggesting the presence of a functional ETC.

We constructed an isogenic mutant of *L. lactis* MG1363 that lacks these characteristics by replacing the *cydA* gene with a chloramphenicol marker-gene (CydAΔ). As anticipated, the increased biomass formation and higher pH observed with aerated heme-grown wild-type cells was absent in the CydAΔ strain, and the growth characteristics resembled that of non-heme grown wild-type cells. Subsequent complementation of the CydAΔ mutant with a vector carrying the (mena)quinoloxidase coding operon (pIL253CydABCD) restored the wild-type-like phenotype when grown aerobically with heme.

Strain	OD600	pH
Wild-type + heme	5.25	5.04
Wild-type	2.60	4.34
CydAΔ + heme	2.57	4.33
CydAΔ	2.66	4.27
CydAΔ + pIL253CydABCD + heme	5.07	5.06
CydAΔ +pIL253CydABCD	2.62	4.30

Table 2. Aerobic growth (OD_{600}) and acidification of *L. lactis* with and without heme. *L. lactis* wild-type, CydAΔ and CydAΔ cells, complemented with the plasmid pIL253CydABCD (carrying the *cydABCD* operon), were grown, aerobically, overnight in M17-medium, at 30^{o}C in the presence/absence of heme.

Measurement of membrane potential 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 $DiSC_3(5)$. The intensity of fluorescence of the probe is sensitive to changes in the membrane potential (ΔΨ), it decreases with increasing ΔΨ and *visa versa*. The proton motive force (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, 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-F0 ATPase, by pumping protons at the expense of metabolic ATP (27) . To discriminate between PMF generation by the ETC and the F1-F0 ATPase, the F1-F0 ATPase-specific inhibitor DCCD (N,N'-dicyclohexylcarbodiimide) was used (48). To further validate PMF formation by the ETC, we used the cytochrome *bd*-negative mutant (CydAΔ) as a control. The changes in membrane potential, $DiSC_3(5)$ fluorescence, were recorded as a function of time (Fig 2).

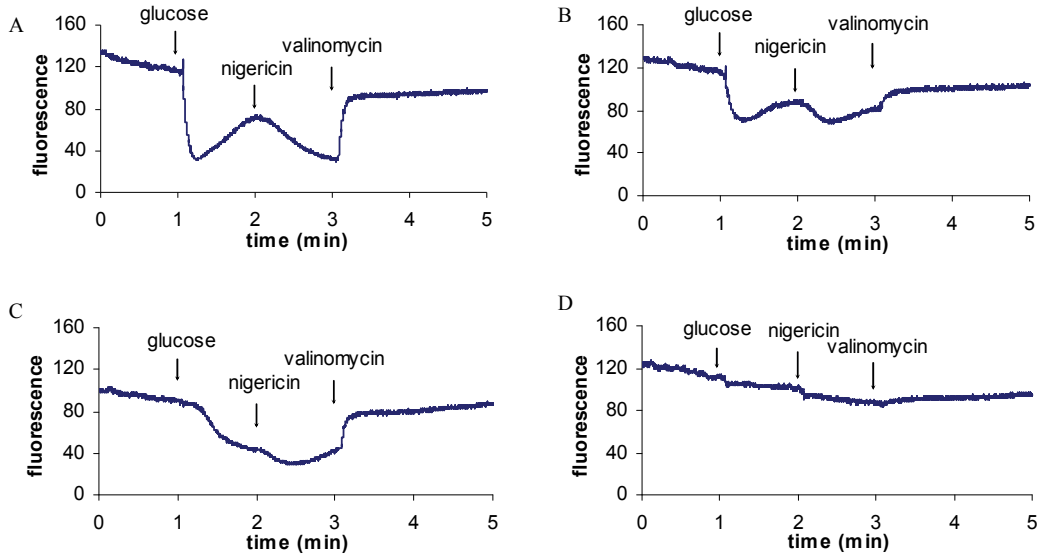

Figure 2. Fluorescence traces of $DiSC_3(5)$ in whole cells of *L.lactis*, showing the generation of a membrane potential. Cell were prepared as described in M&M. A decrease in fluorescence signifies an increase in membrane potential. At t=1 min, glucose (15 mM) was added; at 1=2 min, nigericin (0.1 μM); and at t= 3 min, valinomycin (2 μM). A: wild-type cells; B: wild-type cell treated with DCCD; C: CydAΔ cells; and D: CydAΔ cells treated with DCCD.

In wild-type cells and CydAΔ cells with no DCCD-treatement, addition of glucose led to an increase in ΔΨ. However, for CydAΔ 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 the discussion. Accordingly, the addition of 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 CydAΔ cells (Fig. 2, traces B and C) was caused by the excess of nigericin and could be prevented by using lower nigericin concentrations (data not shown). Subsequent addition of valinomycin dissipated the ΔΨ completely. The fluorescence measurements clearly show that the cytochrome-negative CydAΔ strain is unable to generate a ΔΨ when the F1-F0 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 (10, 13, 55). To investigate this hypothesis, the ETC activity measurements were performed in cells harvested at different phases of growth, corresponding to early-, mid- and late exponential (OD_{600} of ~0.5, ~1.0, and ~1.5, respectively), and the late stationary phase (overnight cultures). A clear build-up of $\Delta\Psi$ was observed upon addition of glucose to DCCD-treated and untreated wild-type cells, and untreated CydAΔ cells but never in DCCD-treated CydAΔ cells, irrespective of the growth phase (table 3). 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 phase.

Strain	OD_{600} (phase)	% fluorescence drop	
		-	+DCCD
wild-type	0.48 (early-)	60.7	26.7
CydAΔ	0.4 (early-)	40.0	5.1
wild-type	1.08 (early-/mid-)[a]	71.8	37.2
CydAΔ	1.04 (early-/mid-)[a]	58.6	4.9
wild-type	1.53 (mid-)	38.0	22.1
CydAΔ	1.49 (mid-)	46.9	7.0
wild-type	4.49 (O/N)[b]	46.9	63.1
CydAΔ	2.6 (O/N)[b]	45.7	5.7

[a] These fluorescence traces are described in detail in figure 2.

[b\] Overnight culture were grown more than 20 hours and considered (late) stationary cells.

Table 3. Relative drop in fluorescence of early-, mid-, and overnight (O/N) (or late-stationary) cultures of DCCD (un)treated wild-type and CydAΔ cells, after glucose addition. The % fluorescence drop compares two fluorescence-values in one trace at one time-point, with 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 M&M.

Rate of Oxygen uptake by whole cells

The terminal electron acceptor of aerobic ETC in *L. lactis* is oxygen, through activity of the oxygen-requiring cytochome *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 on oxygen uptake, aerobically-grown, early exponential phase cells (OD_{600} 0.5-0.58) were used, rather than stationary phase cells to eliminate possible differences in cell viability. Upon addition of glucose, heme-supplemented wild-type cells showed a higher oxygen consumption rate than heme-supplemented CydAΔ cells or wild-type cells grown in the absence of heme (Table 4).

Strain	OD 0.50-0.58	
Wild-type +heme	25.72	± 2.76
Wild-type	13.02	± <0.01
CydAΔ+heme	13.51	± 0.02
Buffer +heme	Nd[a]	

[a]Nd, not detected

Table 4. Oxygen consumption rates (nmol O_2 depletion/min/mg dry weight) of washed cells in 50mM potassium phosphate (pH5.0) at 30 °C, after addition of 13mM glucose. Cells were aerobically grown *L. lactis* wild-type and CydAΔ cells, incubated with heme or without heme. 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 vescicles

We propose a model of a simple ETC in *L. lactis*, which uses NADH as an electron donor and oxygen as electron acceptor (Fig. 1). Oxygen consumption by membrane vesicles of aerobically grown wild-type cells (with and whitout heme) and CydAΔ cells (with heme) was measured (table 5).

Membrane preparation	Addition of	umol O_2 consumption / min/ mg prot.
wild-type +heme	NADH	42.18 ± 0.12
wild-type	NADH	6.62 ± 0.35
CydAΔ+heme	NADH	8.41 ± 1.32
Wild-type +heme	NAD	Nd[a]

[a]Nd, no consumption of oxygen detected after addition of NAD

Table 5. Oxygen consumption by membrane vesicles at 25 °C, after addition of NADH or NAD. Membrane vesicles were prepared from the different cells as decribed in M&M.

After addition of NADH, membrane vesicles prepared from heme-grown wild-type cells showed a more then 6.5 fold increase in oxygen consumption when compared to membrane vesicles from wild-type cells grown without heme and heme-grown CydAΔ cells. The oxygen consumption by the latter two was 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 the nicotinamide adenine dinucleotide. Furthermore, NADH added to buffer without membrane vesicles led only to a small level 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 proton motive force (PMF). A cytochrome *bd*-negative mutant was constructed (CydAΔ), as a negative control lacking the respiratory phenotype. DCCD inhibits the F1-F0 ATPase, the primary PMF-generating system in fermentative LAB using ATP as the energy source (20). DCCD-treated heme-supplemented wild-type cells were still capable of generating a PMF, while in contrast DCCD-treated heme-supplemented CydAΔ cells could not. These two observations demonstrate that heme-supplemented wild-type cells have an additional PMF-generating system other than 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 addition of glucose, to DCCD-treated CydAΔ
cells is explained by incomplete inhibition of the F1-F0 ATPase by DCCD (24). In addition to the F1-F0 ATPase and the ETC, the contribution to the overall energy conservation in *L. lactis* of alternative mechanisms of PMF generation, if present at all, seems minimal (25, 30, 35, 36). What is clearly seen in the fluorescence recordings of the heme-supplemented wild-type cells is that addition of glucose leads to an initial rapid increase in $\Delta\Psi$ and a subsequent conversion into a ΔpH. This conversion in ΔpH is deduced from the increase in $\Delta\Psi$ upon 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 $\Delta\Psi$. To generate a ΔpH of similar size, the cell needs to pump out far more protons and this would lead to a very high $\Delta\Psi$. Therefore, mechanisms are present to increase the ΔpH at the expense of $\Delta\Psi$, i.e., through the electrogenic uptake of K^+ ions which allows more protons to be pumped out (4, 22, 37).

Respiring cells (aerated and heme-supplemented) from an exponentially growing culture showed an increased oxygen consumption rate when compared to similarly grown cells containing a disruption in the cytochrome-genes or when compared to non-heme grown wild-type cells. These results confirm that the *L. lactis* ETC leads to reduction of oxygen. An indication for 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 phases.

In this work and that of others (14), it is proposed that NADH is an important electron donor for the ETC, which explains the observation of mixed fermentation under respiratory conditions. Membrane vesicles prepared from wild-type cells grown with heme showed a more than 6.5 fold increase in oxygen consumption compared to wild-type cells grown without heme and CydAΔ cells with heme. Furthermore, this oxygen consumption was dependent on the reduced form of nicotinamide adenine dinucleotide (NADH). We have thus clearly demonstrated that NADH is a likely electron donor for the ETC in *L. lactis* and a membrane-bound NADH dehydrogenase is present.

When NADH is added to membrane preparations 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 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 in growth and metabolic conversion. Heme-dependent respiration is therefore most likely a trait which 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 the light of this study, a better definition of *L. lactis* would be a facultative aerobe. It is still largely unknown, how many other lactic acid bacteria exist with a similar facultative aerobic metabolism. An extensive screening among the different species (*Pediococci, Lactococci, Lactobacilli*) is required to better define and characterize lactic acid bacteria as a group. Some information on the respiratory capabilities of a limited number of lactic acid bacteria, mostly *streptococci* and *enterococcae*, can already be found in literature (42, 43, 46, 57, 60, 61). Interestingly, analysis of the large 3.3 MB genome of *Lactobacillus plantarum* WCFS revealed the presence of genes coding for a fumurate reductase and heme-dependent nitrate reductase complex, creating a branched ETC capable of oxygen and nitrate respiration (19, 39). 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 lactic acid bacteria could result in improved industrially important traits (higher biomass/gram carbon-source, increased acid stress, oxygen stress, survival rate when stored at low temperatures), making the organisms even more attractive as cell factories.

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1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:**3389-402.
2. **Ames, G. F., K. Nikaido, J. Groarke, and J. Petithory.** 1989. Reconstitution of periplasmic transport in inside-out membrane vesicles. Energization by ATP. J Biol Chem **264:**3998-4002.
3. **Anders, R. F., D. M. Hogg, and G. R. Jago.** 1970. Formation of hydrogen peroxide by group N *streptococci* and its effect on their growth and metabolism. Appl Microbiol **19:**608-12.
4. **Bakker, E. P., and W. E. Mangerich.** 1981. Interconversion of components of the bacterial proton motive force by electrogenic potassium transport. J Bacteriol **147:**820-6.
5. **Blank, L. M., B. J. Koebmann, O. Michelsen, L. K. Nielsen, and P. R. Jensen.** 2001. Hemin reconstitutes proton extrusion in an H^+-ATPase-negative mutant of *Lactococcus lactis*. J. Bacteriol. **183:**6707-9.
6. **Bolotin, A., P. Wincker, S. Mauger, O. Jaillon, K. Malarme, J. Weissenbach, S.**
D. Ehrlich, and A. Sorokin. 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis ssp. lactis* IL1403. Genome Res **11:**731-53.
7. **Christel, G., E. Johansen, M. B. Pedersen, H. Mollgaard, K. I. Sorensen, P. Gaudu, A. Gruss, and G. Lamberet.** 2006. Getting high (OD) on heme. Nat Rev Microbiol **4:**c2; author reply c3.
8. **Cruz-Ramos, H., G. M. Cook, G. Wu, M. W. Cleeter, and R. K. Poole.** 2004. Membrane topology and mutational analysis of *Escherichia coli* CydDC, an ABC-type cysteine exporter required for cytochrome assembly. Microbiology **150:**3415-27.
9. **Duwat, P., S. D. Ehrlich, and A. Gruss.** 1995. The *recA* gene of *Lactococcus lactis*: characterization and involvement in oxidative and thermal stress. Mol Microbiol **17:**1121-31.
10. **Duwat, P., S. Sourice, B. Cesselin, G. Lamberet, K. Vido, P. Gaudu, Y. Le Loir, F. Violet, P. Loubiere, and A. Gruss.** 2001. Respiration capacity of the fermenting

bacterium *Lactococcus lactis* and its positive effects on growth and survival. J Bacteriol **183:**4509-16.

11. **Duwat, P., S. Sourice, and A. Gruss.** 1998. Process for preparing starter cultures of lactic acid bacteria. French patent application FR9809463.
12. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J Bacteriol **154:**1-9.
13. **Gaudu, P., G. Lamberet, S. Poncet, and A. Gruss.** 2003. CcpA regulation of aerobic and respiration growth in *Lactococcus lactis*. Mol Microbiol **50:**183-92.
14. **Gaudu, P., K. Vido, B. Cesselin, S. Kulakauskas, J. Tremblay, L. Rezaiki, G. Lamberret, S. Sourice, P. Duwat, and A. Gruss.** 2002. Respiration capacity and consequences in *Lactococcus lactis*. Antonie Van Leeuwenhoek **82:**263-9.
15. **Gennis, R. B.** 1987. The cytochromes of *Escherichia coli*. FEMS Microbiol. **46:**387-399.
16. **Harold, F. M., J. R. Baarda, C. Baron, and A. Abrams.** 1969. Inhibition of membrane-bound adenosine triphosphatase and of cation transport in *Streptococcus faecalis* by N,N'-dicyclohexylcarbodiimide. J Biol Chem **244:**2261-8.
17. **Jünemann, S.** 1997. Cytochrome *bd* terminal oxidase. Biochimica et Biophysica acta **1321:**107-127.
18. **Kita, K., K. Konishi, and Y. Anraku.** 1986. Purification and properties of two terminal oxidase complexes of *Escherichia coli* aerobic respiratory chain. Methods Enzymol **126:**94-113.
19. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc Natl Acad Sci U S A **100:**1990-5.
20. **Konings, W. N.** 2002. The cell membrane and the struggle for life of lactic acid bacteria. Antonie Van Leeuwenhoek **82:**3-27.
21. **Krasne, S.** 1980. Interactions of voltage-sensing dyes with membranes. II. Spectrophotometric and electrical correlates of cyanine-dye adsorption to membranes. Biophys J **30:**441-62.

22. **Krulwich, T. A.** 1983. Na+/H+ antiporters. Biochim Biophys Acta **726:**245-64.
23. **Lambert, J. M., R. S. Bongers, and M. Kleerebezem.** 2006. A Cre-*lox* based system for multiple gene deletions and selectable marker removal in *Lactobacillus plantarum*. Appl Environ Microbiol **Epub ahead of print**.
24. **Leimgruber, R. M., C. Jensen, and A. Abrams.** 1981. Purification and characterization of the membrane adenosine triphosphatase complex from the wild-type and N,N'- dicyclohexylcarbodiimide-resistant strains of *Streptococcus faecalis*. J Bacteriol **147:**363-72.
25. **Lolkema, J. S., B. Poolman, and W. N. Konings.** 1995. Role of scalar protons in metabolic energy generation in lactic acid bacteria. J Bioenerg Biomembr **27:**467-73.
26. **Lopez de Felipe, F., M. Kleerebezem, W. M. de Vos, and J. Hugenholtz.** 1998. Cofactor engineering: a novel approach to metabolic engineering in *Lactococcus lactis* by controlled expression of NADH oxidase. J Bacteriol **180:**3804-8.
27. **Maloney, P. C.** 1982. Energy coupling to ATP synthesis by the proton-translocating ATPase. J Membr Biol **67:**1-12.
28. **Matsuura, K., and M. Nishimura.** 1977. Sidedness of membrane structures in *Rhodopseudomonas sphaeroides*. Electrochemical titration of the spectrum changes of carotenoid in spheroplasts, spheroplast membrane vesicles and chromatophores. Biochim Biophys Acta **459:**483-91.
29. **Melo, A. M., T. M. Bandeiras, and M. Teixeira.** 2004. New insights into type II NAD(P)H:quinone oxidoreductases. Microbiol Mol Biol Rev **68:**603-16.
30. **Michels PAM, M. J., Boonstra J, Konings WN.** 1979. Generation of a electrochemical proton gradient in bacteria by the excretion of metabolic end products. FEMS Microbiol.Lett. **53:**357-364.
31. **Mickelson, M. N.** 1972. Glucose degradation, molar growth yields, and evidence for oxidative phosphorylation in *Streptococcus agalactiae*. J Bacteriol **109:**96-105.
32. **Miyoshi, A., T. Rochat, J. J. Gratadoux, Y. Le Loir, S. C. Oliveira, P. Langella, and V. Azevedo.** 2003. Oxidative stress in *Lactococcus lactis*. Genet Mol Res **2:**348-59.
33. **MJ Miller, a. R. G.** 1985. The cytochrome *d* complex is a coupling site in the aerobic respiratory chain of *Escherichia coli*. J. Biol. Chem. **260:**14003-14008.

34. **Morishita, T., N. Tamura, T. Makino, and S. Kudo.** 1999. Production of menaquinones by lactic acid bacteria. J Dairy Sci **82:**1897-903.

35. **Otto R, H. J., Konings WN, Veldkamp H.** 1980. Increase of molar growth yield of *Streptococcus cremoris* for lactose as a consequence of lactate consumption by *Pseudomonas stutzeri* in mixed cultures. FEMS Microbiol. Lett.

36. **Otto, R., A. S. Sonnenberg, H. Veldkamp, and W. N. Konings.** 1980. Generation of an electrochemical proton gradient in *Streptococcus cremoris* by lactate efflux. Proc Natl Acad Sci U S A **77:**5502-6.

37. **Padan, E., and S. Schuldiner.** 1987. Intracellular pH and membrane potential as regulators in the prokaryotic cell. J Membr Biol **95:**189-98.

38. **Pedersen, M. B., S. L. Iversen, K. I. Sorensen, and E. Johansen.** 2005. The long and winding road from the research laboratory to industrial applications of lactic acid bacteria. FEMS Microbiol Rev **29:**611-24.

39. **Philippot, L., and O. Hojberg.** 1999. Dissimilatory nitrate reductases in bacteria. Biochim Biophys Acta **1446:**1-23.

40. **Puustinen, A., M. Finel, T. Haltia, R. B. Gennis, and M. Wikstrom.** 1991. Properties of the two terminal oxidases of *Escherichia coli*. Biochemistry **30:**3936-42.

41. **Rezaiki, L., B. Cesselin, Y. Yamamoto, K. Vido, E. van West, P. Gaudu, and A. Gruss.** 2004. Respiration metabolism reduces oxidative and acid stress to improve long-term survival of *Lactococcus lactis*. Mol Microbiol **53:**1331-42.

42. **Ritchey, T. W., and H. W. Seeley.** 1974. Cytochromes in *Streptococcus faecalis* var. *zymogenes* grown in a haematin-containing medium. J Gen Microbiol **85:**220-8.

43. **Ritchey, T. W., and H. W. Seely, Jr.** 1976. Distribution of cytochrome-like respiration in streptococci. J Gen Microbiol **93:**195-203.

44. **Sakamoto, J., E. Koga, T. Mizuta, C. Sato, S. Noguchi, and N. Sone.** 1999. Gene structure and quinol oxidase activity of a cytochrome *bd*-type oxidase from *Bacillus stearothermophilus*. Biochim Biophys Acta **1411:**147-58.

45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed.

46. **Sijpesteijn, A. K.** 1970. Induction of cytochrome formation and stimulation of oxidative dissimilation by hemin in *Streptococcus lactis* and *Leuconostoc mesenteroides*. Antonie Van Leeuwenhoek **36:**335-48.

47. **Simon, D., and A. Chopin.** 1988. Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. Biochimie **70:**559-66.

48. **Simoni, R. D., and P. W. Postma.** 1975. The energetics of bacterial active transport. Annu Rev Biochem **44:**523-54.

49. **Sims, P. J., A. S. Waggoner, C. H. Wang, and J. F. Hoffman.** 1974. Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. Biochemistry **13:**3315-30.

50. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal Biochem **150:**76-85.

51. **Subbarao, N. K., R. I. MacDonald, K. Takeshita, and R. C. MacDonald.** 1991. Characteristics of spectrin-induced leakage of extruded, phosphatidylserine vesicles. Biochim Biophys Acta **1063:**147-54.

52. **Suzuki, H., Z. Y. Wang, M. Yamakoshi, M. Kobayashi, and T. Nozawa.** 2003. Probing the transmembrane potential of bacterial cells by voltage-sensitive dyes. Anal Sci **19:**1239-42.

53. **Thony-Meyer, L.** 1997. Biogenesis of respiratory cytochromes in bacteria. Microbiol Mol Biol Rev **61:**337-76.

54. **Tusnady, G. E., and I. Simon.** 1998. Principles governing amino acid composition of integral membrane proteins: application to topology prediction. J Mol Biol **283:**489-506.

55. **Vido, K., D. Le Bars, M. Y. Mistou, P. Anglade, A. Gruss, and P. Gaudu.** 2004. Proteome analyses of heme-dependent respiration in *Lactococcus lactis*: involvement of the proteolytic system. J Bacteriol **186:**1648-57.

56. **von Wachenfeldt, C., and L. Hederstedt.** 1992. Molecular biology of *Bacillus subtilis* cytochromes. FEMS Microbiol Lett **79:**91-100.

57. **Winstedt, L., L. Frankenberg, L. Hederstedt, and C. von Wachenfeldt.** 2000. *Enterococcus faecalis* V583 contains a cytochrome *bd*-type respiratory oxidase. J Bacteriol **182:**3863-6.

58. **Winstedt, L., and C. von Wachenfeldt.** 2000. Terminal oxidases of *Bacillus subtilis* strain 168: one quinol oxidase, cytochrome aa_3 or cytochrome *bd*, is required for aerobic growth. J Bacteriol **182:**6557-64.

59. **Winstedt, L., K. Yoshida, Y. Fujita, and C. von Wachenfeldt.** 1998. Cytochrome *bd* biosynthesis in *Bacillus subtilis*: characterization of the *cydABCD* operon. J Bacteriol **180:**6571-80.

60. **Yamamoto, Y., C. Poyart, P. Trieu-Cuot, G. Lamberet, A. Gruss, and P. Gaudu.** 2005. Respiration metabolism of Group B *Streptococcus* is activated by environmental haem and quinone and contributes to virulence. Mol Microbiol **56:**525-34.

61. **Yamamoto, Y., C. Poyart, P. Trieu-Cuot, G. Lamberet, A. Gruss, and P. Gaudu.** 2006. Roles of environmental heme, and menaquinone, in *Streptococcus agalactiae*. Biometals **19:**205-10.

Chapter 3

Heme and menaquinone induced electron transport in lactic acid bacteria

R.J.W. Brooijmans, F. Santos, B. A. Smit, W. M. de Vos, J. Hugenholtz. Heme and menaquinone induced electron transport in lactic acid bacteria. Submitted to Applied and Environmental Microbiology

Abstract

Heme- (and menaquinone) induced respiration-like behavior was observed as novel trait for several species and genera of lactic acid bacteria. These include *Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacilllus brevis, Lactobacillus paralimentarius, Streptococcus entericus* and *Lactococcus garviae*. The respiration- associated traits, including increased biomass production without further acidification, are suitable for high-throughput screening as demonstrated by the screening of 8000 *Lactococcus lactis* insertion mutants. Among the respiration-negative insertion-mutants were those that contained NoxA, *bd*-type cytochrome and menaquinol biosynthesis gene-disruptions. Phenotypic screening and *in silico* genome analysis suggest that respiration can be considered characteristic for certain species.

We propose that the *cyd*-genes were present in the common ancestor of lactic acid bacteria, and that multiple gene-loss events best explains the observed distribution of these genes among the species.

Chapter 3 – Heme and menaquinone induced electron transport in LAB

Introduction

Lactic acid bacteria are a group of industrially and economically important bacteria that are extensively used to produce fermented foods. Diverse substrates, such as milk, meat, fruit and vegetables are fermented by certain (consortia that include) lactic acid bacteria to produce foods with improved shelf-life, taste and nutritional properties (2, 14, 28). Furthermore, the consumption of certain lactic acid bacteria themselves may benefit human health by preventing or ameliorate diseases, coined probiotics (13, 19). Lactic acid bacteria are typically cultivated in (micro)anaerobic environments and (historically) classified as obligate fermentative, non-respiring, facultative anaerobes.

Since the early seventies, however, observations were made that heme stimulated growth of several lactic acid bacteria, such as *Lactococcus lactis*, *Enterococcus faecalis*, several *Streptococcus* species and *Leuconostoc mesenteriodes*, when cultivated aerobically. Heme is an essential cofactor of cytochromes, and by extension many bacterial respiratory chains. Indeed in the presence of heme cytochrome formation was observed in these species (5, 22, 23, 25).

Recent experimental work provided conclusive evidence of respiration in *Lactococcus lactis*. The existence of a functional electron transport chain was demonstrated that is capable of generating a proton motive force. This chain included an essential heme-dependent menaquinol-oxidase, a *bd*-type cytochrome encoded by *cydABCD*, (4, 30). Respiration dramatically alters the phenotype, as it improves growth-efficiency and robustness (improved stress resistance) (8, 10). These respiration-associated traits have industrial relevance, as shown by industrial application and patent applications for improved production of starter cultures (7, 20).

Although several lactic acid bacteria were found over the years that show a similar heme response as *Lactococcus lactis* a structured investigation of the distribution of this trait in lactic acid bacteria has remained unpublished (35, 36). It remains unclear, for example, whether respiration is a rare or common trait among the various species and genera of lactic acid bacteria. In this paper we explore the distribution of the respiratory genes (*cydABCD*) and respiration-like behaviour among diverse set of lactic acid bacteria.

Materials and methods

Bacterial strains and growth conditions

For a full list of bacterial strains and media used in this study see table 1.

***Lactobacillus plantarum* strains used in this study**

designation		source	
NCC	**other**	**Isolated from**	**Location**
B253	old3625, Qg	Cheese Q15	
B1836	WCFS1	Human saliva	England
B1837	299	Human colon	UK
B1838	CIP104440	Human stool	France
B1839	SF2A35B	Sour cassava	South America
B1840	NCIMB12120	Ogi	Nigeria
B2029	MLC43	raw cheese with rennet	
B2256	CIP104441	Human stool	France
	CIP104448	Human stool	France
B2257	CIP1044550	Human stool	France
B2258	CIP104451	Human urine	France
B2259	CIP104452	Human tooth abscess	France
B2260	299v	Human intestine	UK
B2261	NC8	Gras silage	
B2262	LM3	Silage	
B2263	LP80	Silage	
B2264	LP85-2	Silage	France
B2457	CHEO3	pork pickled sour sausage	Vietnam
B2484	NCTH19-1	pork pickled sour sausage	Vietnam
B2485	NCTH19-2	pork pickled sour sausage	Vietnam
B2494	NCTH27	pork pickled sour sausage	Vietnam
B2535	LD2	orange fermented	Vietnam
B2726	ATCC8014	Maize ensilage	

designation		source	
NCC	**other**	**Isolated from**	**Location**
B2741	NOS140	cabbage kimchi	Japan
B2753	Q2	sourdough fermented	Italy
B2757	H4	sourdough fermented	Italy
B2766	H14	sourdough fermented	Italy
B2776	old3667; L9	cheese	
B2801	KOG18	turnip pickled with rice bran	Japan
B2802	KOG24	cheese	Japan
B2806	LMG 9208	sauerkraut	UK
B2814	Lp95	wine red grapes	Italy
B2830	BLL(EI31)		
B2831	CECT221 / AATCC14431	Grass silage	Maryland
B2855	N58	pork pickled sour sausage	Vietnam
B2877	X17	hotdogs	Vietnam
B2889	LAC7	banana fermented	Vietnam
B2891	LD3	radish pickled	Vietnam
B2896	ATCC14917	cabbage pickled	Copenhagen
B2897	DKO22	cassava sour	Nigeria
	NCDO1193	Vegetables	
	LMG18021	Milk	Senegal
	CIP102359	Human spinal fluid	France

***Lactococcus lactis* strains used in this study**

designation			designation		
NCC	**other**	**source**	**NCC**	**other**	**source**
B5	R1	cheese prod.	B2199	K335	white kimchi
B20	ML8	commercial starter	B2200	K336	white kimchi
B26	LMG8526 /		B2202	K337	white kimchi
	NCFD2091	chinese radish seeds	B2203	K338	white kimchi
B29	ATCC 19435	unknown	B2206	P7266	litter of pasture grass
B33	AM2	commercial starter	B2207	P7304	litter of pasture grass
B34	P2	unknown	B2211	NCDO 895 / NCIMB 700895	
B32	SK11	hard cheese prod.			unknown

designation			designation		
NCC	**other**	**source**	**NCC**	**other**	**source**
B42	HP	cheese prod.	B2217	KF4	alfalfa sprouts
B48	AM1	commercial starter	B2218	KF5	alfalfa sprouts
B49	KH	unknown	B2219	KF7	alfalfa sprouts
B64	E8	commercial starter	B2220	KF24	alfalfa sprouts
B65	Wg2	unknown	B2221	KF30	mustard and cress
B82	Bos1 (BOZ)	starter Bos	B2222	KF31	mustard & cress
B86	Ru4	starter 4/25	B2223	KF67	grapefruit juice
B87	ZK	Swedish cheese	B2225	KF129	alfalfa & radish sprouts
B643	NCDO 763 / ML3	Dairy	B2226	KF134	alfalfa & radish sprouts
B644	UC317	unknown	B2227	KF138	alfalfa & radish sprouts
B844	M20	soil 13 july ‘92	B2228	KF140	alfalfa & radish sprouts
B848	M9	soil 13 july ‘92	B2229	KF146	alfalfa & radish sprouts
B1154	E7	Soil	B2230	KF147	mung bean sprouts
B1156	Li-1	grass	B2231	KF164	mung bean sprouts
B1157		raw sheep milk	B2232	KF165	mung bean sprouts
B1172	E33	silage	B2233	KF174	orange juice
B1173	E34	Silage	B2234	KF178	alfalfa & onion sprouts
B1174	E32	silage	B2235	KF181	alfalfa & onion sprouts
B1175		soil & grass	B2236	KF196	japanese kaiware shoots
B1230		soil & grass	B2237	KF197	japanese kaiware shoots
B1236	E10	soil & grass	B2238	KF201	sliced mixed vegetables
B1239	E27	Silage	B2239	KF221	mung bean sprouts
B1240	E9	soil & grass	B2240	KF225	mung bean sprouts
B1492	MG1363	plasmid free prophage cured	B2241	KF253	Mustard & cress
B1592	DRA4	Starter A	B2242	KF257	mustard & cress
B1594	C17	cheese prod.	B2243	KF269	japanese kaiware shoots
B1929	B35	Bos starter	B2244	KF337	white kimchi
B2001	LMG 8528 /		B2245	KF292	soya sprouts
	NCFB 2118***	frozen peas	B2246	KF306	pumpkin wedges
B2122	LMG 9446/		B2247	KW2	kaanga wai
	NCFB 1867*	frozen peas	B2248	KW8	kaanga wai
B2123	LMG 9449 /		B2249	KW10	kaanga wai
	NCFB 1868 **	frozen peas	B2250	UC503	Dairy
B2124	NCIMB 702727/		B2251	AM3	Dairy
	NCDO 2727	mung bean	B2252	FG2	Dairy

designation			designation		
NCC	**other**	**source**	**NCC**	**other**	**source**
B2142	P5977	perennial rye grass		E30	silage
B2189	P6056	perennial rye grass		IL1403	soft cheese prod.
B2190	P6284	timothy			
B2191	P7341	roots of pasture grass			
B2192	P7516	litter of pasture grass	* Cavet 21L (1966 Unilever)		
B2193	D53	salad mix	**Cavet 31L (1996 Unilever)		
B2198	K231	white kimchi	***Patel PTC5		

Other lactic acid bacteria used in this study

LAB species	**NCC/ other**	**LAB species**	**NCC/ other**
Carnobacterium divergens	DSM 20589	*Lactobacillus brevis*	B306
Carnobacterium maltaromaticum	DSM 20344	*Lactobacillus rhamnosus*	B637
Carnobacterium gallinarum	DSM 4847	*Lactobacillus delbrueckii del.*	B1799
Enterococcus casseliflavus	DSM 4841	*Lactobacillus gasseri VP*	B872
enterococcus faecalis	B153	*Lactobacillus graminis*	B1356
Enterococcus faecium	B921	*Leuconstoc mesenteroides*	ATCC 8239
Enterococcus faecalis	B145	*Enterococcus faecalis*	B308
Enterococcus flavescens	DSM 7371	*Pediococcus pentosaceus*	DSM 20333
Enterococcus mundii collins	B919	*Pediococcus acidilactici*	B1697
Lactococcus garvieae	DSM 6783	*Streptococcus entericus*	DSM 14446
Lactococcus rafinolactis	DSM 20443	*Streptococcus uberis*	B1118
Lactobacillus sakei sakei	23K	*Vagococcus fluvialis*	B102
Lactobacillus paralimentarius	B1357	*Weissella cibaria*	DSM 14295
Lactobacillus garvieae	DSM 6783	*Weissella halotolerans*	DSM 20190
Lactobacillus coryniformis torg.	B284		

Tables 1. Lactic acid bacterial species and strains used in this study. NCC stands for Nizo culture collection.

Growth medium (MRS-broth or GM17-broth Merck, Amsterdam, the Netherlands (6)) was supplemented, when indicated with heme (hemin) (Sigma, stock solution: 0.5 mg/ml in 0.05M NaOH) to a final conc. of 2 ug/ml or with the equivalent volume of 0.05M NaOH as a control . Furthermore, when indicated vitamin K_2 (menaquinone-4) (Sigma, stock solution: 2 mg/ml in ethanol) or the equivalent volume of ethanol, as a control, was added to a final conc. of 20 ng/ml. Aerobic growth conditions were achieved in shake flask cultivations with a 1:10 medium/volume ratio, at 250 rpm. For high throughput (96-wells micro-titer plates) aerobic cultivations, the plates were filled with 150ul medium/well, covered with breathseals and incubated in a microtron, shaking at 1000 rpm (Greiner Bio-one, Germany). The conditions of cultures grown in stationary tubes were considered anaerobic or micro-aerobic. All strains were grown for 48 hours at 30°C before measuring biomass (optical density at 600nm) and acidification.

Measurement of menaquinone-content of bacterial cells

Overnight cultures were washed twice in phosphate buffer (50mM K_2HPO_4, pH 5.0) and re-suspended to an OD_{600} of 10. Of this cell-suspension 2 ml was lysed by bead beat-ing using 0.1mm silica-beads (Biospec products, Inc) in a Savant Bio 101 FastPrep FP120 and frozen till further use. Menaquinones were extracted by thoroughly mixing 500ul sample with 5 ml extraction buffer (90%hexane, 10% ethanol). After a centrifuge step (10 min at 6000 rpm) the hexane layer was transfered to a new tube. This extraction procedure was repeated twice, and the combined (15ml) hexane layer evaporated under nitrogen-gas. Menaquinones were subsequently re-dissolved in 300ul ethanol. This menaquinone solution was injected in a LS-MC-MC with an APCI probe in the negative mode and analysed for menaquinone content.

Analysis of genomic content of sequenced lactic acid bacteria

The presence of the *cydABCD* and the menaquinone biosynthesis genes were based on annotation of the respective sequence consortia (see table), and by homology analysis with *Lactococcus lactis* MG1363 genes sequences, using BLASTP 2.2.18 (basic local alignment search tool) (34). Comparison of genomic local organization was performed using the KEGG genome map (http://www.kegg.com/), and the pinned region function of ERGO (http://ergo.integratedgenomics.com/ERGO/).

Phylogenetic analysis of cyd genes

Each individual *cydABCD* gene product sequence of *Lactococcus lactis* MG1363 was entered as a string to search for homologues in other lactic acid bacteria using the BLAST algorithm (1). Sequence entries found to be homologous were retrieved (june '08) from GenBank, and separately aligned using the MUSCLE algorithm (9). From the amino acid sequence alignments, bootstrapped neighbor joining trees were obtained using Clustal (29) with default settings, except for the number of iterations, which was set to 1000. Trees were analyzed in LOFT (32) and visualized in MEGA3 (12). An identical exercise was carried out for 16S RNA sequences from the organisms that were found to contain *cyd* genes. Finally, the topology of all the trees was analyzed and compared to the reference tree.

Results

Screening for respiration in lactic acid bacteria

We have screened several lactic acid bacteria, for the heme-stimulated respiratory-response, to examine the match between the genotype and the phenotype and to discover novel respiring lactic acid bacteria. Respiration in *Lactococcus lactis* is easily detected by an increase in growth efficiency that results in a (roughly) doubling of biomass under non-pH controlled batch fermentations (Fig 1).

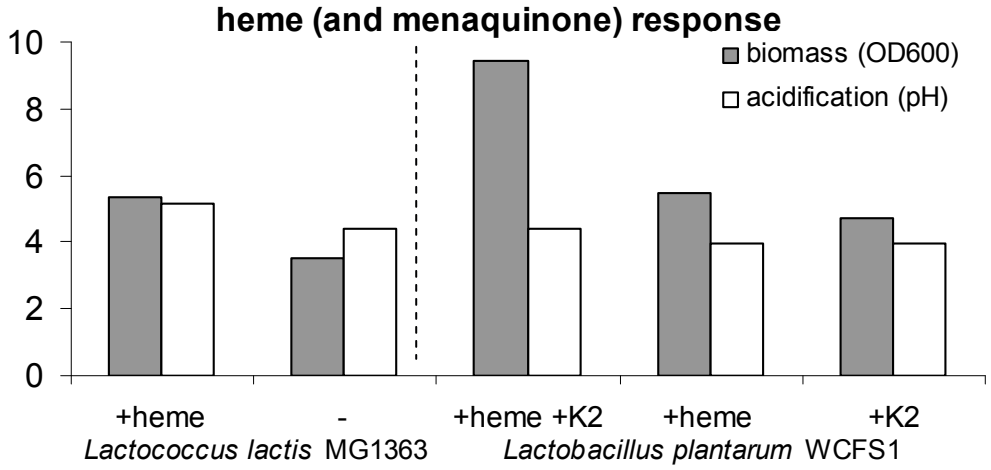

Figure 1. *Lactococcus lactis* MG1363 and *Lactobacillus plantarum* WCFS1 were grown aerobically overnight at 30C in M17-medium and MRS-broth respectively. A large biomass increase, without further acidification, is observed when *Lactococcus lactis* is grown with heme, while *Lactobacillus plantarum* requires menaquinone (K_2) in addition to heme.

This increase in biomass is not associated with a further drop in pH in fact the pH tends to rise, as a results of the conversion of lactate to acetate (8). A further drop in pH would be expected, were it a consequence of more progressive (homo-lactic) fermentation. We have aerobically cultivated 31 different species of lactic acid bacteria both with and without heme. Only two species had a similar response in biomass to addition of heme as *Lactococcus lactis* (Table 2 and 3).

Species		+heme		-	
		OD600	pH	OD600	pH
Lactococcus lactis	MG1636	5.36	5.16	3.52	4.39
Enterococcus casseliflavus	B2376	3.56	4.83	1.13	6.09
Streptococcus uberis	B1118	2.00	5.17	0.91	5.93
Carnobacterium divergens[1]	B2216	2.25	5.48	0.48	6.49

[1]has a high heme-dependent catalase activity

Table 2. Lactic acid bacteria were grown aerobically in the presence (+heme) or absence (-) of heme (2ug/ml). A clear increase in biomass yield is visible after a 48 hour incubation period. The data shown represent averages.

Streptoccocus uberis and *Enterococcus casseliflavus* responded to the addition of heme in a clear increase of biomass, although the pH did drop.

Menaquinones form a part of electron transport chains, facilitating membranous electron transfer (31). The inability of several lactic acid bacteria to produce menaquinones (or vitamin K_2) is in accordance with the absence of a complete menaquinone biosynthesis pathway in their genomes (www.KEGG.com). Addition of both heme and menaquinone to aerated cultures of *Lactobacillus plantarum* WCFS1 leads to an increase in biomass and higher final pH, quite similar to the heme supplemented phenotype of *Lactococcus lactis* (Fig 1). We surveyed our selection of lactic acid bacteria for other members that also require both heme and menaquinone to initiate this respiratory(-like) phenotype. Several species responded in a similar way to addition of these compounds as *Lactobacillus plantarum* WCFS1 (Table 3).

Species		+heme +K2		+Heme		+K2	
		OD600	pH	OD600	pH	OD600	pH
Enterococcus faecalis	B145	7.30	5.82	5.56	5.82	5.27	5.62
Lactobacillus plantarum	WCFS1	9.45	4.39	5.45	3.94	4.71	3.94
Lactobacillus rhamnosus	B637	3.59	4.01	1.51	4.17	1.55	4.15
Lactococcus garviae	B2379	3.57	4.80	2.98	4.77	2.84	4.61
Lactobacillus brevis	B306	10.53	4.17	9.05	4.14	8.43	4.15
Lactobacillus paralimentarius[1]	B1357	1.98	4.37	2.21	4.28	1.17	4.51
Streptococcus entericus[1]	B2339	5.32 g/l	5.49	5.1 g/l	5.48	3.96 g/l	4.42
Streptococcus agalactiae[2]	NEM316	2.1	5.6	1.4	4.8	1.4	4.8

[1]Should be considered heme-stimulated [2]see reference [34]

Table 3. Lactic acid bacteria were grown aerobically in the presence of 2 ug/ml heme (+heme) and/or 20 ug/ml vitamin K_2 (+K2), or with equivalent volumes of ethanol or 0.05M NaOH as control. A clear increase in biomass yield is visible after a 48 hour incubation period when both heme and vitamin K2 are added to the growth medium. The data shown represent averages.

Two *Lactobacillus* species and *Enterococcus faecalis* were found to respire only in the presence of both heme and menaquinone. In addition, there exists evidence for the heme-induced cytochrome formation in *Leuconostoc mesenteriodes* and *Enterococcus faecalis* (V538) (11, 25, 35,).

Distribution of cyd-genes in lactic acid bacteria

Currently there are 62 sequenced genomes of lactic acid bacteria available in the NCBI database of which 45 are complete (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). We examined the potential for respiration in this diverse set of lactic acid bacteria as indicated by the presence of the *cydABCD* genes in their genomes. Using BLASTP and available annotation, we found that in 22 of these genomes al the four *cyd*-genes were present (Table 4) (1).

	locus annotated as *bd*-cytochrome genes or with BLAST similarity%[a]:			
Species	structural subunit I (*cydA*)	structural subunit II (*cydB*)	cytochrome *bd*-linked ABC transporter (*cydC*)	(c*ydD*)
Enterococcus faecalis V583	EF2061	EF2060	EF2059	EF2058
Lactobacillus brevis ATCC 367	LVIS_1642	LVIS_1641	[e]	LVIS_1639
Lactobacillus casei ATCC 334	LSEI_2205 LSEI_0012[b]	LSEI_2204	LSEI_2203	LSEI_2202
Lactobacillus gasseri ATCC 33323	LGAS_1841	LGAS_1842	LGAS_1843	LGAS_1844
Lactobacillus johnsonii NCC 533	LJ1810	LJ1811	LJ1812 (2e-116)	LJ1813 (5e-110)
Lactobacillus plantarum WCFS1	lp_1125	lp_1126	lp_1128	lp_1129
Lactobacillus reuteri 100-23	Lreu23DRAFT_0600	Lreu23DRAFT_0599	Lreu23DRAFT_0598 (3e-140)	Lreu23DRAFT_0597 (3e-120)
Lactobacillus reuteri F275	Lreu_0505	Lreu_0506	Lreu_0507 (1e-136)	Lreu_0508 (6e-121)
Lactobacillus salivarius UCC118	LSL_1032	LSL_1031	LSL_1030 (1e-137)	LSL_1029 (3e-131)
Lactococcus lactis MG1363	llmg_1864	llmg_1863	llmg_1862	llmg_1861
Lactococcus lactis SK11	lacr_0737	lacr_0738	lacr_0739	lacr_0740
Lactococcus lactis Il1403	L107762	L109201	L110479	L112352
Leuconostoc mesenteroides ATCC 8293	LEUM_0560	LEUM_0561	LEUM_0562[d]	LEUM_0563[c]
Oenococcus oeni ATCC BAA-1163	OENOO_65065	OENOO_65064	OENOO_66040	OENOO_66039
Oenococcus oeni PSU-1	OEOE_1837	OEOE_1836	OEOE_0414	OEOE_0415
Streptococcus agalactiae 18RS21	SAJ_1696	SAJ_1694	SAJ_1693[d]	SAJ_1692[c]
Streptococcus agalactiae 2603V/R	SAG1742	SAG1741	SAG1739[d]	SAG1740[c]
Streptococcus agalactiae 515	SAL_1843	SAL_1842	SAL_1841[d]	SAL_1840[c]
Streptococcus agalactiae A909	SAK_1750	SAK_1749	SAK_1748[d]	SAK_1747[c]
Streptococcus agalactiae CJB111	SAM_1705	SAM_1704	SAM_1703[d]	SAM_1702[c]
Streptococcus agalactiae COH1	SAN_1868	SAN_1867	SAN_1866[d]	SAN_1865[c]
Streptococcus agalactiae H36B	SAI_1857	SAI_1856	SAI_1855[d]	SAI_1851[c]
Streptococcus agalactiae NEM316	gbs1787 (2e-140)	gbs1786 (1e-99)	gbs1785 (1e-142)	gbs1784 (5e-132)

[a]Blast against MG1363 *cyd*-genes [b]fusion of subunit 1 and subunit 2 [c]Best hit with MG1363 CydC, annotated as CydD [d]Best hit with MG1363 CydD, annotated as CydC [e]LVIS_1640 is a pseudo gene SAJ_1695 is a cydA pseudo gene SAI_1865 E = 5e-37 annotated as cydA SAI_1866 E=2e-7 annotated as cydA.

Table 4. The locus of genes that are either annotated as cytochromes genes or with high similarity to the *Lactococcus lactis* MG1363 *cydABCD* genes. All *CydABCD* genes were absent in *Lactobacillus sakei* 23K, *Lactobacillus delbrueckii* (strains ATCC 11842, ATCC BAA-365), *Enterococcus faecium* DO, *Lactobacillus acidophilus* NCFM, *Pediococcus pentosaceus* ATCC 25745, *Streptococcus gordonii* CH1, *Streptococcus mutans* UA159, *Streptococcus pneumoniae* (strains D39, R6, SP11-BS70, SP14-BS69, SP18-BS74,SP19-BS75, SP23-BS72, SP3-BS71, SP6-BS73, SP9-BS68, TIGR4), *Streptococcus pyogenes* (strains M1 GAS, M49 591, MGAS10270, MGAS10394, MGAS10750, MGAS2096, MGAS315, MGAS5005, MGAS6180, MGAS8232, MGAS9429, SSI-1, Manfredo), *Streptococcus sanguinis* SK36, Streptococcus suis (strains 05ZYH33, 89/1591, 98HAH33) and *Streptococcus thermophilus* (strains CNRZ1066, LMD-9, LMG 18311).

In the case of *Lactobacillus brevis* ATCC367 the *cydC* gene has degenerated into a pseudo gene. *Streptococcus agalactiae* NEM316 has four open reading frames (gbs1787-1784) that are annotated as hypothetical proteins that have a high similarity to *cydABCD* of *Lactococcus lactis* MG1363 and a similar operon structure. In addition, the *cydABCD* genes are present in all the other *Streptococcus agalactiae* strains. *Lactobacillus casei* ATCC334 possesses besides a *cydABCD*- operon a separate fusion gene of *cydA* and *cydB*. The genome of *Streptococcus agalactiae* H36B contains 3 genes that are annotated as *cydA* (encoding the structural subunit 1 of the *bd*-type cytochrome). Only one (SAI_1857) lies in an operon together with *cydBCD* while SAI_1865 and SAI_1866 are next to each other on the chromosome. The full 489bp length of SAI_1865 matches 100% to the 3' terminus of the SAI_1857 (1428bp), while the 162pb SAI_1866 matches for 95% with a middle part of SAI_1857. Detailed analysis (data not shown) revealed that although SAI_1856 and SAI_1866 have high similarity to parts of the full *cydA* gene, they are truncated versions and likely too short to function as the structural subunit I of the *bd*-type cytochrome. There is confusion in annotation of the *cydC* and *cydD* genes among the lactic acid bacteria. For example the best match to the amino acid sequence of the *Lactococcus lactis* MG1363 *cydC* are the as *cydD* annotated genes of the *Streptococcus agalactiae* strains, while the best match to the amino acid sequence of the *Lactococcus lactis* MG1363 *cydD* are the as *cydC* annotated genes of *Streptococcus agalactiae*. Despite this confusion in annotation, the presence or

absence of all four if the *cyd*-genes could be unambiguously determined for the studied sequenced genomes. Furthermore, we determined the phylogeny of each predicted individual amino acid sequence encoded by *cydABCD*. Overall, the topologies of the separate *cyd*- based trees resemble each other and canonical phylogeny. In all cases the lactic acid bacteria cluster together, and are neighboured by representatives of *Listeria* and *Bacillus*, forming the Firmicutes branch (Fig 2).

We can attempt to relate the observed responses to heme (and menaquinone) to the presence of the *cydABCD* and menaquinone biosynthesis (*preA*, *menABCDEFX*) genes, since several of the sequenced species were present in our survey. At least for the sequenced species in our survey (*Lactococcus lactis* MG1363, SK11, *Lactobacillus plantarum* WCFS1, *Enterococcus faecalis* V583, *Streptococcus agalactiae* NEM316) the correlation between genotype and phenotype matches; in these strains respiration-like behavior could be shown as well as the *cyd*-genes found. Also important in this respect is that we did not find stimulation of biomass formation by heme in *Lactobacillus sakei* 23K, nor in tested (non-sequenced) strains of *Lactobacillus delbreuckii* (B1799) or *Pediococcus pentosaceus* (DSM 20333). There are some discrepancies however, as the sequenced *Leuconostoc mesenteroides* ATCC 8239 has all four *cyd*-genes, but in our hands did not show an increase in biomass in the presence of heme (and menaquinone). Heme-induced cytochrome formation, however, has been reported for certain *Leuconostoc mesenteroides* strains (25).

There are observations that several Lactic acid bacteria, such as *Lactococcus lactis* and *Leuconostoc mesenteroides* produce menaquinones also under non-respiratory (anaeorobic) conditions (18). We have cultivated the respiratory-model lactic acid bacteria, *Lactococcus lactis* MG1363, in respiratory and non-respiratory conditions and measured the menaquinone composition and production levels (Table 5).

Growth condition	Menaquinone content (ug/L)									
	K2(2)	K2(3)	K2(4)	K2(5)	K2(6)	K2(7)	K2(8)	K2(9)	K2(10)	total
Heme O_2	0.0	12.6	3.3	2.9	1.8	3.3	13.7	28.9	0.7	67.0
O_2	0.0	7.1	2.2	2.2	2.5	5.4	16.4	26.8	0.5	63.2
Heme	0.5	74.6	3.5	3.5	2.4	3.2	8.1	12.1	0.1	113.3
-	0.6	77.6	3.9	3.9	3.5	6.9	18.4	21.4	0.2	142.0

Table 5. *Lactococcus lactis* MG1363 was grown overnight with(out) 2 ug/ml heme and with(out) aeration. Washed cells were analyzed for menaquinone content on HPLC.

Assuming that the amounts of menaquinone with a tail-length of 11 or higher is negligible, we observed that, contrary to expectations, the total production of menaquinones decreases in aerated conditions (with or without heme) by roughly 2-fold. Interestingly, aerobic growth conditions did induce an increase the concentration of menaquinone-species with a longer tail-length (mk2(8, 9 and 10)). Furthermore, under anaerobic conditions a short-tailed menaquinone (mk2(3)) was preferentially formed.

Respiration is species-specific

The presence or absence of the *cyd*-genes in various species is remarkably consistent, as shown in table 4 and the corresponding text. The *cyd*-genes were not present in any of the genomes of *Streptococcus pyogenes* (11), *Streptococcus pneumonia* (3), *Streptococcus thermophilus* (3) and *Lactobacillus delbreukii* (2) strains. And as consistently present in all *Streptococcus agalactiae* (8), *Lactaococcus lactis* (3) and *Oenococcus oeni* (2) strains. Furthermore, in two separate studies 43 strain of *Lactobacillus plantarum* were genotyped and their genomic content compared to the genome of *Lactobacillus plantarum* WCFS1. In all these 43 genomes the *cydABCD* operon was present (17)(Tseneva personal communication). To determine if the respiratory-phenotype is as consistent as the genotype, we tested 88 strains of *Lactococcus lactis* and 20 strains of *Lactobacillus plantarum*. We observed that heme (and vitamin K_2) induced the respiratory-(like) response in 84 (out of 88) *Lactococcus lactis* strains, and in 16 (out of 20) *Lactobacillus plantarum* strains respectively (Table 1). One of the 4 *Lactococcus lactis* strains that did not respond to heme was the sequenced strain IL1403. A transposase that is situated directly in front of the *cyd*-genes on the genome could

be responsible for this lack of heme-induced response. Both the genotypic data and the growth experiments indicate that respiration is characteristic for a diverse group of lactic acid bacterial species.

High-throughput respiration screening

By using an insertion knock-out bank of *Lactococcus lactis* B1157, we show the power of a simple, high throughput screening method to isolate heme-stimulated lactic acid bacteria (26). Approximately 8000 insertion mutants were aerobically incubated (with or without heme) and screened on biomass yield.

A total of 73 mutants were found which showed no significant biomass increase upon cultivation with heme. The position of integration of the insertion-knockout vector was determined in 13 of these 74 mutants by analysis of the surrounding genome sequence. The genomic region and the genes of *Lactococcus lactis* B1157 that were disrupted by the insertion event were identified by comparison with *Lactococcus lactis* MG1363 genome sequence (Table 8).

Five of the isolated respiration mutants carried mutations in genes involved in menaquinone biosynthesis. Respiration mutants with disruptions of *aroB* and *aroE* are also likely to suffer from an impaired menaquinone biosynthesis, since these genes are involved in synthesis of chorismate, the menaquinone precursor molecule. Three mutants were found that carried disruptions in the *cyd*-genes that are obviously essential to synthesize the *bd*-type cytochrome. Furthermore, we have now experimental evidence that a disruption of *noxA*, annotated as a NADH-dehydrogenase is directly linked to a respiration negative phenotype. 11 out of 13 respiration mutants carry mutations in genes that can be readily explained and thus validate this high throughput method to screen for respiration in lactic acid bacteria.

Mutant	sequence length (bp)	match Length (bp)	Identities MG1363	locus	Features of genomic sequence annotation
6B-4D	650	649	98%	llmg_1315	putative RNA methyltransferase
7D-9C	800	421	99%	llmg_1607	hypothetical protein
				llmg_1608	putative glycosyl hydrolases
11D-2E	800	547	99%	llmg_1830	menX, menaquinone biosynthesis related protein
1C-6E	750	569	97%	llmg_0197	menA, 4-hydroxybenzoate polyprenyltransferase/ prenyltransferases
737_11	939	114	100%	llmg_0196	Geranylgeranyl pyrophosphate synthase
737_4	750	541	100%	llmg_1861	cydD, cytochrome D ABC transporter ATP binding and permease protein
737_12	700	29	100%	llmg_1863	cydB, cytochrome d ubiquinol oxidase, subunit II
737_16	897	894	100%	llmg_1735	noxA, NADH dehydrogenase, FAD-containing subunit
734_1	750	750	100%	llmg_1939	aroE, hikimate 5-dehydrogenase
				llmg_1938	aroB, 3-dehydroquinate synthase
734_17	600	248	99%	llmg_1833	menC, o-succinylbenzoate synthase
734_18	450	450	91%	llmg_1861	cydD, cytochrome D ABC transporter ATP binding and permease protein
734_24	700	569	99%	llmg_1832	menE, Acyl-CoA synthetases (AMP-forming)/AMP-acid ligases II
734_27	800	155	99%	llmg_1833	menC, o-succinylbenzoate synthase

Table 8. The homologous genes of *Lactococcus lactis* MG1363 that were disrupted in the *Lactoccus lactis* B1157 respiration negative mutants. The sequence length of the genomic region of insertion is shown and the match to known *Lactococcus lactis* MG1363 gene sequences that were present in this region.

Discussion

Lactococcus lactis MG1363 is capable of true respiration, as shown by the formation of a proton motive force by its heme-dependent electron transport chain (4). We have used the characteristic phenotype of respiring *Lactococcus lactis*, a higher biomass with less extensive acidification, to screen for other possibly respiring lactic aid bacteria. Besides increased

biomass production, respiring includes other traits, such as enhanced robustness, which makes the discovery of novel respiring lactic acid bacteria relevant for industrial applications (8, 10). We have discovered respiratory-like behaviour that requires supplementation with heme and menaquinone (vitamin K_2) in several additional species of lactic acid bacteria. The addition of these co-factors, to induce respiration in several lactic acid bacteria, may appear artificial. However, recent unpublished results by A. Gruss et. al, and others suggest that plants can function both as source of heme and menaquinone.

The *cyd*-genes (*cydABCD*) are present in many species of lactic acid bacteria. Most sequenced lactic acid bacteria that were screened showed a match between their genotype (*cyd*-genes, menaquinone biosynthesis genes) and the heme (and menaquinone) induced phenotype. Also the dependence and independence on a menaquinone source in for example the *Lactobacillus* species and *Lactococcus* species correlates well with the presence of a menaquinone biosynthesis pathway.

The *cyd*-genes are not present only in a limited subset of closely related species of lactic acid bacteria. In fact the *cyd*-genes are present in many species that together span the diversity-range found in lactic acid bacteria (16). What is remarkable is that the *cyd*-genes are so consistently present (or absent) in all the (sequenced) strains of a certain species, as can be clearly seen for the members of the genus *Streptococcus*. This uniformity may be the result of a bias in the isolation of the strains from highly similar niches. We have, however, screened a large number of *Lactobacillus plantarum* and *Lactococcus lactis* strains that were isolated from a variety of both industrial and plant-sources (see the materials and methods section). In both cases the induction of respiration by addition of heme and menaquinone was highly uniform with only a few exceptions. We can conclude that respiration is characteristic trait for, at least, *Lactococcus lactis* and *Lactobacillus plantarum*.

The high throughput screening of 8000 insertion mutants of *Lactococcus lactis*, revealed that a high proportion if the respiration-impaired mutants contained insertions in menaquinone-biosynthesis genes. This implies that such methods can be used to screen for menaquinone producers among lactic acid bacteria. Those lactic acid bacteria that are stimulated by heme alone are potential producers of menaquinones.

Roughly half of the lactic acid bacteria, whose genome has been sequenced, have at least one copy of all the *cydABCD* genes. We investigated whether such a distribution could

best be explained by horizontal gene transfer or, in opposition, by gene loss. The phylogenetic tree, constructed with the *cyd* genes sequences in this study (Fig 2), is highly similar to the canonical (16s rRNA) evolutionary tree.

All lactic acid bacteria group nicely together in one separate branch. This indicates ancient origins of the *bd*-type cytochrome. The results presented here not only support the idea that the *cyd* genes were present in the common ancestor of lactic acid bacteria, but in fact of all Firmicutes.

That gene loss best explains the observed *cyd*-gene distribution amongst lactic acid bacteria is in line with their highly auxotrophic nature. Lactic acid bacteria as a group have a history of adaptation to nutrient rich food-environments and progressive gene-loss that was nicely visualised by Makarova et.al. (15). A typical example of this process is the extensive gene decay (high abundance of pseudo-genes) in the yoghurt-bacterium *Streptococcus thermophilus* (3).

(Mena)quinones are best known as cofactors of bacterial respiratory chains, shuttling electrons from dehydrogenases to the terminal oxidase. Menaquinone production in anaerobic, non-heme supplemented conditions have been reported in literature before for other *Lactococcus lactis* strains and *Leuconostoc* sp. (18). Several groups have proposed an additional role of menaquinones in offering protection against oxidative stress (27, 33). Recently it has been shown that quinones of *Lactcoccus lactis* can reduce metal-ions such as Fe^{3+} and Cu^{2+}, which may facilitate their assimilation (21). Still, contrary to expectations when *Lactococcus lactis* was grown aerobically (both with and without heme), the total amount of menaquinones (MK 2-10) that was produced was almost two-fold lower compared with anaerobic conditions. Furthermore we observed that aerobic cultivation induces an altered composition of the menaquinone pool, with a shift towards menaquinones with longer tail lengths (mk2(8-10 isoprenoid residues). This study reports that respiration in several lactic acid bacteria can be induced by a combination of heme and vitamin K_2 (a MK-4). It is not known what function the observed shift, in the composition of the menaquinone-pool to menaquinones with a longer tail-length, serves in bacteria. In humans, however, menaquinones with longer tail-lengths remain detectable for longer times in the blood stream and may form a more constant form of vitamin K_2 (24).

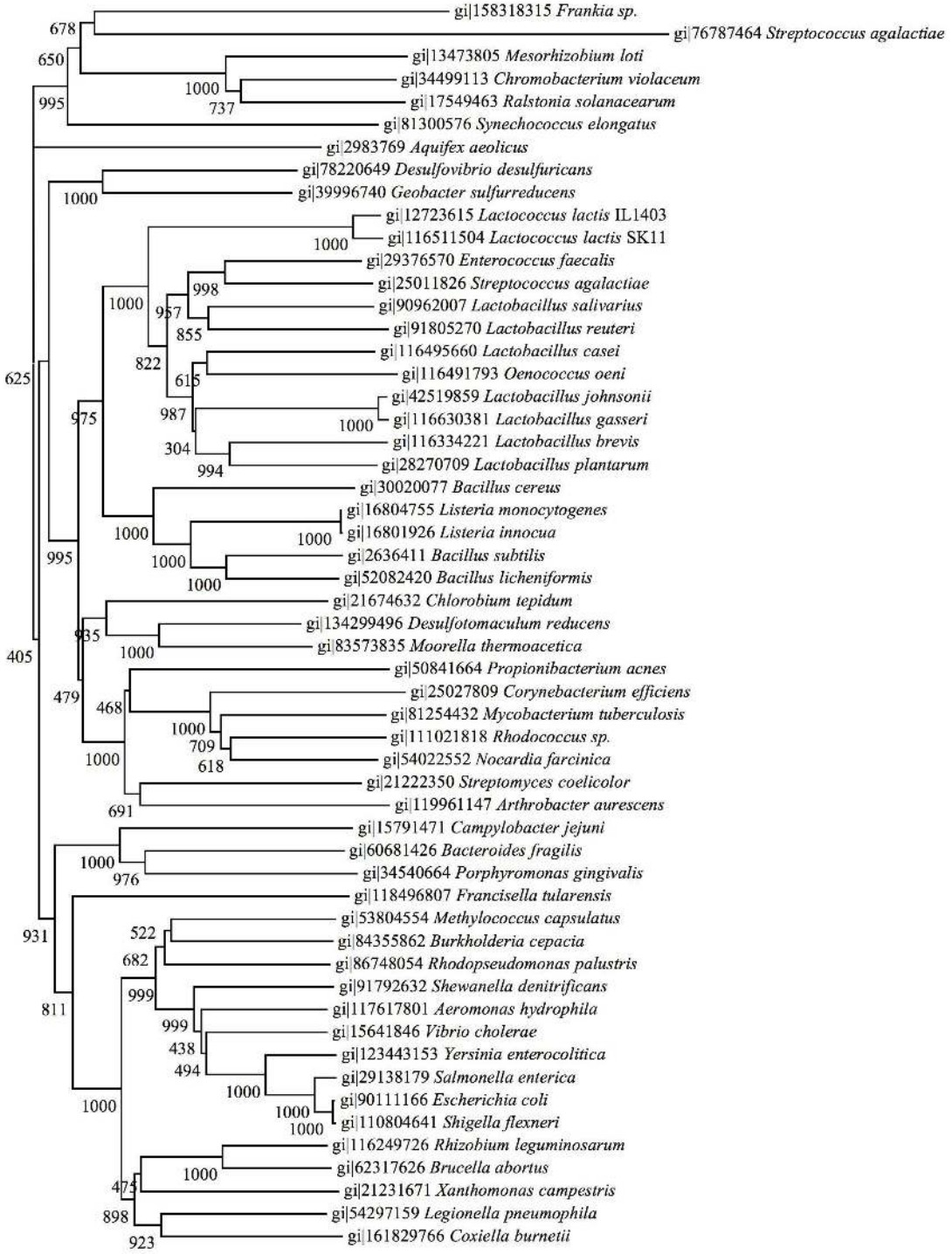

Figure 2. Phylogenetic tree based on *cydA* sequences, constructed as explained in the materials and methods section.

Therefore, induction of the production of specifically longer-tailed menaquinone species by lactic acid bacteria may better fulfil human vitamin K_2 requirements.

The function of menaquinones, and especially of the various tail- lengths, in the (anaerobic) metabolism of *Lactcoccus lactis*, is unclear. For example, the various menaquinone mutants of *Lactococcus lactis* grew well anaerobically and aerobically (table 8). In fact many species of lactic acid bacteria grow well both anaerobically as aerobically, although not all of these produce menaquinones. Since, lactic acid bacteria do not strictly depend on menaquinone for growth they make ideal organisms to study the influence of the various tail-lengths on (respiratory) metabolism.

This work has revealed a number of novel lactic acid bacteria with potential respiratory capacity which, as in the case of *Lactococcus lactis*, could be targeted for future industrial exploitation.

Acknowledgements

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1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:**3389-402.
2. **Bernardeau, M., M. Guguen, and J. Vernoux.** 2006. Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. FEMS Microbiol Rev **30:**487-513.
3. **Bolotin, A., B. Quinquis, P. Renault, A. Sorokin, S. D. Ehrlich, S. Kulakauskas, A. Lapidus, E. Goltsman, M. Mazur, G. D. Pusch, M. Fonstein, R. Overbeek, N. Kyprides, B. Purnelle, D. Prozzi, K. Ngui, D. Masuy, F. Hancy, S. Burteau, M. Boutry, J. Delcour, A. Goffeau, and P. Hols.** 2004. Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. Nat Biotechnol **22:**1554-8.
4. **Brooijmans, R. J., B. Poolman, G. K. Schuurman-Wolters, W. M. de Vos, and J. Hugenholtz.** 2007. Generation of a membrane potential by *Lactococcus lactis* through aerobic electron transport. J Bacteriol.
5. **Bryan-Jones, D. G., and R. Whittenbury.** 1969. Haematin-dependent oxidative phosphorylation in Streptococcus faecalis. J Gen Microbiol **58:**247-60.
6. **De Man, J. C., M. Rogosa, and M. E. Sharpe.** 1960. A medium for the cultivation of *Lactobacilli*. J. Appl. Bacteriol. **23:**130-135.
7. **Duwat P, G. A., LeLoir Y, Gaudu P.** 1999. Bactéries lactiques transformées pour leur conférer un métabolisme respiratoire, et levains comprenant lesdites bactéries. French patent application FR2798670.
8. **Duwat, P., S. Sourice, B. Cesselin, G. Lamberet, K. Vido, P. Gaudu, Y. Le Loir, F. Violet, P. Loubiere, and A. Gruss.** 2001. Respiration capacity of the fermenting bacterium *Lactococcus lactis* and its positive effects on growth and survival. J Bacteriol **183:**4509-16.
9. **Edgar, R. C.** 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics **5:**113.
10. **Gaudu, P., K. Vido, B. Cesselin, S. Kulakauskas, J. Tremblay, L. Rezaiki, G. Lamberret, S. Sourice, P. Duwat, and A. Gruss.** 2002. Respiration capacity and consequences in *Lactococcus lactis*. Antonie Van Leeuwenhoek **82:**263-9.

11. **Huycke, M. M., D. Moore, W. Joyce, P. Wise, L. Shepard, Y. Kotake, and M. S. Gilmore.** 2001. Extracellular superoxide production by *Enterococcus faecalis* requires demethylmenaquinone and is attenuated by functional terminal quinol oxidases. Mol Microbiol **42:**729-40.
12. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform **5:**150-63.
13. **Ljungh, A., and W. T.** 2006. Lactic acid bacteria as probiotics. Curr Issues Intest Microbiol **7:**73-89.
14. **Lonvaud-Funel, A.** 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. Antonie Van Leeuwenhoek **76:**317-31.
15. **Makarova, K., A. Slesarev, Y. Wolf, A. Sorokin, B. Mirkin, E. Koonin, A. Pavlov, N. Pavlova, V. Karamychev, N. Polouchine, V. Shakhova, I. Grigoriev, Y. Lou, D. Rohksar, S. Lucas, K. Huang, D. M. Goodstein, T. Hawkins, V. Plengvidhya, D. Welker, J. Hughes, Y. Goh, A. Benson, K. Baldwin, J. H. Lee, I. Diaz-Muniz, B. Dosti, V. Smeianov, W. Wechter, R. Barabote, G. Lorca, E. Altermann, R. Barrangou, B. Ganesan, Y. Xie, H. Rawsthorne, D. Tamir, C. Parker, F. Breidt, J. Broadbent, R. Hutkins, D. O'Sullivan, J. Steele, G. Unlu, M. Saier, T. Klaenhammer, P. Richardson, S. Kozyavkin, B. Weimer, and D. Mills.** 2006. Comparative genomics of the lactic acid bacteria. Proc Natl Acad Sci U S A **103:**15611-6.
16. **Makarova, K. S., and E. V. Koonin.** 2007. Evolutionary genomics of lactic acid bacteria. J Bacteriol **189:**1199-208.
17. **Molenaar, D., F. Bringel, F. H. Schuren, W. M. de Vos, R. J. Siezen, and M. Kleerebezem.** 2005. Exploring *Lactobacillus plantarum* genome diversity by using microarrays. J Bacteriol **187:**6119-27.
18. **Morishita, T., N. Tamura, T. Makino, and S. Kudo.** 1999. Production of menaquinones by lactic acid bacteria. J Dairy Sci **82:**1897-903.
19. **Parvez, S., K. A. Malik, S. Ah Kang, and H. Y. Kim.** 2006. Probiotics and their fermented food products are beneficial for health. J Appl Microbiol **100:**1171-85.

20. **Pedersen, M. B., S. L. Iversen, K. I. Sorensen, and E. Johansen.** 2005. The long and winding road from the research laboratory to industrial applications of lactic acid bacteria. FEMS Microbiol Rev **29:**611-24.
21. **Rezaiki, L., G. Lamberet, A. Derre, A. Gruss, and P. Gaudu.** 2008. *Lactococcus lactis* produces short-chain quinones that cross-feed Group B *Streptococcus* to activate respiration growth. Mol Microbiol **67:**947-57.
22. **Ritchey, T. W., and H. W. Seeley.** 1974. Cytochromes in Streptococcus faecalis var. zymogenes grown in a haematin-containing medium. J Gen Microbiol **85:**220-8.
23. **Ritchey, T. W., and H. W. Seeley.** 1975. Distribution of cytochrome-like respiration in Streptococci. J. Gen. Microbiol. **93:**195-203.
24. **Schurgers, L. J., and C. Vermeer.** 2002. Differential lipoprotein transport pathways of K-vitamins in healthy subjects. Biochim Biophys Acta **1570:**27-32.
25. **Sijpesteijn, A. K.** 1970. Induction of cytochrome formation and stimulation of oxidative dissimilation by hemin in Streptococcus lactis and Leuconostoc mesenteroides. Antonie Van Leeuwenhoek **36:**335-48.
26. **Smit, B. A., J. E. van Hylckama Vlieg, W. J. Engels, L. Meijer, J. T. Wouters, and G. Smit.** 2005. Identification, cloning, and characterization of a *Lactococcus lactis* branched-chain alpha-keto acid decarboxylase involved in flavor formation. Appl Environ Microbiol **71:**303-11.
27. **Soballe, B., and R. K. Poole.** 2000. Ubiquinone limits oxidative stress in *Escherichia coli*. Microbiology **146:**787-796.
28. **Steinkraus, K. H.** 1983. Lactic acid fermentation in the production of foods from vegetables, cereals and legumes. Antonie Van Leeuwenhoek **49:**337-48.
29. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res **22:**4673-80.
30. **Thony-Meyer, L.** 1997. Biogenesis of respiratory cytochromes in bacteria. Microbiol Mol Biol Rev **61:**337-76.

31. **Unden, G., and J. Bongaerts.** 1997. Alternative respiratory pathways of *Escherichia coli*: energetics and transcriptional regulation in response to electron acceptors. Biochim Biophys Acta **1320:**217-34.

32. **van der Heijden, R. T., B. Snel, V. van Noort, and M. A. Huynen.** 2007. Orthology prediction at scalable resolution by phylogenetic tree analysis. BMC Bioinformatics **8:**83.

33. **Vido, K., H. Diemer, A. van Dorsselaer, E. Leize, V. Juillard, A. Gruss, and P. Gaudu.** 2005. Roles of Thioredoxin Reductase during the Aerobic Life of *Lactococcus lactis*. J Bacteriol **187:**601–610.

34. **Wegmann, U., M. O'Connell-Motherway, A. Zomer, G. Buist, C. Shearman, C. Canchaya, M. Ventura, A. Goesmann, M. J. Gasson, O. P. Kuipers, D. van Sinderen, and J. Kok.** 2007. Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* MG1363. J Bacteriol **189:**3256-70.

35. **Winstedt, L., L. Frankenberg, L. Hederstedt, and C. von Wachenfeldt.** 2000. *Enterococcus faecalis* V583 contains a cytochrome bd-type respiratory oxidase. J Bacteriol **182:**3863-6.

36. **Yamamoto, Y., C. Poyart, P. Trieu-Cuot, G. Lamberet, A. Gruss, and P. Gaudu.** 2006. Roles of environmental heme, and menaquinone, in *Streptococcus agalactiae*. Biometals **19:**205-10.

Chapter 4

The electron transport chains of *Lactobacillus plantarum* WCFS1

R. J. W. Brooijmans, J. Hoolwerf, W. M. de Vos, J. Hugenholtz. The electron transport chains of *Lactobacillus plantarum* WCFS1. A modified version of this chapter was submitted to Applied and Environmental Microbiology

Abstract

The aerobic response of *Lactobacillus plantarum* WCFS1 to the presence of both heme and menaquinone resemble that of respiring *Lactococcus lactis*. In addition, *L. plantarum* WCFS1 reduced nitrate when both heme- and menaquinone were present. The *L. plantarum* genome contains the *cydABCD* and *narGHJI* operons, coding for a *bd*-type cytochrome and nitrate reductase A respectively. The functional connection between the *nar* operon and nitrate reduction was demonstrated by absence of nitrate reduction in a *narG*-knockout. (High) glucose concentrations negatively affected nitrate reductase activity, while mannitol did not. Active nitrate reduction provided additional oxidizing power and allowed anaerobic growth on mannitol, as carbon source. Several other genes with a proposed function in the reduction of nitrate were found in the vicinity of the *narGHJI*-operon. Nitrate reduction led to an increased biomass production and to transient nitrite production. The nitrite was found to be ultimately converted into ammonia in a non-heme, non-menaquinone-dependent reaction. We propose the existence of a non-redundant branched electron transport chain in *L. plantarum* WCFS1 that generates proton motive force and is capable of using oxygen or nitrate as terminal electron acceptor.

Introduction

Lactic acid bacteria (LAB) are a group of industrially important bacteria that are extensively used for production of fermented foods from dairy, meat, fruit and vegetables with improved organoleptic, structural and nutritional properties. LAB have been used for their fermentative capacity for thousands of years and have long been classified as obligate fermentative, non-respiring, facultative anaerobes. In 1970 it was reported that when *Lactococcus lactis* (formerly known as *Streptococcus lactis*) and *Leuconostoc mesenteroides* were grown in the presence of heme, they formed cytochromes (48). Further investigation of this phenomenon led to the discovery of a more robust phenotype of cells grown in the presence of heme such as increased biomass yield, increased resistance to oxygen- and acid-stress and prolonged survival at low temperatures (21, 24, 42). Recently, the presence of a functional aerobic electron transport chain, leading to energy (proton motive force) generation, was demonstrated in heme-grown *Lactococcus lactis* (13). This respiratory-like behavior has also been reported in a number of other LAB such as *Enterococcus faecalis* and *Streptococcus agalactiae*, but not yet in any representative of the genus *Lactobacillus* (54, 55).

In this work we have looked for the presence of respiratory-like behavior in *Lactobacillus plantarum*. This lactic acid bacterium is economically important since it is utilized, as starter bacterium, in many food fermentations and is merchandised as a probiotic since it is an important inhabitant of the human gastrointestinal tract (1, 2, 19, 52). We demonstrate induction of aerobic and anaerobic respiration in *L. plantarum* opening several possibilities for more efficient and more robust production of *L. plantarum* as starter culture and as probiotic.

Materials and Methods

Cultures and growth conditions

The two strains used in this study are *Lactobacillus plantarum* WCFS1, an isolate from NCIMB8826, and an isogenic strain (NarGΔ) carrying a chloramphenicol replacement of *narG*. *L. plantarum* strains were cultivated on MRS broth (Difco) or chemically defined media (CDM) (50). When mentioned citrate, acetate were omitted, glucose titrated or replaced

by mannitol. NarGΔ was grown in the presence of 5 ug/ml chloramphenicol. For the induction of nitrate-reductase activity heme (heme or hemin) was added to a final conc. of 2.5 ug/ml (stock 0.5 mg/ml in 0.05 M NaOH Sigma-Aldrich), vitamin K_2 (or menaquinone 4) to a final concentration of 1 ug/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 ug/ml chloramphenicol and/or 10 ug/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 K2 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 (46). 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 (32). The plasmid pNZ5319_NarG_KO contains two ~1kb regions that are identical to DNA sequences that flank *narG*, 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).

PCR primers used in this study

PCR-amplify	Forward primer	Reverse primer
1kb upstream *narG*	P101: CCAGTCAGTAATAGCTGCTAA	P100: CGATAAGACCTCCTTTATCAC
1kb downstream *nar*G	P102: CGGAAGTTAAAGAAGGTGAAC	P103: CGAATTCTGAGCAGCTTCCA
moaA Q-PCR	GCAAAATGATGACGAAGTCCTAGA	TATTCTTTTTGCCAGGTCTTTAATGA
moeA Q-PCR	GTCGTCGTGATGCTCGAAAA	TCGGGAACCACGATGTTGAT
narG Q-PCR	GTTTGCGGACAACTGGTTAGC	TCTTGCAAAATAACGTGGGTCAT
narK Q-PCR	GCCACAAGTAACAGCAGGCTTA	CCCCCAATTGGTCGAACA
groES Q-PCR	CCCAAAGCGGTAAGGTTGTT	CTTCACGCTGGGGTCAACTT
gyrB QPCR	GGAATTGATGAAGCCCTAGCAG	GAATCCCACGACCGTTATCA
ldhL Q-PCR	TGATCCTCGTTCCGTTGATG	CCGATGGTTGCAGTTGAGTAAG
pfk Q-PCR	GTGGCGACGGTTCTTACCAT	CCCTGGAAGACCAATCGTGT
recA Q-PCR	GGCAGAACAGATCAAGGAAGG	TATCCACTTCGGCACGCTTA
rpoB Q-PCR	CACCGTACCCGTAGAAGTTATGC	GGAGACCTTGATCCAAGAACCA
fusA Q-PCR	CCCATGATGGTGCTTCACAA	TCGTGGCAGCAGAGGTAATG
16S Q-PCR	TGATCCTGGCTCAGGACGAA	TGCAAGCACCAATCAATACCA

Table 1. Primers used in this study.

Transcriptional analysis

Q-RT-PCR techniques were applied to assess active transcription and differential expression of genes. Amplification was carried out in 96-well plates in an ABI Prism 7700 from Applied Biosystems using the fluorescent agent SYBR Green for detection. Reactions were set up using the SYBR Green Master Mix from the same manufacturer following its recommendations. Specificity and product detection were checked after amplification by determining the temperature dependent melting curves. Primers were designed with the Primer Express software package (Applied Biosystems, The Netherlands) to have a Tm between 59 and 61ºC and an amplicon size of 100 ± 20 bp (Table 1).

Analytical determinations

Nitrate and nitrite were determined by photometric endpoint determination, using a "Nitrite/nitrate, colorimetric method" kit (Roche Diagnostics GmbH, Mannheim Germany) as described (3, 10, 31). Nitrate concentrations were also determined using a UV-method "Nitrate NO_3^-" from (Roche Diagnostics GmbH, Mannheim Germany) (3). Acetic acid was determined by a UV-method using the "Acetic acid" kit from Boehringer Mannheim / R-biopharm as described (6, 8, 9).

L-lactate acid was determined by a UV-method using the "D-Lactic acid / L-Lactic acid" kit from Boehringer Mannheim / R-biopharm as described (25, 40, 41)
Ammonia was determined by a UV-method using the "Ammonia" kit from Boehringer Mannheim / R-biopharm as described (5, 7).

HPLC analyses

Organic acids were also measured by HPLC techniques (49). 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).

Api-50 screen for nitrate reduction at high sugar concentrations

The API 50 test kit (bioMérieux, inc. Lyon), is developed to test the fermentation capacity of bacteria on 49 sugars. Cells were grown overnight in modified MRS broth

(without acetate, citrate and 10mM glucose) in the presence of heme, menaquinone and nitrate. These cells were washed twice in 50mM KPi buffer (pH 5.5) and re-suspended, in the same buffer, to an optical density (OD_{600}) of 5. In each well of the test-strip, 110 µl of cell suspension was added and incubated overnight under N_2-headspace. The presence of nitrite was quantified with the colorimetric method (see above).

Results

Aerobic respiration by L. plantarum WCFS1

The genome of *Lactobacillus plantarum* WCFS1 contains the *cydABCD* operon that encodes a *bd*-type cytochrome, with menaquinol-oxidase activity. However, supplementation with heme does not result in a respiratory phenotype, as reported for some other LAB. Heme supplementation does induce (heme-dependent) catalase activity (data not shown), possibly protecting cells against oxidative stress (30). Catalase-induction, however, has no significant impact on biomass formation. Apparently, heme-cultivated *L. plantarum* still lacks essential components for restoration of electron transport and energy restoration through respiration. The genome of *L. plantarum* however does not contain a full complement of genes to synthesize (mena)quinones, which are essential components for (aerobic) electron transport. Only when both heme and a menaquinone source (vitamin K_2) were supplemented during growth to *L. plantarum* an altered (respiratory) phenotype is found under aerobic conditions (Table 2).

Similar to *Lactococcus lactis*, the respiring phenotype of *L. plantarum* shows a significant increase in biomass while the final pH is higher compared to non-respiring conditions. The higher final pH, under respiratory conditions, coincides with a pronounced shift from lactic to acetic acid production (data not shown).

Lb. plantarum WCFS1		Biomass	Acidity
Heme	Vit. K_2	(OD_{600})	(pH)
+	+	9.45 (± 0.16)	4.39 (± 0.06)
+	-	5.45 (± 0.07)	3.94 (± 0.01)
-	+	4.71 (± 0.21)	3.94 (± 0.01)

Table 2. Effect of heme and/or menaquinone (vitamin K_2) supplementation on biomass and acidification of *Lactobacillus plantarum* WCFS1. Cells were aerobically grown in non-pH controlled batch fermentations on MRS-broth for 24 hours.

The production of acetate requires oxidizing power. Aerobically grown cells possess NADH-oxidase enzymes that regenerate NAD^+, using dissolved oxygen. Respiring cells, in addition may re-oxidize NADH via the (heme and menaquinone activated) electron transport chain.

***L. plantarum* WCFS1 genome codes for nitrate reductase genes**

Micro-array based experiments, to elucidate the genome-wide effects of respiration on gene expression, showed transcription of genes involved in nitrate-reduction (Chapter 6). The *narGHJI* genes that appeared to be transcribed in *L. plantarum*, have homologues in *B. subtilis* and *E. coli* coding for nitrate reductase A, which is involved in anaerobic nitrate respiration. This type of nitrate-reductase requires various cofactors to function: a heme-moiety, a molybdenum-pterin cofactor and iron-sulfur clusters. The heme-moiety is characteristically missing in LAB but genes coding for molybdo-pterin cofactor biosynthesis (*moaABEDA*,*mobAB*,*moeAB*) were found in close proximity of the *narGHJI* operon(Fig 1). 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 this vicinity (12, 47). The gene product of *narK*, besides its role in nitrite extrusion may also promote nitrate import into the cytoplasm (15).

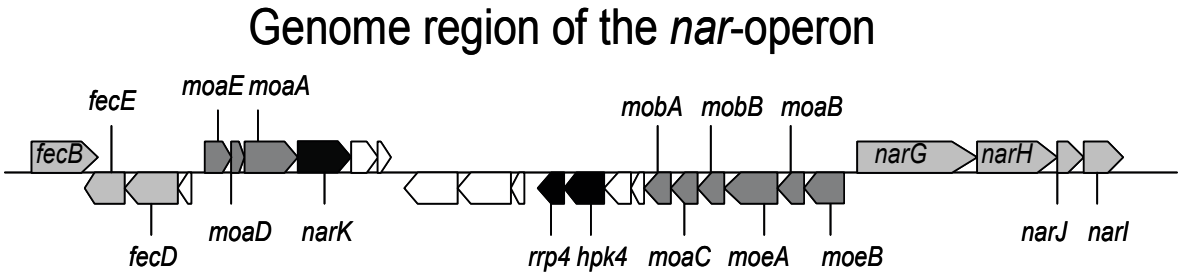


Figure 1. The genomic region upstream of the *nar*-operon (*narGHJI*) in *Lactobacillus plantarum* WCFS1. Genes coding for molybdo-pterin cofactor biosynthesis (*moaABEDA*,*mobAB*,*moeAB*), iron transport (*fecBED*), nitrite extrusion (*nark*) and a kinase/response regulator system (*rrp4*, *hpk4*) are found in this region. Putative genes are indicated with white arrows.

Growth conditions that allow nitrate reduction

The active transcription of the nitrate reductase genes, suggesting actual occurrence of this process in *L. plantarum*, prompted us to look for direct products of nitrate reduction, i.e. nitrite. An initial experiment in which *L. plantarum* WCFS1 was cultivated overnight in MRS-broth, supplemented with nitrate did not lead to production of detectable amounts of nitrite. The *narGHJI* operon is predicted to encode for a nitrate reductase A-type of enzyme which in other microorganisms is associated with electron transport (11, 23). Reconstitution of electron transport by addition of both heme and vitamin K_2, did lead to significant, but small, production of nitrite.

Higher levels of nitrite were formed, when in the presence of both heme and vitamin K_2, glucose concentration (normally present in 20 g/L = 110 mM in MRS-broth) was reduced (Fig 2).

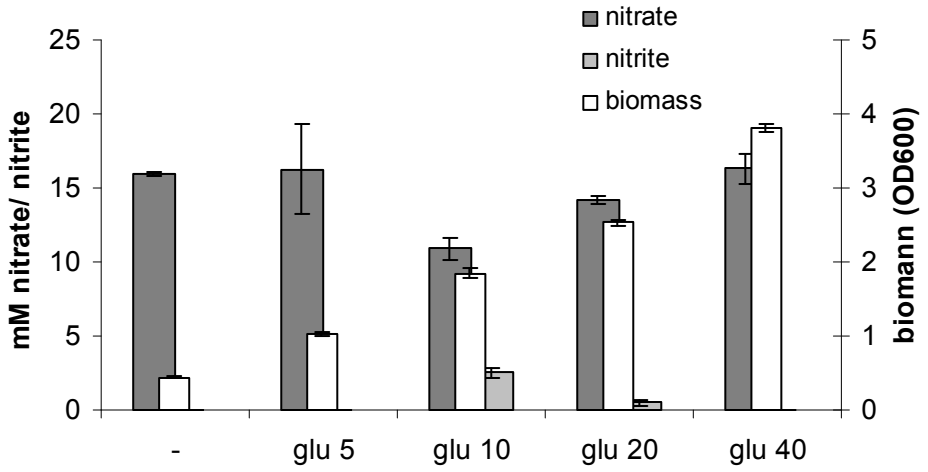


Figure 2. Nitrate respiration of *Lactobacillus plantarum* WCFS1 grown in the presence of various concentrations of glucose. Wild-type cells were grown overnight in anaerobic batch cultures in modified MRS broth (no acetate, no citrate, no glucose). Medium was supplemented with heme, menaquinone, nitrate and a variable concentration of glucose (mM).

Using 10 mM glucose in chemically defined medium it was demonstrated that both heme and vitamin K_2 have to be added simultaneously to induce nitrite production (Table 3).

	Nitrite mg/L		
Culture, additions	Average		stdev
wild-type, heme, vit. K2	33.54	±	7.11
wild-type, heme	1.13	±	0.08
wild-type,vit. K2	1.31	±	0.04
narGΔ, heme, vit. K2	Nd		

Nd, not detected

Table 3. Nitrite production by wild-type *Lactobacillus plantarum* WCFS1 and cells containing a *narG* deletion (narGΔ) after overnight growth in chemically defined medium containing 40mM Nitrate and 10 mM glucose. When both heme and vitamin K2 are supplemented a 33-fold increase in nitrite production is observed.

To establish the involvement of the *narGHJI* operon in the reduction of nitrate, a *narG* knockout (narGΔ) was constructed. NarGΔ was unable to produce nitrite under the optimal conditions for nitrate-reduction in the wild-type, which is growth medium with low glucose and nitrate, heme and menaquinone added.

Impact of nitrate-reduction on bioenergetics

Nitrate-reductase activity is associated in many bacteria with energy-production via two mechanisms (23). First, by generation of proton motive force (PMF) via an electron transport chain that terminates in the nitrate reductase A complex. The PMF generated logically would prevent utilization of ATP via the F_1-F_0 ATPase, which is the usual mechanism by which LAB create a PMF (29, 36). Secondly, the electron transport chain induces an altered redox balance in the cell, allowing for a more complete catabolism of substrates under anaerobic conditions. With a more favorable redox balance L-lactate, for example, can be converted to acetate via the acetate-kinase enzyme, generating ATP. Washed *L. plantarum* cells, pre-cultured under nitrate reducing conditions, converted L-lactate to acetate in a phosphate buffer. This conversion coincides with formation of nitrite and ammonia (Table 4).

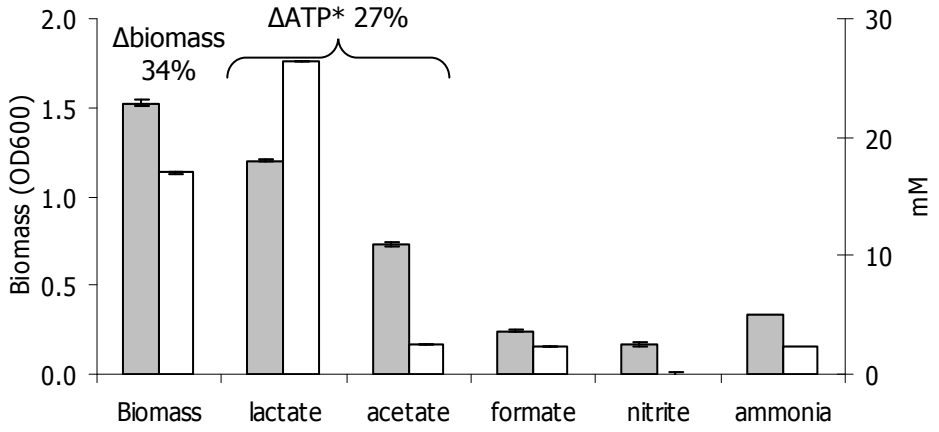
Strain	addition	Nitrite (mM)	ammonia (μM)	L-lactate (mM) consumed	Acetate (mM)
wild-type	NO_3	4.36 (±0.18)[a]	1002 (±27)	7.49 (±0.22)	4.51 (±0.26)
narGΔ	NO_3	Nd	44 (±0.67)	3.67 (±2.21)	0.96 (±0.51)
wild-type	-	Nd	50 (±1.35)	1.03 (±0.52)	1.02 (±0.08)
wild-type	NO_2	1.72 (±1.23)[b]	448 (±89)	2.96 (±0.58)	1.56 (±0.12)
-	NO_3	Nd	Nd	Nd	Nd

Nd, not detected

[a]nitrite, produced [b] nitrite, consumed

Table 4. Metabolite formation by *Lactobacillus plantarum* WCFS1 and its narGΔ derivative after an overnight incubation in buffer containing lactate and nitrate. The cells were pre-cultured in conditions that induced nitrate reduction. Cells were washed in 50mM KPi-buffer (pH 5.0), re-suspended to OD_{600} of 2.0, in buffer containing 10mM L-lactate and, where indicated, 20mM nitrate or 20mM nitrite was added.

Nitrate-reducing conditions stimulate the formation of biomass, in non-pH controlled batch cultivations. The wild-type culture showed a clear increase in biomass, compared to narGΔ, or a wild-type culture that was not supplemented with nitrate (data not shown).
To establish the exact biomass increase as a result of nitrate reduction, additional growth experiments were performed under pH-controlled conditions. The impact of nitrate-reduction on acidification patterns and energy metabolism was studied (Fig 3).



* Calculated, based on substrate level phosphorylation

Figure 3. Biomass and metabolite formation of *Lactobacillus plantarum* WCFS1 grown in pH-controlled batch conditions. Cells were grown in MRS with 10mM glucose, but without acetate and ammonium citrate (white bars). Nitrate reduction was induced by addition of heme, menaquinone and nitrate (gray bars). Measured concentrations were corrected by subtracting initial medium concentrations.

Cultures that actively reduced nitrate formed less lactate and significantly more acetate and formic acid. In these pH-controlled conditions nitrate reduction also lead to formation of more biomass (34%), ammonia and production of nitrite. Assuming a substrate-level phosphorylation efficiency of 2 ATP per glucose converted to lactate, and 4 ATP per glucose converted to acetate via glycolysis, it could be calculated that ATP generation under nitrate-reducing conditions was increased by 27% using substrate level phosphorylation (acetate production from 2 to 11 mM, lactate from 17 to 9 mM). This additional ATP-generation however is insufficient to explain the observed 34% increase in biomass (assuming a linear

correlation between ATP production and biomass). Hence, an other energy conserving mechanism may be active, such as an electron transport chain that generates a PMF. This chain may translocate 2 protons at the point of the nitrate reductase complex A (per molecule nitrate reduced). The formed proton gradient can subsequently prevent expenditure of ATP by the F_1-F_0 ATPase. Assuming that the F_1-F_0 ATPase has a proton pumping efficiency in the order of 3 H^+/ATP, we deduce that per nitrate molecule reduced by the electron transport chain 2/3 or 0.67 ATP/ (nitrate reduced) is conserved (13, 23). The catabolism of glucose towards lactate is redox balanced and surplus NADH, available for the electron transport chain, is only generated in the production of acetate (1 NADH/acetate).

The electron transport chain reduces nitrate to nitrite, which in turn is further converted and is associated with increased ammonia yields. The combined nitrite production (2.5 mM) and increase in ammonia concentration (Δ 2.7 mM), suggested the total reduction of 5.2 mM nitrate, well within the range of the (additionally) produced acetate. We can deduce that reduction of 5.2 mM nitrate could lead to an additional increase in ATP production of 5.5% ATP, which would add up to an ATP increase of 33%, which is very close to the observed 34% biomass increase.

Active degradation of nitrite

Growth of *L. plantarum* under nitrate-reducing conditions shows that nitrite initially accumulates in the medium and is rapidly removed later in growth (Fig 4).

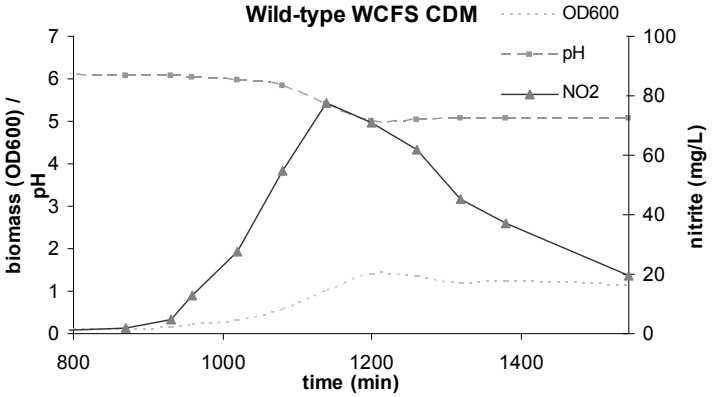


Figure 4. Growth of *Lb. plantarum* WCFS1 on chemically defined medium supplemented with heme, vitamin K2, 20mM nitrate and 10mM glucose (nitrate-CDM) followed in time.

We have experimentally verified that *L. plantarum* cells actively removed nitrite and that the contribution of chemical degradation to this removal is minimal. A positive correlation exists between the amount of biomass formed and the absolute amount of nitrite that is removed from the medium (data not shown). The reduction of nitrite seems not to be negatively affected by high glucose concentration in contrast to the reduction of nitrate. As mentioned, when nitrate reducing cells in a nitrate-containing buffer converted L-lactate to acetate, a substantial production of ammonia was also observed. This ammonia production was also observed when cells were incubated with only nitrite (Table 4). The degradation of nitrite was not dependent on the presence of heme, menaquinone or a functional *narG* (data not shown).

Effect of sugar source on nitrate-reductase activity

High glucose levels in the medium resulted in a clear decrease in nitrate reduction (Fig 2). We performed a qualitative screen, using a modified Api-50 test kit, to discover substrates which do not inhibit nitrate reduction at high concentrations. Significant nitrite production could be detected when cells were incubated overnight in the presence of high D-mannitol concentrations (50mM). Growth on MRS-broth (no acetate, no citrate) with mannitol instead of glucose, requires heme, vitamin K_2, nitrate and a functional *narG* gene (Table 5).

Strain	Additions			Residual NO_3 (mM)		Biomass (OD600)	
wild-type	heme	K2	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	-	K2	NO_3	18.94	±1.59	0.85	±0.03
wild-type	heme	K2	-	0.46	±0.46	0.95	±0.02
NarGΔ	heme	K2	NO_3	19.34	±0.53	0.91	±0.02

Table 5. Residual nitrate concentrations and biomass formation after overnight growth of *Lb. plantarum* WCFS1 and its derivative NarGΔ, in medium containing mannitol (50mM) and combinations of nitrate (20mM), heme and vitamin K_2.

Growth in these conditions therefore depends on active nitrate reduction. Mannitol, a reduced sugar, is taken up while being phosphorylated similar to glucose. Mannitol-P can be catabolised via normal glycolysis only after conversion to fructose-6-P, a reaction that reduces one NAD^+. Conversion of mannitol to lactate as the main energy producing pathway is therefore redox-unbalanced. A high flux through this pathway requires dissipation of excessive reducing power by the nitrate-reductase activity or citrate metabolism (http://bamics1.cmbi.ru.nl:1555/).

When citrate is used as redox-sink by *L. plantarum,* citrate is first converted into acetate and oxaloacetate (citrate lyase). Oxaloacetate can be subsequently oxidized, via malate (malate dehydrogenase) and fumarate (fumarase), to succinate (fumarate reductase). In the conversion of one mole oxaloacetate into one mole of succinate two moles of NADH are oxidized.

In contrast to glucose, high concentrations of mannitol do not seem to repress nitrate-reduction (Fig 5). The decrease in nitrate levels initially coincides with increase in biomass before leveling off. At that point nitrite levels appear to decrease significantly, suggesting that the catabolism of nitrite takes over as redox sink in the late growth phases.

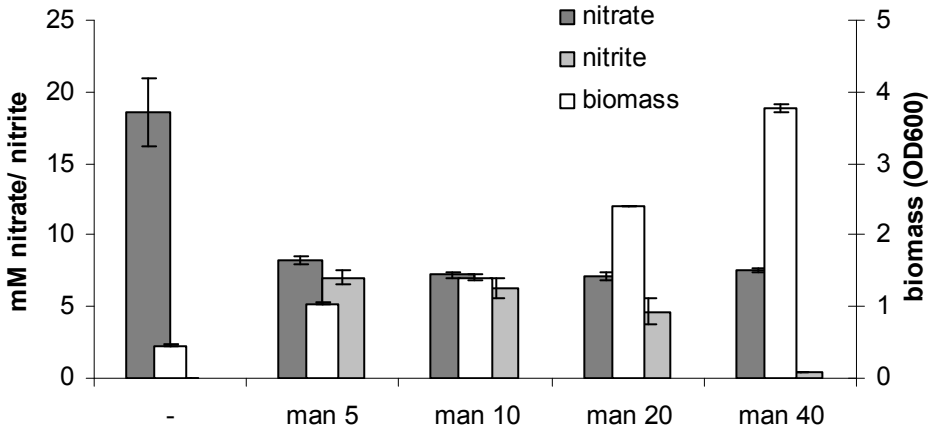


Figure 5. Wild-type cells were grown overnight in anaerobic batch cultures on modified MRS broth (no acetate, no citrate, no glucose). Medium was supplemented with heme, menaquinone, nitrate and variable mM mannitol. Nitrate-reduction is not repressed at high mannitol concentrations.

Active transcription of the nitrate reductase gene cluster

The narGΔ strain is unable to produce nitrite from nitrate. The *narG* gene encodes the subunit that contains the molybdenum-pterin cofactor and 4 iron-sulfur clusters in the assembled nitrate reductase complex. We analyzed the effect of the *narG* deletion on expression of a few selected genes of molybdenum-pterin biosynthesis and the nitrite extrusion protein by Q-RT-PCR techniques (Fig 6).

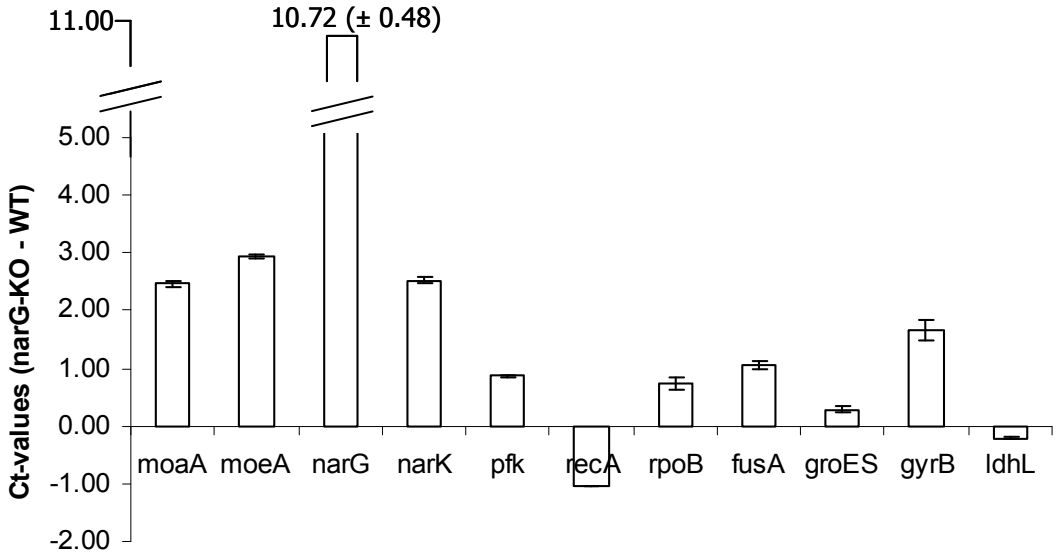


Figure 6. The difference in cycle-time (Ct) values of selected mRNA transcripts, between *Lactobacillus plantarum* WCFS1 wild-type cells and its derivative narGΔ. *MoaA*, *moeA*, *narG* and *narK* are involved in nitrate reduction and *recA*, *rpoB*, *fusA*, *GroES*, *gyrB* and *ldhL* are selected housekeeping genes. Both cell types were grown in conditions that allow reduction of nitrate by the wild-type cells. High cycle-time values indicate a low abundance of mRNA transcript. A difference in Cycle-time value of 1 indicates a log-2 difference in the ratio of mRNA transcript abundance.

When both wild-type and narGΔ cells were grown in medium containing heme, menaquinone and nitrate, nitrite accumulation was only observed in cultures containing wild-type cells. The transcription levels of the molybdenum-pterin coding genes *moeA* and *moaA*, and the nitrite extrusion protein *narK* were down-regulated in narGΔ. The observed differential expression suggests operation of a regulatory system. A kinase/response regulator

gene pair (*rrp4 hpk4*) is positioned in the centre of the nitrate reductase gene cluster (Fig 1). This kinase/response regulator forms a likely candidate system to regulate expression of genes involved in nitrate reduction.

Discussion

L. plantarum, as a typical lactic acid bacterium, has traditionally been considered an obligate fermentor. Here we propose that *L. plantarum* contains a branched electron transport chain, capable of using oxygen or nitrate as terminal electron acceptor. This proposal is compatible with the fact that the genome of *L. plantarum* WCFS1 contains the *cyd*-operon (*cydABCD*) and the *nar*-operon (*narGHJI*) (28). This oxygen and nitrate-dependent respiration has not been reported before for *Lactobacillus plantarum* WCFS1 or any other *Lactobacillus* sp. Both the *bd*-type cytochrome and the nitrate reductase A that are encoded by these genes, have menaquinol-oxidase activity and contain heme moieties. Most LAB, with the exception of the vitamin B12-producing *L. reuteri*, do not produce porphyrins and are dependent on external sources for their supply of heme. *L. plantarum*, in addition, lacks a full complement of genes to synthesize (mena)quinones. Menaquinones can be supplied in the form of menaquinone 4 (vitamin K_2).

The aerobic branch terminates in a *bd*-type cytochrome, encoded by *cydABCD*. This (mena)quinoloxidase is a membrane-bound, PMF generating, enzyme consisting of 2 subunits (cydA and cydB). CydC and cydD are required for assembly of the oxidase (18). This *bd*-type cytochrome is found in various (facultative) aerobic bacteria (26), where it functions as an (alternative) terminal electron acceptor capable of working (and activate) under low-oxygen conditions (27, 45). Aerobic respiration by *L. plantarum* induces a significant increase in biomass formation in non-pH controlled batch cultures. This biomass increase does not coincide with a further drop in pH and requires both heme and menaquinone. The proposed aerobic electron transport chain is simple, non-redundant and consists of a NADH-dehydrogenase, menaquinone pool and a *bd*-type cytochrome comparable to the *Lactococcus lactis* chain (Fig 7).

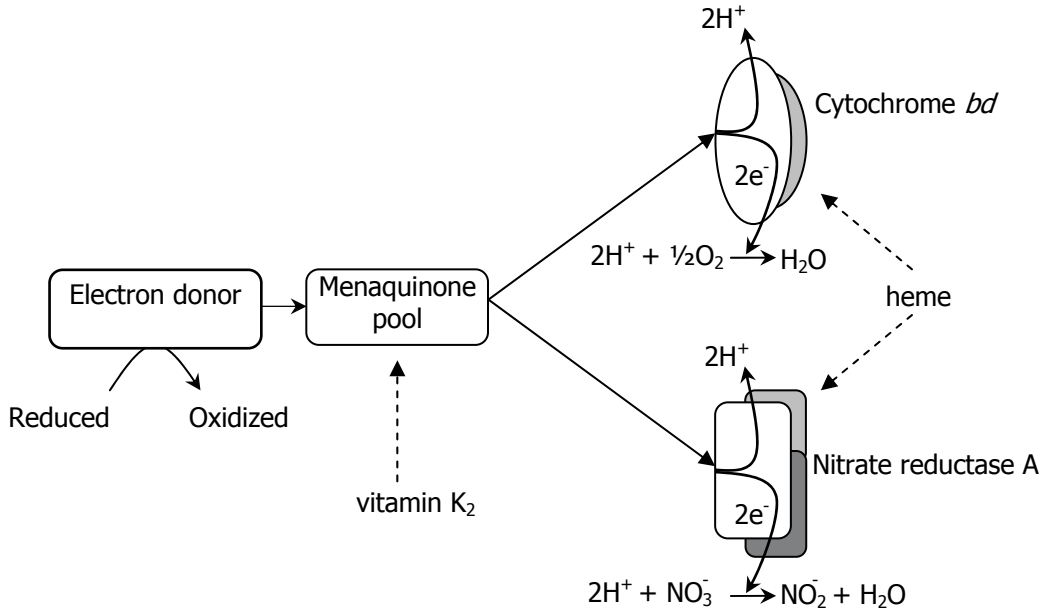


Figure 7. Proposed branched electron transport chain of *Lactobacillus plantarum* WCFS1 that terminates in either a nitrate reductase A or a *bd*-type cytcochrome complex. Dashed (- - -) lines represent the extra cellular origin of menaquinone (vitamin K_2) and heme.

The aerobic chain in *L. lactis*, the only respiratory system studied in LAB, was demonstrated to generate a PMF, and to use NADH as electron donor (13). The second branch of the electron transport chain works under anaerobic conditions and terminates in a nitrate-reductase A complex (encoded by *narGHJI*), that uses nitrate as final electron acceptor. Such a respiratory membrane-bound nitrate reductase A complex 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 (4, 35). The *narI* subunit contains two heme moieties, provides binding to the membrane and interacts with the quinone pool (44, 56). *NarJ* is required for full activity of the membrane-bound nitrate reductase (20). It has an established function in an anaerobic electron transport chain and generates PMF (33, 51, 53, 57). Both the nitrate-reductase complex and the *bd*-type cytochrome 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 (23, 39). We propose a simple non-redundant, structure of the nitrate reducing chain in *L. plantarum*, consisting of three parts, a dehydrogenase, menaquinol pool

and the nitrate reductase complex (Fig 7). We have attempted to measure PMF generation in whole cells of *L. plantarum* as described for *Lactococcus lactis* (14). However, DCCD was unable to completely inhibit the PMF generation by the ATPase, in these conditions. Inside-out membrane vesicles of *L. plantarum* may be more sensitive to DCCD treatment, and provide evidence for PMF generation by the anaerobic ETC.

The *nar*-operon seems unique for *L. plantarum* WCFS1 and is not found in any other lactic acid bacterium with the exception of *Lactobacilllus reuteri* 100-23. However the genome of *Lactobacillus reuteri* 100-23 is not yet published and an accurate (published) annotation of the open reading frames is not yet available. Genes that code for nitrate reductase associated proteins - molybdenum-pterin biosynthesis, a nitrite extrusion protein (*narK*), a flavodoxin, an iron chelating ABC transporter, a response regulator with a (putative) sensor protein - are found in the vicinity of the *narGHJI*-operon on the *L. plantarum* WCFS1 genome. Seven genes coding for proteins of unknown function are present within this genomic region. We speculate that these proteins are important for nitrate-reductase activity. They could include flavodoxin containing dehydrogenases that are known to donate electrons to the menaquinone pool (38). The response regulator and the sensor protein (*rrp4*, *hpk4*) are interesting targets to study in the elucidation of transcriptional regulation of nitrate reductase activity. We confirmed the active transcription of key-genes (*narG*, *narK*, *moeA*, *moaA*) involved in nitrate reduction by Q-PCR.

The ability of *L. plantarum* to reduce nitrate was previously undetected, presumably because of the dependency on heme and menaquinone, and because of the inhibition at high glucose concentrations. At low glucose levels nitrite is produced, possibly late in growth using lactate as electron donor, when glucose is depleted. The presence of high mannitol concentrations, however, does not inhibit nitrate reductase activity. This sensitivity to specifically glucose suggests involvement of a glucose-mediated regulatory system.

Fermentative growth with mannitol as main carbon source requires dissipation of excessive reducing power, when lactate and acetate are the main products. Active nitrate reduction (or citrate catabolism) acted as redox sink and restored growth on mannitol. Nitrate reduction may enable *L. plantarum* to grow anaerobically on a wider variety of substrates, such as reduced sugars (sugar alcohols and other polyols). Nitrate reduction in pH-controlled conditions leads to production of more biomass, than could be accounted for by substrate

level phosphorylation (Fig 3). This strongly suggests that an electron transport chain is operating leading to the generation of a PMF, just as reported for aerobic electron transport in *Lactococcus lactis* (13).

The ability of various *Lactobacillus* sp. to reduce nitrate was observed as early as 1955 (17). However this is the first report on nitrate reductase activity in LAB that is heme- and menaquinone dependent. A functional *narG* gene is also a requisite for nitrate reduction, as shown by the inability of the *narG*-mutant (narGΔ) to reduce nitrate. The inhibitory effect of nitrite on growth of various undesirable microorganisms such as *Clostridiae* during cheese production is well documented (43). Interestingly, the nitrate reduction and nitrite production is determined by presence of the different co-factors and components of the electron transport chain (heme, menaquinone and nitrate) and, thus, should be easily manipulated by varying these concentrations. As demonstrated in this work, the nitrite production is transient and is eventually converted to ammonia and possibly NO and N_2O (55-57).

The ability of *L. plantarum* to respire, using either oxygen or nitrate as final electron acceptor, could be relevant during the fermentation of plant and meat -based (food) fermentations, and in the human GI-tract. First, nitrate is a natural compound in green plants and drinking water, it is also used as a curing salt in meat fermentations (22, 34, 37). Secondly, live *L. plantarum*, 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 (16). The combination of nitrate (or maybe also oxygen), live *Lactobacillus* sp., heme and menaquinones in the human GI-tract is a reality. It would be extremely interesting and relevant to study the (positive or negative) effect of respiration on the (probiotic) effect of *Lactobacillus* strains on human health.

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1. **Adawi, D., S. Ahrne, and G. Molin.** 2001. Effects of different probiotic strains of *Lactobacillus* and *Bifidobacterium* on bacterial translocation and liver injury in an acute liver injury model. Int J Food Microbiol **70:**213-20.
2. **Ahrne, S., S. Nobaek, B. Jeppsson, I. Adlerberth, A. E. Wold, and G. Molin.** 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J Appl Microbiol **85:**88-94.
3. **Arneth, W., and B. Herold.** 1988. Nitrat/Nitrit-Bestimmung in Wurstwaren nach enzymatischer Reduction. Fleischwirtschaft **68:**761-764.
4. **Augier, V., M. Asso, B. Guigliarelli, C. More, P. Bertrand, C. L. Santini, F. Blasco, M. Chippaux, and G. Giordano.** 1993. Removal of the high-potential [4Fe-4S] center of the beta-subunit from *Escherichia coli* nitrate reductase. Physiological, biochemical, and EPR characterization of site-directed mutated enzymes. Biochemistry **32:**5099-108.
5. **Bartels, U.** 1991. Die enzymatische Bestimmung von Ammonium in Niederschlagswasser. CLB Chemie in Labor und Biotechnik **42:**377-382.
6. **Bergmeyer, H. U.** 1974. Methods of Enzymatic Analysis, 2nd. ed, vol. 1. Weinheim/Academic Press Inc. , New York and London.
7. **Bergmeyer, H. U., and H.-O. Beutler.** 1985. Methods of Enzymatic Analysis, 3rd ed, vol. VIII. Verlag Chemie, Weinheim.
8. **Bergmeyer, H. U., and H. Möllering.** 1974. Methods of Enzymatic Analysis, 2nd. ed, vol. 3. Weinheim/Academic Press Inc. , New York and London.
9. **Beutler, H.-O.** 1984. Methods of Enzymatic Analysis, 3rd ed. ed, vol. VI. Deerfield Beach/Florida, Basel.
10. **Beutler, H.-O., B. Wurst, and S. Fisher.** 1986. Eine neue Methode zur enzymatischen Bestimmung van Nitrat in Lebensmitteln. Deutsche Lebensmittel-Rundschau **82:**283-289.
11. **Bonnefoy, V., and J. A. Demoss.** 1994. Nitrate reductases in *Escherichia coli*. Antonie Van Leeuwenhoek **66:**47-56.

12. **Braun, V., and C. Herrmann.** 2007. Docking of the periplasmic FecB binding protein to the FecCD transmembrane proteins in the ferric citrate transport system, of *Escherichia coli*. J Bacteriol.
13. **Brooijmans, R. J., B. Poolman, G. K. Schuurman-Wolters, W. M. de Vos, and J. Hugenholtz.** 2007. Generation of a membrane potential by *Lactococcus lactis* through aerobic electron transport. J Bacteriol.
14. **Brooijmans, R. J., B. Poolman, G. K. Schuurman-Wolters, W. M. de Vos, and J. Hugenholtz.** 2007. Generation of a membrane potential by *Lactococcus lactis* through aerobic electron transport. J Bacteriol **189:**5203-9.
15. **Clegg, S., F. Yu, L. Griffiths, and J. A. Cole.** 2002. The roles of the polytopic membrane proteins NarK, NarU and NirC in *Escherichia coli* K-12: two nitrate and three nitrite transporters. Mol Microbiol **44:**143-55.
16. **Conly, J. M., and K. Stein.** 1992. The production of menaquinones (vitamin K2) by intestinal bacteria and their role in maintaining coagulation homeostasis. Prog Food Nutr Sci **16:**307-43.
17. **Costilow, R. N., and T. W. Humphreys.** 1955. Nitrate reduction by certain strains of *Lactobacillus plantarum*. Science **121:**168.
18. **Cruz-Ramos, H., G. M. Cook, G. Wu, M. W. Cleeter, and R. K. Poole.** 2004. Membrane topology and mutational analysis of *Escherichia coli* CydDC, an ABC-type cysteine exporter required for cytochrome assembly. Microbiology **150:**3415-27.
19. **Cunningham-Rundles, S., S. Ahrne, S. Bengmark, R. Johann-Liang, F. Marshall, L. Metakis, C. Califano, A. M. Dunn, C. Grassey, G. Hinds, and J. Cervia.** 2000. Probiotics and immune response. Am J Gastroenterol **95:**S22-5.
20. **Dubourdieu, M., and J. A. DeMoss.** 1992. The *narJ* gene product is required for biogenesis of respiratory nitrate reductase in *Escherichia coli*. J Bacteriol **174:**867-72.
21. **Duwat, P., S. Sourice, B. Cesselin, G. Lamberet, K. Vido, P. Gaudu, Y. Le Loir, F. Violet, P. Loubiere, and A. Gruss.** 2001. Respiration capacity of the fermenting bacterium *Lactococcus lactis* and its positive effects on growth and survival. J Bacteriol **183:**4509-16.

22. **Gangolli, S. D., P. A. van den Brandt, V. J. Feron, C. Janzowsky, J. H. Koeman, G. J. Speijers, B. Spiegelhalder, R. Walker, and J. S. Wisnok.** 1994. Nitrate, nitrite and N-nitroso compounds. Eur J Pharmacol **292:**1-38.
23. **Garland, P. B., J. A. Downie, and B. A. Haddock.** 1975. Proton translocation and the respiratory nitrate reductase of *Escherichia coli*. Biochem J **152:**547-59.
24. **Gaudu, P., K. Vido, B. Cesselin, S. Kulakauskas, J. Tremblay, L. Rezaiki, G. Lamberret, S. Sourice, P. Duwat, and A. Gruss.** 2002. Respiration capacity and consequences in *Lactococcus lactis*. Antonie Van Leeuwenhoek **82:**263-9.
25. **Gutmann, I., and A. W. Wahlefeld.** 1974. Methoden der enzymatischen Analyse, 2nd ed. ed, vol. 3. Weinheim/Academic Press Inc. , New York and London.
26. **Jünemann, S.** 1997. Cytochrome *bd* terminal oxidase. Biochimica et Biophysica acta **1321:**107-127.
27. **Kita, K., K. Konishi, and Y. Anraku.** 1986. Purification and properties of two terminal oxidase complexes of *Escherichia coli* aerobic respiratory chain. Methods Enzymol **126:**94-113.
28. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc Natl Acad Sci U S A **100:**1990-5.
29. **Konings, W. N.** 2002. The cell membrane and the struggle for life of lactic acid bacteria. Antonie Van Leeuwenhoek **82:**3-27.
30. **Kono, Y., and I. Fridovich.** 1983. Functional significance of manganese catalase in *Lactobacillus plantarum*. J Bacteriol **155:**742-6.
31. **Kretzschmar, R., and T. Kretschmar.** 1988. Enzymatische Nitrat-Bestimmung in kommunalen Abwasser. Vom Abwasser **70:**119-128.
32. **Lambert, J. M., R. S. Bongers, and M. Kleerebezem.** 2007. Cre-lox-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. Appl Environ Microbiol **73:**1126-35.
33. **Lanciano, P., A. Magalon, P. Bertrand, B. Guigliarelli, and S. Grimaldi.** 2007. High-stability semiquinone intermediate in nitrate reductase A (NarGHI) from

Escherichia coli is located in a quinol oxidation site close to heme bD. Biochemistry **46:**5323-9.

34. **Linseisen, J., S. Rohrmann, T. Norat, C. A. Gonzalez, M. Dorronsoro Iraeta, P. Morote Gomez, M. D. Chirlaque, B. G. Pozo, E. Ardanaz, I. Mattisson, U. Pettersson, R. Palmqvist, B. Van Guelpen, S. A. Bingham, A. McTaggart, E. A. Spencer, K. Overvad, A. Tjonneland, C. Stripp, F. Clavel-Chapelon, E. Kesse, H. Boeing, K. Klipstein-Grobusch, A. Trichopoulou, E. Vasilopoulou, G. Bellos, V. Pala, G. Masala, R. Tumino, C. Sacerdote, M. Del Pezzo, H. B. Bueno-de-Mesquita, M. C. Ocke, P. H. Peeters, D. Engeset, G. Skeie, N. Slimani, and E. Riboli.** 2006. Dietary intake of different types and characteristics of processed meat which might be associated with cancer risk--results from the 24-hour diet recalls in the European Prospective Investigation into Cancer and Nutrition (EPIC). Public Health Nutr **9:**449-64.
35. **Magalon, A., M. Asso, B. Guigliarelli, R. A. Rothery, P. Bertrand, G. Giordano, and F. Blasco.** 1998. Molybdenum cofactor properties and [Fe-S] cluster coordination in *Escherichia coli* nitrate reductase A: investigation by site-directed mutagenesis of the conserved his-50 residue in the NarG subunit. Biochemistry **37:**7363-70.
36. **Maloney, P. C.** 1982. Energy coupling to ATP synthesis by the proton-translocating ATPase. J Membr Biol **67:**1-12.
37. **McKnight, G. M., C. W. Duncan, C. Leifert, and M. H. Golden.** 1999. Dietary nitrate in man: friend or foe? Br J Nutr **81:**349-58.
38. **Melo, A. M., T. M. Bandeiras, and M. Teixeira.** 2004. New insights into type II NAD(P)H:quinone oxidoreductases. Microbiol Mol Biol Rev **68:**603-16.
39. **Miller, M. J., and R. B. Gennis.** 1985. The cytochrome *d* complex is a coupling site in the aerobic respiratory chain of *Escherichia coli* J. Biol. Chem. **260:**14003-14008.
40. **Noll, F.** 1966. Methoden zur quantitativen Bestimmung von L(+)-Lactat mittels Lactat-Dehydrogenase und Glutamat-Pyruvat-Transaminase. Biochem Z **346:**41-49.
41. **Piendl, A., and I. Wagner.** 1983. Physiologischen Eigenschaften der organischen Säuren des Bieres. Brauindustrie **68:**1520-1528.

42. **Rezaiki, L., B. Cesselin, Y. Yamamoto, K. Vido, E. van West, P. Gaudu, and A. Gruss.** 2004. Respiration metabolism reduces oxidative and acid stress to improve long-term survival of *Lactococcus lactis*. Mol Microbiol **53:**1331-42.

43. **Robach, M. C., F. J. Ivey, and C. S. Hickey.** 1978. System for evaluating clostridial inhibition in cured meat products. Appl Environ Microbiol **36:**210-1.

44. **Rothery, R. A., F. Blasco, A. Magalon, M. Asso, and J. H. Weiner.** 1999. The hemes of *Escherichia coli* nitrate reductase A (NarGHI): potentiometric effects of inhibitor binding to narI. Biochemistry **38:**12747-57.

45. **Sakamoto, J., E. Koga, T. Mizuta, C. Sato, S. Noguchi, and N. Sone.** 1999. Gene structure and quinol oxidase activity of a cytochrome *bd*-type oxidase from *Bacillus stearothermophilus*. Biochim Biophys Acta **1411:**147-58.

46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed.

47. **Shanmugam, K. T., V. Stewart, R. P. Gunsalus, D. H. Boxer, J. A. Cole, M. Chippaux, J. A. DeMoss, G. Giordano, E. C. Lin, and K. V. Rajagopalan.** 1992. Proposed nomenclature for the genes involved in molybdenum metabolism in *Escherichia coli* and *Salmonella typhimurium*. Mol Microbiol **6:**3452-4.

48. **Sijpesteijn, A. K.** 1970. Induction of cytochrome formation and stimulation of oxidative dissimilation by hemin in *Streptococcus lactis* and *Leuconostoc mesenteroides*. Antonie Van Leeuwenhoek **36:**335-48.

49. **Starrenburg, M. J., and J. Hugenholtz.** 1991. Citrate Fermentation by *Lactococcus* and *Leuconostoc* spp. Appl Environ Microbiol **57:**3535-3540.

50. **Teusink, B., F. H. van Enckevort, C. Francke, A. Wiersma, A. Wegkamp, E. J. Smid, and R. J. Siezen.** 2005. In silico reconstruction of the metabolic pathways of *Lactobacillus plantarum*: comparing predictions of nutrient requirements with those from growth experiments. Appl Environ Microbiol **71:**7253-62.

51. **Unden, G., and J. Bongaerts.** 1997. Alternative respiratory pathways of *Escherichia coli*: energetics and transcriptional regulation in response to electron acceptors. Biochim Biophys Acta **1320:**217-34.

52. **Vesa, T., P. Pochart, and P. Marteau.** 2000. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Aliment Pharmacol Ther **14:**823-8.

53. **Wang, H., C. P. Tseng, and R. P. Gunsalus.** 1999. The *napF* and *narG* nitrate reductase operons in *Escherichia coli* are differentially expressed in response to submicromolar concentrations of nitrate but not nitrite. J Bacteriol **181:**5303-8.

54. **Winstedt, L., L. Frankenberg, L. Hederstedt, and C. von Wachenfeldt.** 2000. *Enterococcus faecalis* V583 contains a cytochrome *bd*-type respiratory oxidase. J Bacteriol **182:**3863-6.

55. **Yamamoto, Y., C. Poyart, P. Trieu-Cuot, G. Lamberet, A. Gruss, and P. Gaudu.** 2006. Roles of environmental heme, and menaquinone, in *Streptococcus agalactiae*. Biometals **19:**205-10.

56. **Zhao, Z., R. A. Rothery, and J. H. Weiner.** 2003. Effects of site-directed mutations on heme reduction in Escherichia coli nitrate reductase A by menaquinol: a stopped-flow study. Biochemistry **42:**14225-33.

57. **Zumft, W. G.** 1997. Cell biology and molecular basis of denitrification. Microbiol Mol Biol Rev **61:**533-616.

Chapter 5

Heme and menaquinone induced aerobic response in *Lactobacillus plantarum* WCFS1

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Chapter 5 – Heme and menaquinone induced response in *L. plantarum*

Abstract

Aerobic growth in the presence of a heme and menaquinone source (vitamin K_2) leads to a drastic alteration in fermentation patterns, with several distinct phases. Biomass formation and aerobic cell viability are positively affected by these cofactors. Changes in global gene-expression levels suggest alterations of the pyruvate metabolism and oxidative stress. Supplementation with heme and menaquinone induced a strong down-regulation of the F_1F_0 ATPase genes as well. Several genes are required to induce this altered aerobic phenotype, such as *cydA* that codes for the structural subunit I of the menaquinol oxidase and *ndh1* (NADH-dehydrogenase).

Introduction

Lactic acid bacteria, and more specifically members of the genus *Lactobacillus*, have an important role in the lives of many people. They are necessary for the production of many fermented food products with improved shelf-life, enhanced taste en economic value. Several members of this genus are also marketed as health promoting (e.g. probiotics) (13, 16, 21, 31). *Lactobacillus plantarum* is a typical *Lactobacillus* sp. which has been isolated from a variety of fermented food products, plants (silage) and humans (1, 12, 17). *L. plantarum* WCFS1 is the model strain that has been intensively studied, is completely sequenced and has an extensive genetic toolbox (10).

Lactic acid bacteria in general are considered non-respiring fermentors that do not have the genetic capacity to produces mature cytochromes. However, supplementation with heme to aerobic cultures of *Lactococcus lactis* mg1363 induces respiration. Respiration markedly alters the phenotype as it increases biomass production and robustness (4-6, 23, 30). Addition of both heme and vitamin-K_2 (a menaquinone source) to aerated cultures of *L. plantarum* induced a similar respiratory-like response: the biomass increases while the pH tends to be higher (3).

In this study we will characterize the aerobic response of *L. plantarum* WCFS1 to heme and menaquinone in more detail. As lactic acid bacteria have no citric acid cycle, even in aerobic conditions, glucose is mainly converted to acids. By studying the changes in the production levels of the different acids (such as lactate, acetate and formate) the underlying metabolic pathways can be deduced. This may reveal how more biomass is produced from glucose catabolism, while not further acidifying the medium, as a result of heme and menaquinone supplementation.

Heme enhances robustness of *Lactococcus lactis* cells that includes a higher oxidative stress resistance. Perhaps a combination of heme and menaquinone induces oxidative stress resistance in *L. plantarum* as well. In any case, the suspected large impact on cell behavior warrants an analysis of the impact of these cofactors on the metabolism and global genome expression levels.

Materials and methods

Culture and growth conditions

The sequenced strain *L. plantarum* WCFS1 (10) or its derivatives, a cytochrome *bd* negative mutant (cydAΔ Cm^r) and a NADH-dehydrogenase mutant (ndh1Δ Cm^r), were used in this study. Cells were aerobically grown in MRS medium or MRS medium without acetate medium at 30C, by shaking at 300rpm in a shake flask. *Escherichia coli*, used as cloning host was cultivated on TYB medium. When indicated chloramphenicol and/ or erythromycin were added to a final concentration of 10 µg/ml. Chloramphenicol was added to a concentration of 100 ug/ml to *Escherichia coli* cultures.

Mutant construction

Molecular cloning techniques were carried out in accordance with standard laboratory procedures (25). The construction of cydAΔ has been described (Chapter 4). Ldh1Δ is a *L. plantarum* isogenic mutant in which the *ldh1* gene (lp_0313) has been replaced with a choramphenicol resistance marker-gene. A knock-out plasmid was constructed, a pNZ5317 derivative, that carried ~1kb *ldh1* flanking sequences to facilitate the double crossover event as described previously (11). For the construction of the Ldh1Δ, primers were designed based on *L. plantarum* WCFS1 genome sequence data. A 1 kb fragment upstream (forward primer p93 GGCAAATCAG CATAGTGTTC CTG, reverse primer p92 GGCTCGCATC CCTGCGTAAC) and downstream of *ndh1* (forward primer p94 GGAACGGTCT TCAGTAAAGG, reverse primer p95 CGCATCAATG AATCAGTCCA TG) were amplified by PCR-techniques. The flanking fragments were cloned blunt-ended in vector pNZ5317 digested with SwaI (upstream fragment) and Ecl36II (downstream fragment) to produce the knock-out vector pRB6671_ndh1_KO. The plasmid was constructed in *Escherichia coli* as host, and subsequently transformed into *L. plantarum* competent cells. Ldh1Δ was identified as a chloramphenicol resistant and erythromycin sensitive isolate and further analyzed by PCR analysis of its chromosome.

Analysis of genome-wide mRNA transcription levels

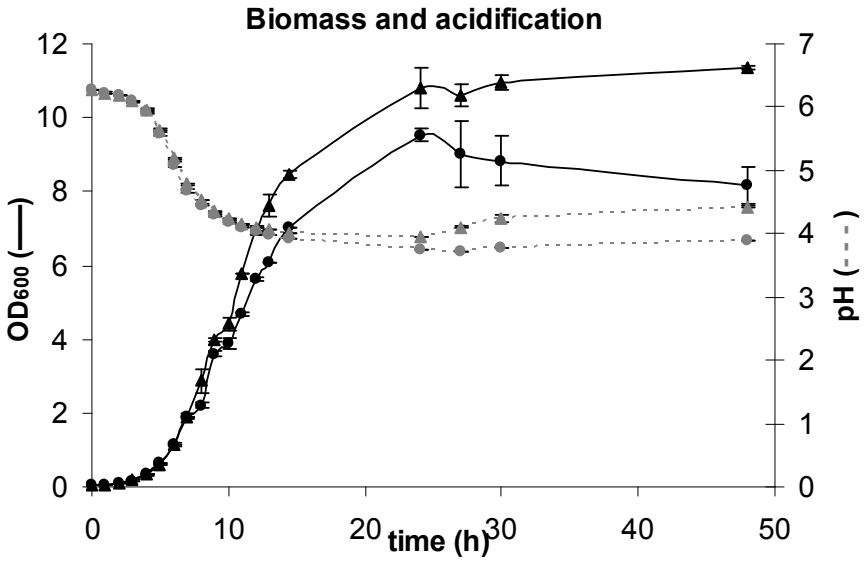
All the procedures, from RNA-quenching to the statistical analysis of the spot luminescence, including the types of kits and equipment used, were performed as described previously (27).

Results

Heme and menaquinone alter aerobic metabolism.

Wild-type cells were cultivated in non-pH controlled batch conditions, supplemented with heme and menaquinone or without these components. Key-metabolites involved in primary metabolism, biomass and acidification were measured for 48 hours (Fig. 1). The heme and menaquinone cells will be referred to as respiring cells, while the cells grown without these supplements will be named aerobic cells. For the first 10 hours no significant differences are seen between the two growth conditions. In the second phase (after 10 hours) respiring cells consumed lactate with a concomitant increase in acetate production. During this batch cultivation more biomass was produced and maintained in the supplemented cultures. The difference in fermentation pattern between the two growth conditions was exuberated over time. After 48 hours virtually all the lactate was converted to acetate in the supplemented cells, while the aerobically grown cells managed to convert roughly half. Respiring cells produced acetic acid as main acid, while aerobic cultures remained mixed-acid producers.

A



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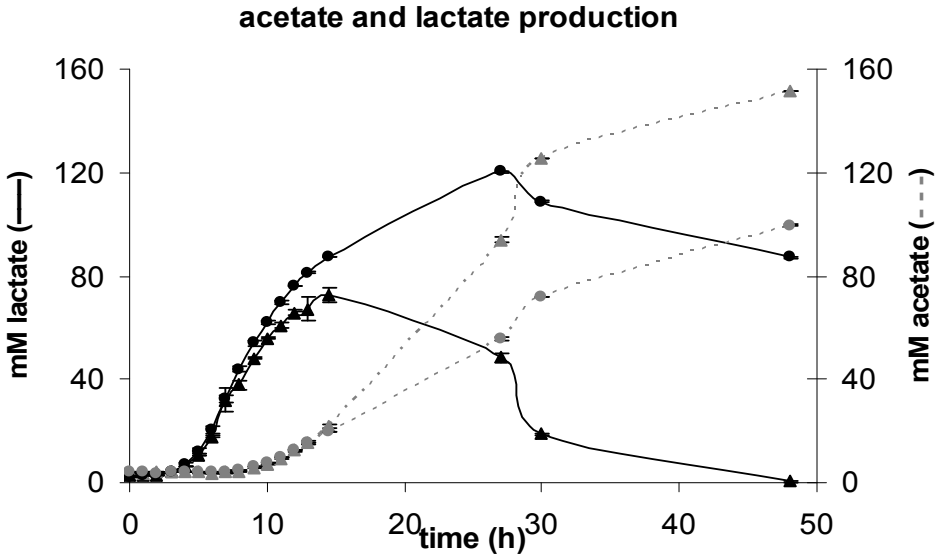

Figure 1. Altered metabolism induced by oxygen respiration in *Lb. plantarum*. Wild-type cells were aerobically grown in shake flasks in MRS at 30C, with no pH-control. Respiration was induced by addition of heme and menaquinone. NaOH and ethanol were added as a control (aerobic cultures). (A) biomass and acidification is higher after 10 h in the respiring cultures (▲) compared to aerobic cultures (●). (B) respiration (▲) induced a higher and more complete turnover over lactate to acetate compared to aerobic cultures (●).

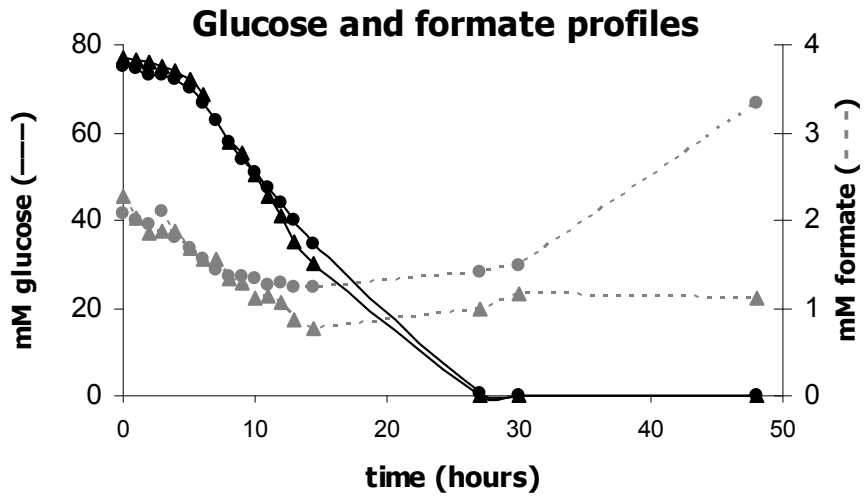

Figure 2. Glucose and formate metabolism in aerobic and respiring *Lb. plantarum* cultures. Glucose is depleted after 27 hours in both conditions. After depletion of glucose formate levels increase in aerobic- (●), but are constant in respiring cultures (▲).

After the first 10 hours the acidification rate of the two cultures became different. The pH of the respiring culture first started to decrease more slowly and eventually increased from pH 4 (at the lowest level) to pH 4.4. This is a consequence of the overall conversion of lactate to acetate, since acetate has a higher pK_a-value than lactate. In both cultures conditions glucose was no longer measurable in the medium after 27 hours of cultivation (Fig. 2). Upon this depletion of glucose, the rate of conversion of lactate to acetate increased in both conditions. Another striking difference between the fermentation patterns of the respiring and aerobic cultures was the late-growth phase formation of formate. After the depletion of glucose, acetate increased rapidly in the respiring culture while formate levels remained stable, while in aerobic conditions formate levels clearly increased. This suggests different routes for the conversion of lactate to acetate. Aerobic cells partially converted lactate to acetate using pyruvate formate lyase, while respiring cells did not. Respiring cells apparently relied more on the, NADH producing (pyruvate dehydrogenase) or non-producing (pyruvate oxidase) route (Fig. 3).

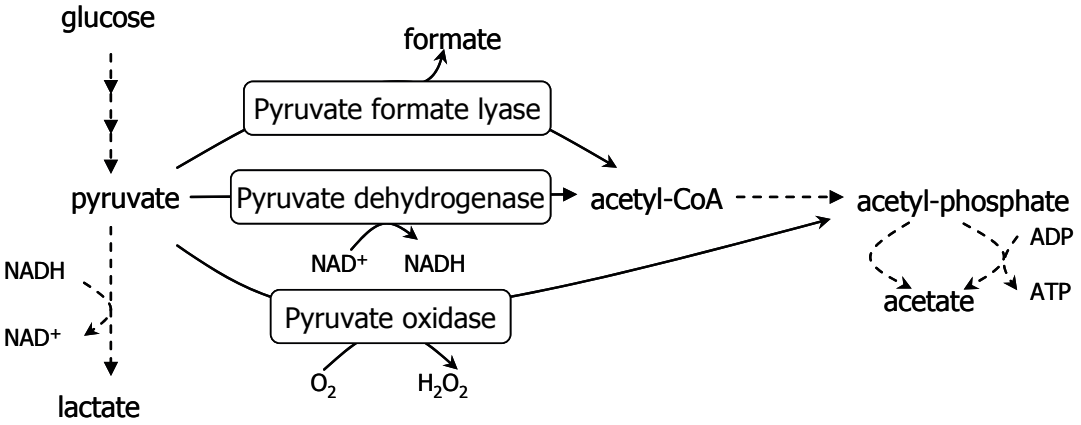

Figure 3. Schematic representation of the conversion of pyruvate to acetyl-CoA in context of primary metabolism.

Global gene expression

The impact of respiration on the global gene-expression was studied with micro-array techniques. Heme and menaquinone supplementation induces a respiration-like response late in the exponential growth phase, a condition that is difficult to reproduce in continuous fermentation. RNA samples were therefore taken (from non-pH controlled batch cultivations) well after start of the second growth phase (after 10 hours), but before entering into stationary phase, at 19 hours after start of cell growth. Many genes were differentially expressed in these aerobic conditions, in response to the heme and menaquinol supplementation (Table. 1). About 216 were 2-fold (or more) up-regulated and 258 down-regulated, constituting about 13% of the genes. Many genes involved in cell growth and protein-synthesis were down-regulated, in the cells of the respiring culture (compared to the aerobic culture) at the moment of RNA-extraction. Furthermore, many hypothetical genes were up-regulated.

Functional categories	Up	down
Amino acid biosynthesis	4	7
Biosynthesis of cofactors, prosthetic groups, and carriers	3	9
Cell envelope	11	24
Cellular processes	13	9
Central intermediary metabolism	3	2
DNA metabolism	5	11
Energy metabolism	12	19
Fatty acid and phospholipid metabolism	1	13
Hypothetical proteins	85	38
Other categories	10	4
Protein fate	2	4
Protein synthesis	3	56
Purines, pyrimidines, nucleosides and nucleotides	16	13
Regulatory functions	24	8
Transcription	2	9
Transport and binding proteins	22	30
sugar metabolism		2
Total	**216**	**258**

Table 1. Number of genes that were significantly higher (Up) or lower expressed (Down), more than 2-fold, as a result of heme and menaquinone supplementation. These genes are divided into functional categories.

Electron transport chain related genes

Several genes that are typically related with the aerobic electron transport chain (ETC) and respiration in *Lactococcus lactis* have homologous in *Lactobacillus plantarum*. A recent study of the effect of heme-induced respiration in *Lactococcus lactis* showed a lack of differential regulation of genes involved in respiration (22). A similar lack of differential regulation of many of such genes was observed (Table 2). Of all four the cytochrome genes only *cydA* and *cydB* were significantly up-regulated during respiration, but less than two-fold differentially expressed (1.9 and 1.5 times respectively). Genes that belong to the *fhu*-operon, or the proposed NADH-dehydrogenase genes were also not differentially expressed.

Locus	Gene	Putative function	^{2}log ratio	p-value
				(< 0.05)
lp_1125	*cydA*	*bd*-type cytochrome subunit I	0.91	0.037
lp_1126	*cydB*	*bd*-type cytochrome subunit II	0.59	0.039
lp_1296	*hemH*	Ferrochelatase	1.30	0.006
lp_2377	*hemK*	protoporphyrinogen oxidase (putative)	-0.89	0.014
				(> 0.05)
lp_0055		fumarate RD, flavoprotein subunit precursor	0.70	0.336
lp_0271	*vdcB*	3-octaprenyl-4-hydroxybenzoate carboxy-lyase	-0.88	0.082
lp_0313	*ndh1*	NADH dehydrogenase	0.70	0.057
lp_1069	*ndh2*	NADH dehydrogenase	-0.18	0.514
lp_1128	*cydC*	*bd*-type cytochrome ABC transporter, subunit	0.55	0.053
lp_1129	*cydD*	*bd*-type cytochrome ABC transporter, subunit	0.35	0.350
lp_3103	*fhuD*	ferrichrome ABC transporter, substr. binding prot.	0.48	0.082
lp_3104	*fhuC*	ferrichrome ABC transporter, ATP-binding prot.	0.20	0.337
lp_3105	*fhuB*	ferrichrome ABC transporter, permease prot.	0.011	0.317
lp_3106	*fhuG*	ferrichrome ABC transporter, permease prot.	-0.005	0.545
lp_3431	*ubiE*	menaquinone/ubiquinone biosynthesis methylase	0.21	0.294
lp_3464		protein kinase, ABC1 family	0.53	0.086

Table 2. Log-2 expression ratio (^{2}log) and significance (p-value) of selected genes associated with the aerobic ETC, heme and menaquinone metabolism (sensu lato). Abbreviations: RD (reductase), substr. (substrate), prot. (protein).

All genes coding for the F_1F_0 ATPase were down-regulated by more than two-fold (Fig 3). In non-respiring lactic acid bacteria the F_1F_0 ATPase is the main mechanism that generates proton motive force (PMF) at the expense of ATP (15). The active ETC with the *bd*-type cytochrome does this while consuming NADH, forming a more efficient alternative, as shown in *Lactococcus lactis* (2). For the ETC to generate part of the PMF, the F_1F_0 ATPase activity must be down-regulated. It is to be expected that the ATPase activity is quite sensitive to changes in growth conditions, as it responds to transport needs (proton motive force dissipation) and ATP-availability. However the average of more than ~2.8 fold higher expression of the ATPase genes was the highest differential expression seen among almost 70 different micro-array experiments of *Lactobacillus plantarum* (https://bamics3.cmbi.kun.nl/correlex/).

The higher rate of acetate production in respiring cells was not clearly reflected by changes in expression of genes involved in primary metabolism. Genes of a metabolic pathway that convert gluconate to acetate were up-regulated. More specifically genes of the operon (lp_1249-lp1251) encoding a D-gluconate uptake, gluconate kinase, phosphogluconate dehydrogenase and phosphoketolase were up-regulated (Table. 3) (Fig. 4). However, gluconate was not added as carbon source, but perhaps gluconate (an oxidized form of glucose) was chemically formed in presence of oxygen and free heme. Its catabolism includes a decarboxylation step and reduction of an electron carrier. As the aerobic ETC functions as redox-sink, theoretically gluconate will be more readily catabolized in the presence of heme and menaquinone.

The conversion of pyruvate to acetyl-CoA by either the pyruvate oxidase, pyruvate dehydrogenase or the pyruvate formate lyase was unclear (based on differences in gene expression). In the respiring cultures *pox5*, was strongly down-regulated, while *pox4* was up-regulated. Strong (more than 2-fold) down-regulation of specifically *pox5* expression in anaerobic (vs aerobic) conditions has been observed before (26). Furthermore two genes of the pyruvate dehydrogenase operon were down-regulated in the presence of heme and menaquinone, while NADH production would seem favorable in combination with an ETC (Table. 3). Genes that encode the pyruvate formate lyase were not significantly affected. As formate production differs only after glucose is depleted, this is therefore not expected at the time point of RNA isolation.

A final observation is the up-regulation of the *ldh1* gene in supplemented cells by more than 2-fold, while the lactate production was decreasing. *ldh2*, encoding a second lactate dehydrogenase, was not differentially regulated. A specific role of Ldh1 for lactate to pyruvate conversion, rather then the opposite reaction, would provide a rational for its up-regulation in supplemented cultures. In fact up-regulation of the other NADH dehydrogenase *ldh2* has been associated with higher lactate production levels (Chapter 6).

Locus	Gene	Putative function	^{2}log ratio	p-value
		Gluconate catabolism		
lp_1249	*gntP*	gluconate transport protein	1.372	0.006
lp_1250	*gntK*	gluconokinase	1.365	0.005
lp_1251	*gnd1*	phosphogluconate DH (decarboxylating)	1.403	0.005
lp_2659	*xpk1*	phosphoketolase	1.147	0.008
		Pyruvate to acetyl-CoA conversion		
lp_3587	*pox4*	pyruvate oxidase	0.621	0.031
lp_3589	*pox5*	pyruvate oxidase	-1.154	0.007
lp_2153	*pdhB*	pyruvate DH complex, E1 comp., β-subunit	-0.865	0.013
lp_2154	*pdhA*	pyruvate DH complex, E1 comp., α-subunit	-0.593	0.039

Table 3. Significantly differentially expressed genes, involved in gluconate catabolism and pyruvate to acetyl-CoA conversion. Log-2 ratio (^{2}log) and statistical significance (p-value) are given.

Responses to oxidative stress

The genome of *L. plantarum* WCFS1 encodes several NADH-oxidase (NOX) genes. By removing oxygen NOX-activity not only regenerates NAD^{+} but also provides a level of oxidative stress resistance. The aerobic ETC catalyses this same reaction while conserving energy (4). We observed that in heme and menaquinone supplemented conditions *nox6* was significantly down-regulated ~2.5 fold. While, in *Escherichia coli* respiration (with the *bo*-cytochrome as major respiratory cytochrome) provokes oxygen stress by creating reactive oxygen species (ROS) (7, 8, 20) The *bd*-type cytochrome, in contrast, reduces oxygen stress as its activity as high affinity oxygen scavenger maintains low intracellular oxygen levels. Activity of specifically the *bd*-type cytochrome protects against H_2O_2 and ROS (9, 14). Therefore ETC activity in *Lactococcus lactis* and *L. plantarum* reduces oxidative stress (19, 23). Indeed aerobic incubation of *L. plantarum*, without ETC activity produces H_2O_2 (18). In agreement with a decrease of oxygen stress in the supplemented culture we observed a reduced expression of the NADH-peroxidase gene *npr1*. A clear example that supplementation *L. plantarum* reduces oxidative stress is the drastically higher number of colony forming units after 48 hours of aerobic cultivation, compared with non-supplemented cultures ($2.17x10^{7}$ $\pm 0.61x10^{7}$ vs. $<1x10^{4}$).

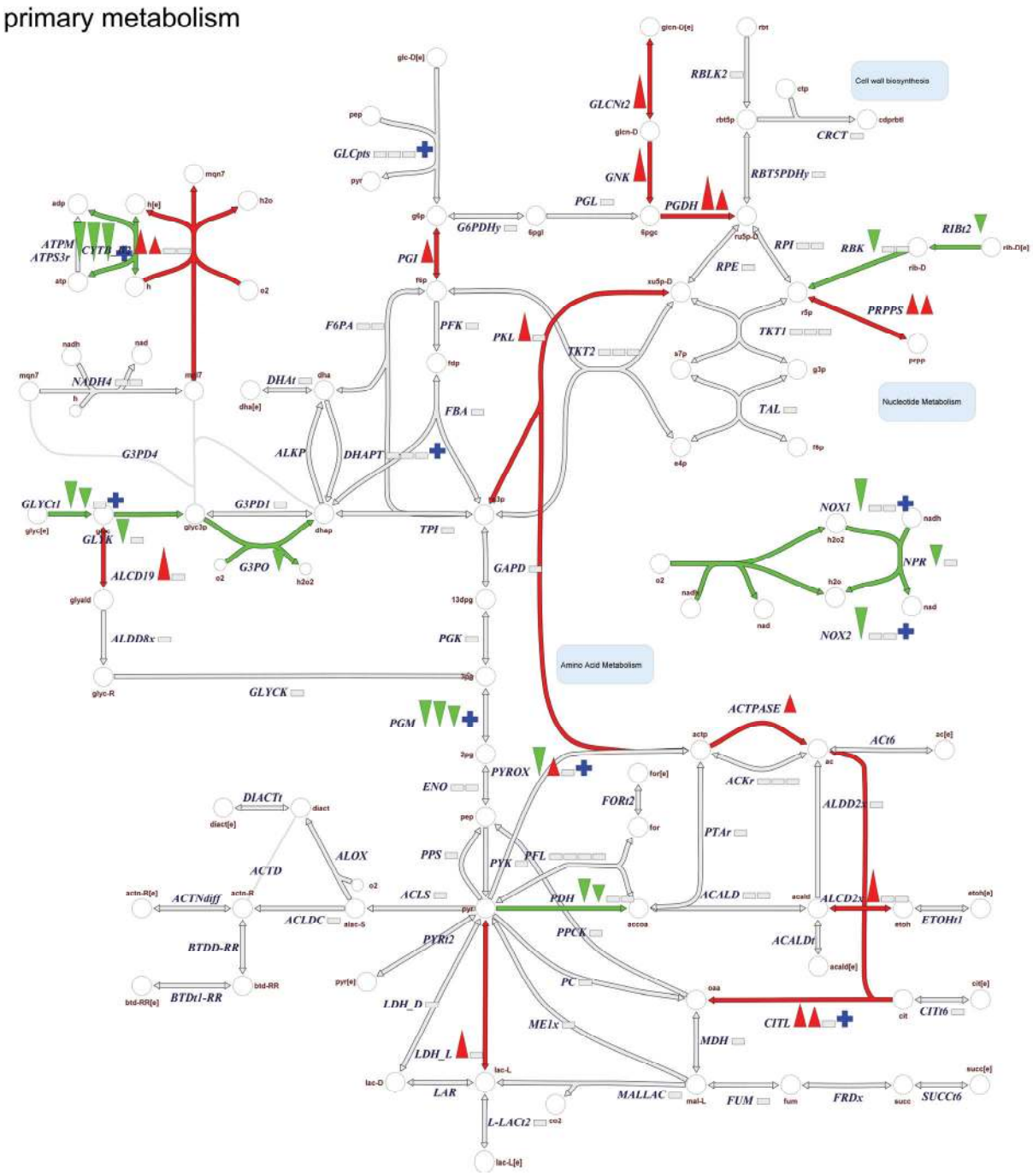

Figure 4. Visualization of genes involved in primary metabolism that were differentially expressed. Red signifies up-regulation by 2-fold or more, green down-regulation by 2-fold or more, and yellow signifies differentially expressed by less then 2-fold. Blank pathways indicate that those associated genes were not significantly regulated. All abbreviations are explained in the supplementary material of Teusink *et. al.* (29). The size of the triangles signifies the relative up- or- down-regulation of the genes associated

with the metabolic pathway. Square boxes indicates those genes were not affected by more then 2-fold. Blue plus sign indicate more genes are associated with those reactions then are shown.

Phenotype of selected mutants

The aerobic branch of the ETC uses a *cydABCD* encoded menaquinol oxidase (cytochrome *bd*). In *Lactococcus lactis* a mutation in one of the structural genes (*cydA*) leads to a loss of the respiratory phenotype (4, 5). A mutant of *L. plantarum* WCFS1, of whom the *cydA* gene was mutated, failed to demonstrate the respiratory-like phenotype, when grown with heme and menaquinone (Table 1) (Chapter 4). Furthermore, the aerobic ETC in *Lactococcus lactis* starts with a membrane-bound NADH-dehydrogenase that oxidizes NADH with the reduction of menaquinone. The genes that encode the NADH-dehydrogenase in *L. plantarum* have thus far not been identified. In the genome of *L. plantarum* two NADH-dehydrogenase genes are annotated (*ndh1* and *ndh2*) that are likely candidates for this function. The predicted *ndh1* gene product has no membrane spanning helices, although it lies in a operon-like structure upstream of a gene encoding a integral membrane protein (d), with which it may be associated (Fig 5).

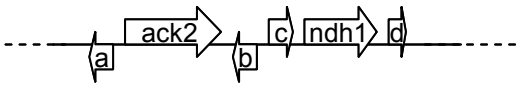

Figure 5. The genomic region of *ndh1* and *ack2*. *ndh1* (NADH-dehydrogenase) forms part of an operon-like structure with a transcription factor (c) and an integral membrane protein (d) that neighbors the acetate kinase gene (*ack2*). *ack2* itself is flanked by another integral membrane protein (a) and a putative acetyl transferase (b).

Up-stream of *ndh1* is positioned the acetate kinase gene (*ack2*). Activation of the aerobic ETC induced a higher metabolite flux toward acetate. The close proximity of *ack2* and *ndh1* on the genome may indicate functional association. We have constructed a mutant lacking a functional ndh1 gene, which indeed fails to show respiratory behavior upon addition of heme and menaquinone to the culture medium (Table. 1).

Cells	Heme	Vit-K_2	OD_{600}	pH
Wild-type	+	+	9.88 ±0.25	4.51 ±<0.01
Wild-type	-	-	7.04 ±0.08	3.96 ±0.01
CydAΔ	+	+	5.56 ±0.17	3.94 ±0.02
CydAΔ	-	-	5.69 ±0.13	3.96 ±0.01
Ndh1Δ	+	+	7.22 ±0.2	3.96 ±0.01
Ndh1Δ	-	-	6.79 ±0.1	3.95 ±<0.01

[1]Nd.: Not determined

Table 1. Phenotype of respiring wild-type cells and respiration-negative *cydA* and *ndh1* mutants. Cells were incubated for 48 hours in MRS-broth in aerated conditions. *CydA* and *ndh1* mutants lack the heme and menaquinone induced response (higher final biomass and pH) as shown in the wild-type.

Wild-type cells respond to the addition of heme and menaquinone in aerobic conditions by a clear increase in biomass and a higher final pH. The two mutants (CydAΔ and Ndh1Δ) do not respond in this way. Their final biomass at most equals that of non-supplemented wild-type cells, while their final pH is unaffected by supplementation.

Discussion

Heme and menaquinone supplementation in *Lactobacillus plantarum* gives rise to a respiratory-like phenotype, as seen in *Lactococcus lactis*. The increase in biomass formation, without more extensive acidification can largely be contributed to shift from lactate to acetate as main catabolic end product. Acetate production can yield more substrate level ATP and has a higher pK_a-value than lactate. As in *Lactococcus lactis* heme and menaquinone supplementation improves resistance against oxidative stress. The cell viability was greatly improved after an aerobic incubation of several days. Furthermore, the *cydA* (*bd*-type cytochrome) and *ndh1* (NADH-dehydrogenase) genes were also shown to be essential for the manifestation of the supplemented aerobic phenotype. That a functional aerobic ETC is involved in the observed heme- and menaquinone-induced phenotype of *L. plantarum* is convincingly shown.

Fermentation profiles are suggestive that respiring cells (supplemented with heme and menaquinone) are somewhat more efficient in generating biomass. Assuming that for every lactate one ATP is produced, and for every acetate 2 ATP, we can approximate (or at least compare) the ATP-yield of substrate level phosphorylation. At every time-point the

ATP-yield is calculated as the amount of lactate formed, plus twice the amount of acetate formed. When we then divide the biomass (optical density measured by absorbance at 600nm) by this ATP-yield we approximate the biomass/ATP-yield, or biomass generating efficiency (Fig 6).

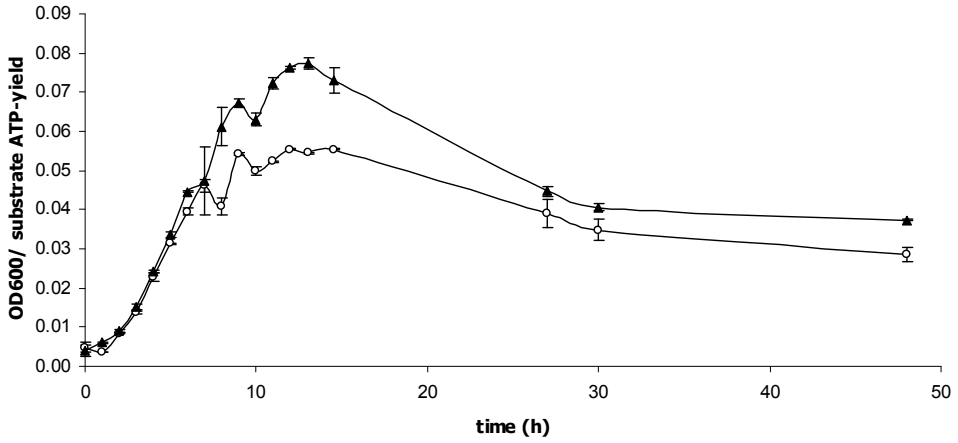

Figure 6. The ratio between the biomass yield (OD_{600}) and the substrate-level ATP-yield. After ~10 hours respiring cultures (▲) have a higher biomass generating efficiency than aerobic cultures (ם).

This graph shows that respiring cells generate more biomass then aerobic cells with the same amount of substrate-level ATP after 10 hours. This is highly suggestive that starting at this time-point respiring cells conserve additional energy, likely via proton motive force formation with the aerobic ETC. Nevertheless non-supplemented aerobic cultures do apparently suffer from more oxidative stress as is also shown by the up-regulation of several genes (*nox6* and *npr1*). Oxidative stress resistance may put a higher ATP demand on cell maintenance, and thus reallocate ATP (away from biomass formation).

In two recent studies the effect of aerobic cultivation on the transcriptome was compared with anaerobic cultivation (26, 28). Strong up-regulation of *pox3* (^{2}log 1.8 – 3.8 or fold-change 3.2 - 13.9) and *nox5* (^{2}log 0.5 – 2.7 or fold-change 1.4 – 6.5) by aerobiosis was observed in one study, up-regulation of *pox3* (^{2}log 4.32 or fold-change 20) and *pox5* (^{2}log 2.7 or fold-change 6.5) in the other. In our experiment addition of heme and menaquinone to aerobic cultures induced a significant down-regulation of *nox6* and *pox5* (^{2}log -1.15 or fold-

change 0.5), and up-regulation of *pox4* (^{2}log 0.621 or fold-change 1.5). To conclude, there is quite a bit of variability in the response to oxygen, even in highly similar experiments. The rough "trend" in the responses to oxygen appears to be strong up-regulation of individual *pox*-genes and *nox*-genes. In this sense down-regulation of *nox6* and strong down-regulation of *pox5* suggest a lower oxygen-response in the heme and menaquinone supplemented cells. This in turn supports the idea that the *bd*-type cytochrome maintains low intracellular oxygen levels, which has so suggested for *Lactococcus lactis* (23).

The *cydAB* genes were differentially up-regulated, but not strongly. The *cydCD* genes that are involved in maturation of the cytochrome were not differentially expressed at all. The lack of apparent strict regulation of several electron transport associated genes, among which *ndh1* has been noted in a recent paper that focused on the respiratory response *Lactococcus lactis* (22).

In this paper we have given a thorough characterization of the effect of heme and menaquinone on aerobic fermentation patterns. We have also shown the involvement of an ETC. Interestingly *L. plantarum* possesses and alternative cytochrome: a nitrate reductase A. This reductase is found in other prokaryotes where it is active in the anaerobic ETC that uses nitrate as electron sink. Furthermore, recently in *Lactococcus lactis* reduction of Cu^{2+} and Fe^{3+} by the endogenous quinine pool was shown (24). The ETC of *L. plantarum* that is established by addition of heme and menaquinone may perhaps allow for more metabolic flexibility then we are currently aware of.

1. **Ahrne, S., S. Nobaek, B. Jeppsson, I. Adlerberth, A. E. Wold, and G. Molin.** 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J Appl Microbiol **85:**88-94.
2. **Blank, L. M., B. J. Koebmann, O. Michelsen, L. K. Nielsen, and P. R. Jensen.** 2001. Hemin reconstitutes proton extrusion in an H^+-ATPase-negative mutant of *Lactococcus lactis*. J. Bacteriol. **183:**6707-9.
3. **Brooijmans, R. J. W., W. M. de Vos, and J. Hugenholtz.** manuscript in preparation (Chapter 4). Respiration in *Lactobacillus plantarum* WCFS1
4. **Brooijmans, R. J. W., B. Poolman, G. K. Schuurman-Wolters, W. M. de Vos, and J. Hugenholtz.** 2007. Generation of a membrane potential by *Lactococcus lactis* through aerobic electron transport. J Bacteriol.
5. **Duwat, P., S. Sourice, B. Cesselin, G. Lamberet, K. Vido, P. Gaudu, Y. Le Loir, F. Violet, P. Loubiere, and A. Gruss.** 2001. Respiration capacity of the fermenting bacterium *Lactococcus lactis* and its positive effects on growth and survival. J Bacteriol **183:**4509-16.
6. **Gaudu, P., K. Vido, B. Cesselin, S. Kulakauskas, J. Tremblay, L. Rezaiki, G. Lamberret, S. Sourice, P. Duwat, and A. Gruss.** 2002. Respiration capacity and consequences in *Lactococcus lactis*. Antonie Van Leeuwenhoek **82:**263-9.
7. **Gonzalez-Flecha, B., and B. Demple.** 1995. Metabolic sources of hydrogen peroxide in aerobically growing *Escherichia coli*. J Biol Chem **270:**13681-7.
8. **Imlay, J. A., and I. Fridovich.** 1991. Assay of metabolic superoxide production in *Escherichia coli*. J Biol Chem **266:**6957-65.
9. **Kita, K., K. Konishi, and Y. Anraku.** 1986. Purification and properties of two terminal oxidase complexes of *Escherichia coli* aerobic respiratory chain. Methods Enzymol **126:**94-113.
10. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003.

Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc Natl Acad Sci U S A **100:**1990-5.

11. **Lambert, J. M., R. S. Bongers, and M. Kleerebezem.** 2007. Cre-lox-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. Appl Environ Microbiol **73:**1126-35.
12. **Leroy, F., and L. De Vuyst.** 2004. Lactic acid bacteria as functional starters for the fermentation industry. Trends Food Sci Technol **15:**67-78.
13. **Lin, W. H., B. Yu, S. H. Jang, and H. Y. Tsen.** 2007. Different probiotic properties for *Lactobacillus fermentum* strains isolated from swine and poultry. Anaerobe.
14. **Lindqvist, A., J. Membrillo-Hernandez, R. K. Poole, and G. M. Cook.** 2000. Roles of respiratory oxidases in protecting *Escherichia coli* K12 from oxidative stress. Antonie Van Leeuwenhoek **78:**23-31.
15. **Maloney, P. C.** 1982. Energy coupling to ATP synthesis by the proton-translocating ATPase. J Membr Biol **67:**1-12.
16. **Medellin-Pena, M. J., H. Wang, R. Johnson, S. Anand, and M. W. Griffiths.** 2007. Probiotics Affect Virulence-related Gene Expression of *Escherichia coli* O157:H7. Appl Environ Microbiol.
17. **Messens, W., and V. L. De.** 2002. Inhibitory substances produced by *Lactobacilli* isolated from sourdoughs--a review. Int J Food Microbiol **72:**31-43.
18. **Murphy, M. G., and S. Condon.** 1984. Correlation of oxygen utilization and hydrogen peroxide accumulation with oxygen induced enzymes in *Lactobacillus plantarum* cultures. Arch Microbiol **138:**44-8.
19. **Nguyen, P. T., J. Abranches, T. N. Phan, and R. E. Marquis.** 2002. Repressed Respiration of Oral Streptococci Grown in Biofilms. Curr Microbiol **44:**262-6.
20. **Nystrom, T., C. Larsson, and L. Gustafsson.** 1996. Bacterial defense against aging: role of the *Escherichia coli* ArcA regulator in gene expression, readjusted energy flux and survival during stasis. Embo J **15:**3219-28.
21. **Pathmakanthan, S., C. K. Li, J. Cowie, and C. J. Hawkey.** 2004. *Lactobacillus plantarum* 299: beneficial in vitro immunomodulation in cells extracted from inflamed human colon. J Gastroenterol Hepatol **19:**166-73.

22. **Pedersen, M. B., C. Garrigues, K. Tuphile, C. Brun, K. Vido, M. Bennedsen, H. Mollgaard, P. Gaudu, and A. Gruss.** 2008. Impact of aeration and heme-activated respiration on *Lactococcus lactis* gene expression: Identification of a heme-responsive operon. J Bacteriol.

23. **Rezaiki, L., B. Cesselin, Y. Yamamoto, K. Vido, E. van West, P. Gaudu, and A. Gruss.** 2004. Respiration metabolism reduces oxidative and acid stress to improve long-term survival of *Lactococcus lactis*. Mol Microbiol **53:**1331-42.

24. **Rezaiki, L., G. Lamberet, A. Derre, A. Gruss, and P. Gaudu.** 2008. *Lactococcus lactis* produces short-chain quinones that cross-feed Group B *Streptococcus* to activate respiration growth. Mol Microbiol **67:**947-57.

25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed.

26. **Serrano, L. M.** 2008. Oxidative stress response in *Lactobacillus plantarum* WCFS1: A functional genomics approach. University of Wageningen, Wageningen.

27. **Serrano, L. M., D. Molenaar, M. Wels, B. Teusink, P. A. Bron, W. M. de Vos, and E. J. Smid.** 2007. Thioredoxin reductase is a key factor in the oxidative stress response of *Lactobacillus plantarum* WCFS1. Microb Cell Fact **6:**29.

28. **Stevens, M. J., A. Wiersma, W. M. De Vos, O. P. Kuipers, E. J. Smid, D. Molenaar, and M. Kleerebezem.** 2008. Improvement of *Lactobacillus plantarum* Aerobic Growth, Directed by Comprehensive Transcriptome Analysis. Appl Environ Microbiol.

29. **Teusink, B., A. Wiersma, D. Molenaar, C. Francke, W. M. de Vos, R. J. Siezen, and E. J. Smid.** 2006. Analysis of growth of *Lactobacillus plantarum* WCFS1 on a complex medium using a genome-scale metabolic model. J Biol Chem **281:**40041-8.

30. **Vido, K., D. Le Bars, M. Y. Mistou, P. Anglade, A. Gruss, and P. Gaudu.** 2004. Proteome analyses of heme-dependent respiration in *Lactococcus lactis*: involvement of the proteolytic system. J Bacteriol **186:**1648-57.

31. **Zhang, L., N. Li, R. Caicedo, and J. Neu.** 2005. Alive and dead *Lactobacillus rhamnosus* GG decrease tumor necrosis factor-alpha-induced interleukin-8 production in Caco-2 cells. J Nutr **135:**1752-6.

Chapter 6

The anaerobic electron transport chain of *Lactobacillus plantarum* is an efficient redox sink

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Chapter 6 – The anaerobic ETC of *L. plantarum* is an efficient redox sink

Abstract

Lactobacillus plantarum WCFS1 can reduce nitrate only when supplied with heme and menaquinone. The presence of nitrate, heme and menaquinone allows anaerobic growth on the reduced sugar mannitol, with the reduction of nitrate as redox sink. Equimolar concentrations of nitrate or citrate were equally effective as redox sink, without a clear impact on the final biomass-level. In these conditions genes corresponding to the metabolic conversion of citrate conversion to succinate, nitrate-reductase genes, and many other genes, were differentially expressed. The genes that encode the nitrate-reductase proteins (*nar*-genes), molybdo-pterin co-factor, nitrite extrusion and others, are found in a genetic region of 28 genes and were co-conserved in many *L. plantarum* strains. Nitrate (and not heme and menaquinone) was found to stimulate transcription of *moaA*, *moeA*, *narG*, *narK*, *hpK4* and *rrp4*.

Introduction

Lactic acid bacteria form an important group of related Gram-positive of bacteria that are widely used in the production of fermented foods (12, 17). A *bd*-type cytochrome enables heme-dependent aerobic respiration in *Lactococcus lactis* (1). Lactic acid bacteria do not produce hemes, which has to be supplied. *L. plantarum* does not have a complete biosynthetic pathway to produce menaquinone(s), another component of electron transport chains (4). However, supplementation with both these cofactors induced an aerobic and anaerobic electron transport chain activity in *L. plantarum* WCFS1 (2). This anaerobic electron transport chain can reduce nitrate to nitrite.

In fact *L. plantarum* WCFS1, not only has genes that encode the aerobic *bd*-type cytochrome, as *Lactococcus lactis*, but a nitrate-reductase A as well (9). The *narGHJI* genes encoded nitrate reductase A generates proton motive force in *Escherichia coli* (7). In fact the *bd*-type cytochrome and the nitrate reductase A are alike in that they are proposed to generate proton motive force with a similar efficiency (6, 8, 14, 16). Furthermore, the nitrate reductase A complex, just like the *bd*-type cytochrome, is a menquinol oxidase that contains heme-cofactors (15). As the aerobic electron transport chain has a huge impact on metabolism, a huge impact of nitrate-reductase activity on metabolism could be expected as well (3).

As mentioned, supplementation of heme and menaquinones activated the anaerobic electron transport chain of *L. plantarum*. The presence of these cofactors and nitrate allowed anaerobic growth on the reduced sugar mannitol that requires an extracellular redox sink (13). Citrate can also be supplied as extracellular redox sink to sustain anaerobic growth on mannitol, but which does not involve direct ATP or proton motive generation (5, 20). As anaerobic growth on mannitol forces cells to use either citrate or nitrate as redox-sink we can distill additional effects of nitrate reduction on cell behavior and energy metabolism, besides its role as redox-sink. Here, we investigate the impact, of the anaerobic electron transport chain that reduces nitrate, on metabolism in more detail. We will study its impact on growth, fermentation-profiles and on global gene expression levels in comparison with citrate as redox sink.

Materials and methods

Cultures and growth conditions

L. plantarum WCFS1 was the principal strain used in this study, an isolate from NCIMB8826 (9). *L. plantarum* was Cells were cultured in chemically defined medium (CDM) as described or Man Rogosa Sharpe (MRS) broth (22) (Difco). The CDM recipe was modified as acetate was omitted and glucose replaced by mannitol (50mM). When mentioned, heme was added to a final conc. of 2.5 ug/ml (stock 0.5 mg/ml in 0.05 M NaOH Sigma-Aldrich), vitamin K_2 (or menaquinone-4) to a final conc. of 1 ug/ml (stock 2mg/ml in ethanol Sigma-Aldrich) or the equivalent volumes of ethanol and 0.05 M NaOH. $NaNO_3$ (Sigma-Aldrich) was added as nitrate source to a concentration of 20mM.

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 K2 or ethanol, were added when mentioned. 1L fermentors were filled with 500ml medium and the headspace flushed with 20ml/min N_2 (g).

Q-RT-PCR procedures

Q-RT-PCR techniques were applied to assess differential expression of genes. Amplification was carried out in 96-well plates in an ABI Prism 7700 from Applied Biosystems using the fluorescent agent SYBR Green for detection. Reactions were set up using the SYBR Green Master Mix from the same manufacturer following its recommendations. Specificity and product detection were checked after amplification by determining the temperature dependent melting curves. Primers were designed with the Primer Express software package (Applied Biosystems, The Netherlands) to have a Tm between 59 and 61ºC and an amplicon size of 100 ± 20 bp.

Nitrate and nitrite determination

Nitrate was analyzed by 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.

Nitrite was also measured determined by photometric endpoint determination, using a "Nitrite/nitrate, colorimetric method" kit (Roche Diagnostics GmbH, Mannheim Germany).

Analysis of genome-wide mRNA transcription levels

All the procedures, from RNA-quenching to the statistical analysis of the spot luminescence, including the types of kits and equipment used, were performed as described previously (21).

Results

Growth on mannitol using NO_3 or citrate as electron acceptor

L. plantarum cells are able to reduce nitrate in the presence of heme and menaquinone in certain conditions. This ability is suppressed by high concentrations of glucose, but not by high mannitol concentrations. Mannitol is a reduced sugar and anaerobic catabolism of mannitol to lactate is not redox balanced. Growth on mannitol requires additional oxidizing power. To examine the impact of nitrate reduction on fermentation patterns we cultivated wild-type cells (anaerobically) with mannitol as carbon source. Cells were supplied with citrate (citrate cells) or nitrate (nitrate cells) as redox sink (Fig 1). Nitrate cells were supplemented with heme and menaquinone to activate the electron transport chain and an equal volume of ethanol 0.05 M NaOH were added to citrate cells as control. Cells were grown in modified chemically defined medium in pH-controlled batch conditions (22). When the stationary phase was reached, no significant difference in the maximum biomass level was observed between the citrate or nitrate consuming cells. The maximal optical density (600nm) achieved was roughly ~3.5 in both conditions. Growth of nitrate reducing cells started after a prolonged (>17 hours) lag-phase. In both culture conditions mannitol was completely consumed by the start of stationary phase.

Initially citrate was consumed and converted to mainly acetate and lactate. This is nicely visualized in graphs that shows the biomass (as measured by optical density at 600nm) and lactate concentrations (Fig 1).

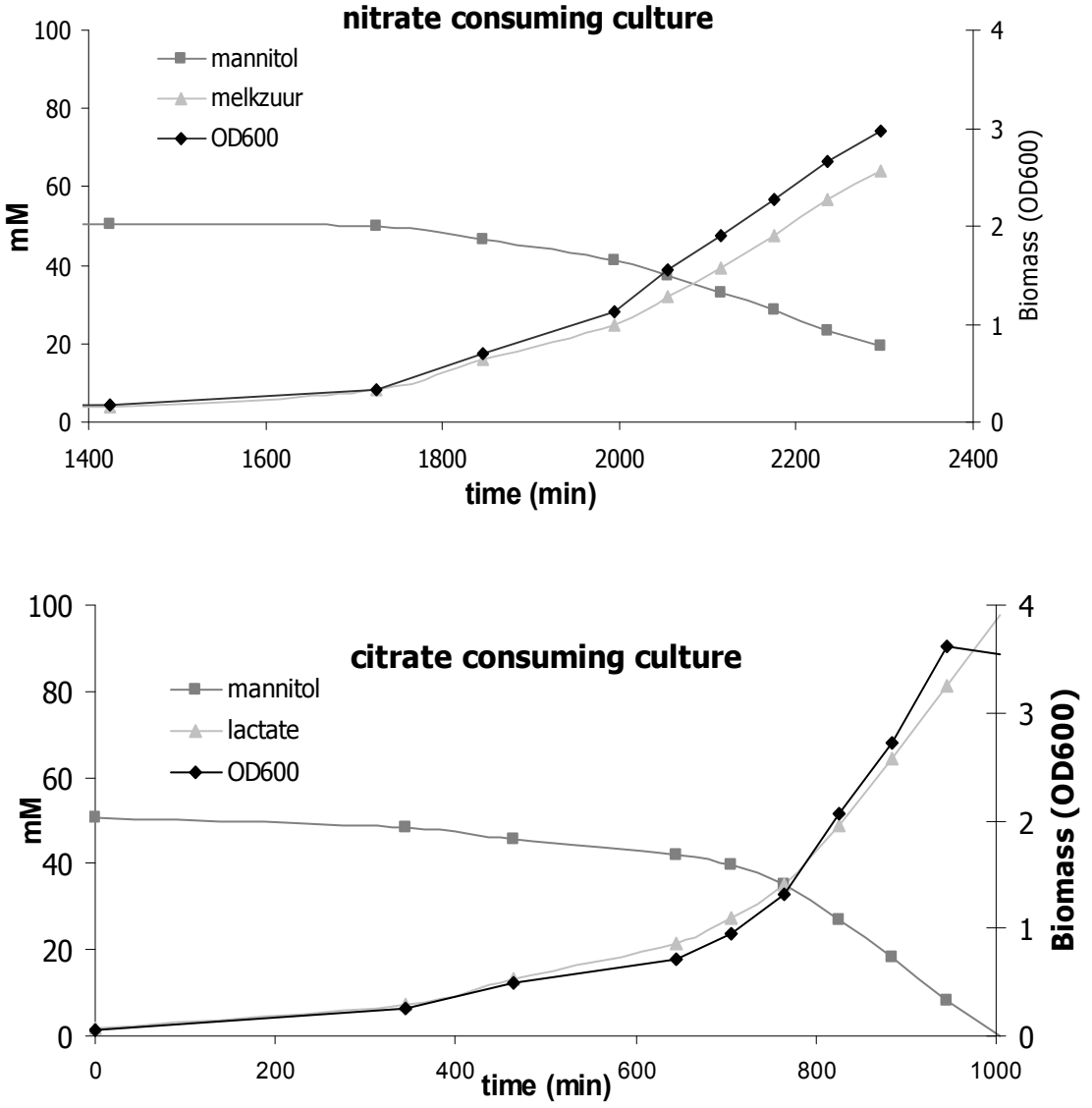

Figure 2. Biomass (OD_{600}), mannitol and lactate levels during growth with citrate or nitrate as redox sink.

During exponential growth citrate was therefore partially converted also into lactate (without yielding substrate-level ATP). When mannitol and citrate became limited, more

succinate was formed. In the final balance, after four days of incubation, citrate was completely consumed and acetate and succinate produced in molar ratios of roughly 1:1:1. This shows that at this time point the lactate was almost completely derived from mannitol. The conversion of citrate to succinate regenerates 2 NAD^+, see also discussion. In these pH-controlled batch cultures mannitol was thus, almost exclusively catabolised to lactate. Nitrate-reducing cells converted mannitol to virtually exclusively lactate as main product as well.

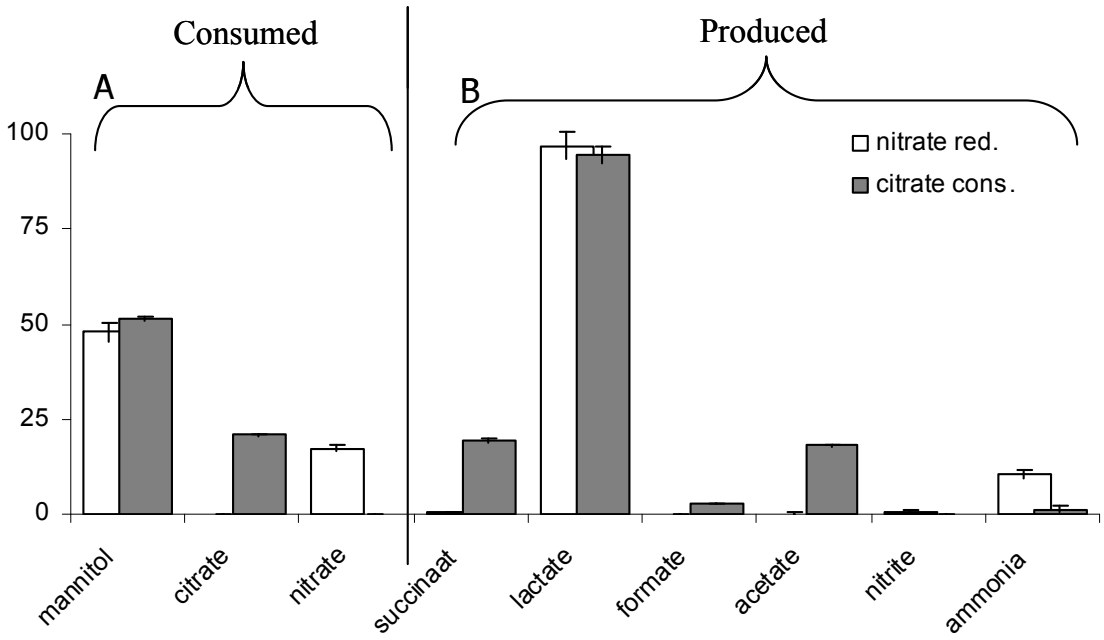

Figure 2. Consumption (A) and production (B) of metabolites after long-term incubation (4 days) Nitrate reducing cells (white bars) completely consumed 20mM of nitrate and produce exclusively lactate. Citrate consuming cells (dark bars) converted 20mM of citrate to equimolar ratios of acetate and succinate.

High levels of nitrate (18mM) were reduced and small transient levels of nitrite, of up to 3 mM were formed. A major difference in these two cultures is the production of about 10.7 mM ammonia by nitrate reducing cells (compared to only 1.4 mM by citrate reducing cells). Even when assuming that this net ammonia formation of approximately 9mM is derived from nitrite, another 9mM of degraded nitrite cannot be accounted for. In any case,

since the nitrate and citrate allowed the comparable conversion of mannitol into lactate, apparently nitrate and citrate are equally effective as redox sink (Fig 2).

Nitrate reductase induces changes in global gene transcription

We isolated RNA from exponentially growing citrate or nitrate reducing cells. About 220 genes were significantly up-regulated more then 2-fold in nitrate-reducing cells of which many were prophages (~70). A hundred or so genes were significantly down-regulated, by more then two-fold (or up-regulated in citrate consuming cells) (Table 1).

Functional categories	**Up**	**down**
Amino acid biosynthesis	8	7
Cell envelope	2	8
Cellular processes	6	3
Central intermediary metabolism	4	3
Energy metabolism	18	19
Fatty acid and phospholipid metabolism	16	1
Hypothetical proteins	41	23
Prophages	69	0
Purines, pyrimidines, nucleosides and nucleotides	15	18
Regulatory functions	10	8
Transport and binding proteins	29	24
Other	4	5
Total	**222**	**119**

Table 1. Numbers of 2-fold or more significantly differentially expressed genes, divided into functional categories. (Up) implies the number of genes that are differentially up-regulated in nitrate-reducing cells, or down-regulated (Down), in comparison with citrate consuming cells. A clear difference is the large number of prophage genes that are up-regulated in the nitrate-reducing culture.

Many genes of the citrate-succinate pathway were strongly up-regulated in citrate reducing cells. The gene encoding for the malic enzyme was also strongly induced, while the phosphoenolpyruvate carboxykinase was down-regulated (Table 2). Shunting of part of the malate pool towards lactate, via pyruvate, may constitute an overflow mechanism. It is consistent with the observation that during exponential growth citrate cells have a higher

lactate/OD_{600} ratio and that the acetate vs succinate ratio is only 2:1. During exponential growth with citrate malate is apparently partly converted to lactate.

Many genes on the nitrate-reductase island (as described in Chapter 4) were up-regulated in nitrate-reducing cells. They had all *narGHIJ*-genes and 8 (out of 9) molybdopterin synthesis genes significantly up-regulated, although not strongly. The highest differentially expressed genes in this region are *rrp4* and *hpk4* that encode a response regulator protein and a histidine protein kinase respectively. The gene *nark*, coding for a nitrite extrusion protein, and the gene product of lp_0912, coding for a nitropropane dioxygenase were both up-regulated. Where the extrusion of nitrite prevents accumulation of intracellular nitrite levels, dioxygenase activity will degrade NO_2, without formation of ammonia. The activity of nitropropane dioxygenase may help to explain why the ammonia levels formed by nitrate-reducing cells fall short of the assumed levels of degraded nitrite.

Locus	Gene	function	ratio (log2)	p-value
		Citrate metabolism		
lp_0594	*mleP1*	malate/citrate transport protein	-0.60	0.001
lp_1101	*ldhL2*	L-lactate dehydrogenase	-3.90	>0.001
lp_1102		cation transport protein	-4.10	>0.001
lp_1103	*citR*	citrate lyase regulator	-3.36	>0.001
lp_1105	*mae*	malic enzyme, NAD-dependent	-3.38	>0.001
lp_1106	*citC*	[citrate (pro-3S)-lyase] ligase	-3.51	>0.001
lp_1107	*citD*	citrate lyase, acyl carrier protein	-3.62	>0.001
lp_1108	*citE*	citrate lyase, beta chain	-3.66	>0.001
lp_1109	*citF*	citrate lyase, alpha chain	-3.91	>0.001
lp_1112	*fum*	fumarate hydratase	-3.41	>0.001
lp_1113		fumarate reductase, flavoprotein subunit precursor,N-term truncated	-2.96	>0.001
lp_1114	*citX*	apo-citrate lyase phosphoribosyl-dephospho-CoA transferase	-1.04	>0.001
lp_1115	*mleR2*	malolactic regulator (putative)	-1.04	>0.001
lp_1116	*mleR1*	malolactic regulator	-0.65	>0.001
lp_1118	*mleS*	malolactic enzyme	-1.56	0.007
lp_1119	*mleP2*	malate transport protein	-0.96	>0.001
lp_2057	*ldhD*	D-lactate dehydrogenase	-1.26	0.000
lp_3418	*pck*	phosphoenolpyruvate carboxykinase (ATP)	1.56	0.000

Locus		Gene	function	ratio (log2)	p-value
		Nitrate metabolism			
lp_1500	*narI*	nitrate reductase, gamma chain		0.91	0.000
lp_1498	*narH*	nitrate reductase, beta chain		0.75	0.003
lp_1489		unknown		3.45	0.000
lp_1488	*hpk4*	histidine protein kinase; sensor protein (putative)		3.99	0.000
lp_1487	*rrp4*	response regulator		3.84	0.000
lp_1483		(none)		0.99	0.000
lp_1482		(none)		0.92	0.000
lp_1480	*moaA*	molybdopterin precursor synthase MoaA		0.75	0.019
lp_1478	*moaE*	molybdopterin biosynthesis protein, E chain		0.48	0.041
lp_1477		flavodoxin		-0.77	0.016
lp_1473	*fecB*	iron chelatin ABC transporter, substrate binding protein (putative)		0.76	0.001
lp_1472		ABC transporter component, iron regulated (putative)		0.85	0.000
lp_1471	*nifU*	NifU-like protein		0.90	0.001

Table 2. Differentially expressed genes involved in citrate metabolism and nitrate reduction (sensu stricto).

Previously we observed that a mutant strain that carried a chloroamphenicol replacement of the *ndh1* gene, was defective in aerobic heme and menaquinone stimulation, but not in nitrate reduction (Chapter 5). In other words, Ndh1 is the likely catalyst for the electron transfer from NADH to the aerobic ETC, while the anaerobic ETC that uses nitrate as terminal electron acceptor operates with another dehydrogenase. In fact Ndh1 was not differentially regulated. There were several dehydrogenases and uncharacterized oxidoreductases that were quite highly differentially expressed, more than 4-fold even (Table 3).

The nitrate reductase gene-island of *Lactobacillus plantarum* strains

The *nar*-genes are found in the fully sequenced genomes of *L. plantarum* WCFS1 and *Lactobacillus reuteri* 100-23. The active nitrate-reductase complex requires a molybdenum-pterin cofactor (11). All 9 genes that are required to synthesize the molybdenum-pterin are present on the genome of *L. plantarum* (9). Furthermore a protein coding for a nitrite extrusion system is present. On the genome of *L. plantarum* WCFS1 these genes are located

in close proximity to each other and form essentially a gene-island or nitrate-reductase island (lp_1473-lp1503) (9).

Locus	Gene	function	ratio (log2)	p-value
lp_3069		oxidoreductase	3.371	>0.001
lp_2732		oxidoreductase	3.014	>0.001
lp_3662	*adhE*	alcohol DH & acetaldehyde DH	2.747	>0.001
lp_0371	*glpD*	glycerol-3-phosphate DH	2.182	>0.001

Table 3. Several oxidoreductases and dehydrogenases (DH) were highly expressed in nitrate reducing cells (or highly repressed in citrate consuming cells).

Distribution of the nitrate reductase and associated genes

In a previous study, the genome content of 24 strains of *Lactobacillus plantarum* was compared with that of *Lb. plantarum* WCFS1 (genotyping) (Tseneva et. al., manuscript in preparation). 28 genes that neighbor each other in the nitrate-reductase island of *Lb. plantarum* WCFS1 were all present in 9, but all completely absent in the other 12 (Table 4).

The complete absence or presence of all these 28 genes suggests a similar island-like topography in those 9 strains. The co-conservation indicates a functional role of these genes in nitrate-reduction *sensu lato*. Of these 28 genes 14 have function that can be associated with nitrate-reduction. Examples are the molypdoterin co-factor biosynthesis, flavodoxin protein, structural nitrate-reductase and the nitrite-extrusion protein encoding genes. The other co-conserved genes code for an iron chelating ABC transporter, a response regulator with a (putative) sensor protein and 7 genes coding for proteins of unknown function. When we tested these 24 strains for the ability to reduce nitrate, three produced nitrite, all of whom possessed these genes. Two strains of *L. reuteri* have been completely sequenced. The genome of *L. reuteri* 100-23 harbours *nar*-genes, and can reduce nitrate in the presence of heme and menaquinone. *L. reuteri* F275 neither contains *nar*-genes nor reduces nitrate.

locus Id	gene	Product
lp_1473	*fecB*	iron chelatin ABC transporter, substrate binding protein (putative)
lp_1475	*fecE*	iron chelatin ABC transporter, ATP-binding protein
lp_1476	*fecD*	iron chelatin ABC transporter, permease protein
lp_1477	lp_1477	Flavodoxin
lp_1478	*moaE*	molybdopterin biosynthesis protein, E chain
lp_1479	*moaD*	molybdopterin biosynthesis protein, D chain
lp_1480	*moaA*	molybdopterin precursor synthase MoaA
lp_1481	*narK*	nitrite extrusion protein
lp_1483	lp_1483	Unknown
lp_1484	lp_1484	Unknown
lp_1485	lp_1485	Unknown
lp_1486	lp_1486	Unknown
lp_1487	*rrp4*	response regulator
lp_1488	*hpk4*	histidine protein kinase; sensor protein (putative)
lp_1489	lp_1489	Unknown
lp_1490	lp_1490	Unknown
lp_1491	*mobA*	molybdopterin-guanine dinucleotide biosynthesis protein MobA (putative)
lp_1492	*moaC*	molybdopterin precursor synthase MoaC
lp_1493	*mobB*	molybdopterin-guanine dinucleotide biosynthesis protein MobB
lp_1494	*moeA*	molybdopterin biosynthesis protein MoeA
lp_1495	*moaB*	molybdopterin biosynthesis protein MoaB
lp_1496	*moeB*	molybdopterin biosynthesis protein MoeB
lp_1497	*narG*	nitrate reductase, alpha chain
lp_1498	*narH*	nitrate reductase, beta chain
lp_1499	*narJ*	nitrate reductase, delta chain
lp_1500	*narI*	nitrate reductase, gamma chain
lp_1502	lp_1502	Unknown
lp_1503	lp_1503	Unknown

Table 4. Genes present in the *Lactobacillus plantarum* WCFS1 nitrate-reductase island. Lp_1473 to lp_1503 were all present or all absent in 9 out of 24 strains of *Lb. plantarum*.

Regulation of nitrate-island genes

Nitrate reduction in *L. plantarum* requires heme, menaquinone and addition of nitrate. These chemicals may be sensed by cells and positively influence the transcription of genes on the nitrate-reductase island. At least the repression of nitrate reductase activity at high glucose levels, but not high mannitol levels suggest that this activity is actively regulated. A gene coding for a histidine protein kinase (*hpk4*, lp_1488) and a response regulator protein (*rrp4*,

lp_1487) are positioned in the middle of the nitrate gene cluster and may provide transcriptional regulation. We have grown *Lactobacillus plantarum* on MRS medium (no acetate, no citrate and with 10mM glucose) for ~24hours in the presence of heme, menaquinone, NO_3, high glucose and/or high mannitol (Table 1). The batch cultivation in each condition was done in triplicate. RNA was isolated from these batch cultures and the expression of *narG*, *moeA*, *moeE*, *hpk4*, *rrp4*, *fecD* and *narK* was measured with Q-PCR techniques. The average expression of 3 housekeeping genes (*pfk*, *rpoB*, *groES*) was subtracted from the expression of these genes (Table 5). The expression of *moeA*, *moeE* and *narG* appears to be strongly induced by the presence of nitrate. *narK* was strongly induced when nitrate was actively reduced to nitrite (condition A), but also by nitrate alone. This is similar to what is known of *nark* regulation in *Escherichia coli* (10, 18). *Hpk4* was most strongly expressed in nitrate-reducing conditions as well as in the presence of high mannitol (but not high glucose) levels. In other words, *hpk4* was significantly higher expressed in conditions were nitrate reduction took place when compared to all the other conditions. The gene product of *rrp4*, that forms the second part of the putative regulator system, was also differentially expressed in these same conditions but less pronounced. Finally, *fecD* that encodes part of an iron-chelating ABC transporter did not appear to be differentially regulated.

	Corrected Ct-values						
Supplements	**moaA**	**moeA**	**narG**	**narK**	**hpk4**	**rrp4**	**fecD**
+heme +K_2 +NO_3 (A)	6.0 ± 0.1	3.5 ± >0.1	4.9 ± 0.1	3.5 ±0.1	4.7 ± 0.1	7.0 ± 0.4	8.2 ± 0.4
+heme	11.8 ± 0.4	8.3 ± 0.2	9.6 ± 0.1	8.4 ±0.4	8.6 ± 1.1	9.0 ± 0.7	9.8 ± 0.8
+K_2	10.5 ± 0.6	7.7 ± 0.2	9.4 ± 0.8	7.7 ± 0.6	7.4 ± 1.1	8.8 ± 0.2	9.3 ± 0.1
+NO_3	7.2 ± 0.4	3.6 ± 0.4	4.6 ± 0.4	5.7 ±0.5	6.6 ± 0.4	7.4 ± 0.4	8.7 ± 0.2
(A) +50mM glucose	12.3 ± 0.4	11.9 ± 0.1	9.7 ± 0.4	10.3 ±0.1	8.6 ± 0.2	9.4 ± 0.1	8.7 ± 0.1
(A) +50mM mannitol	13.2 ± 0.1	10.9 ± >0.1	10.6 ± 0.1	11.3 ±>0.1	6.0 ± 0.2	6.6 ± 0.2	9.2 ± 0.1

Table 5. Corrected Ct-values of selected genes on the nitrate reductase island. Cells were cultivated for 24 hours in the presence of several compounds essential for nitrate-reduction in *Lactobacillus plantarum* WCFS1.

Discussion

Anaerobic growth on the reduced sugar mannitol requires cells to use a redox-sink (13). We provided citrate or nitrate (together with heme and menaquinone) to the cultures for this purpose. We have observed that when nitrate was used as redox-sink that, in these specific conditions, nitrite is almost as quickly degraded as it is formed. We can compare the efficiencies of citrate and nitrate as redox sink. Based on the metabolic routes of *Lactobacillus plantarum* WCFS1 for the conversion of citrate we can deduce how much NAD^+ is regenerated (Fig 3).

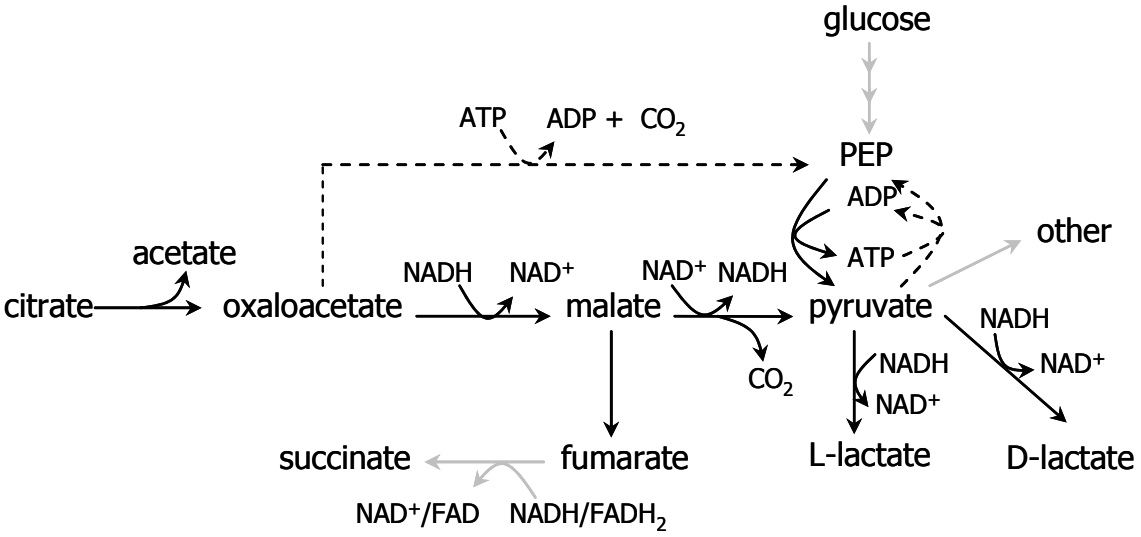

Figure 3. Schematic representation of citrate reduction and the interaction with pyruvate metabolism (based on Ferain *et. al.*) (5). When citrate is converted to succinate (and acetate) 2 NAD^+ are regenerated, when converted to lactate (and acetate) only 1 NAD^+ is regenerated. Up-regulation (solid lines), down-regulation (Dashed lines) or other (and non-regulated) (gray lines) of genes in citrate consuming cells (compared to nitrate reducing cells is shown).

Since 20mM of citrate was reduced with the concomitant production of 20mM of succinate and 20mM of acetate (after 4 days) ~40mM equivalence of NAD^+ was likely regenerated. When 18M nitrate was supplied as redox sink, during growth nitrite was formed, as high as 3mM and largely consumed again in latter growth phases. The reduction of 18mM nitrate (and thus 18mM nitrite) allowed a similar level of mannitol breakdown and thus provided the

same level of reducing power. We propose on this basis that the reduction of one nitrate and the reduction of one nitrite each regenerated 1 NAD^+. We have observed an increase in ammonia production in nitrate reducing cells by 9mM. The reduction of nitrite directly to ammonia could regenerate 3 NAD^+, far more then what is likely based on the comparison with citrate as redox sink. The removal of nitrite, together with acetone (although the enzyme has broad substrate specificity) by activity of the nitropropane dioxygenase that consumes 2 H^+ is more line with the observed efficiency. In any case the acetone that is for example co-consumed with nitrite must be generated by altered internal metabolism and may account for the observed ammonia production. To determine whether nitrite ends up as ammonia or nitropopane, the labeling of nitrite should prove conclusive.

The heme-containing nitrate-reductase A complex, encoded by *narGHJI*, generates proton motive force in *Escherichia coli* and oxidizes menaquinol (6, 7). Despite these facts *L. plantarum* does not show an improvement in biomass formation when nitrate is used as redox sink, as opposed to citrate. This does not necessarily mean that the *L. plantarum* nitrate reductase does not translocate protons. As our discussion of possible nitropropane dioxygenase activity shows, it may co-consume nitrite with another substrate. In the example, acetone is converted together with nitrite to nitropropane. Therefore there may be energetic considerations, in the formation of the co-consumed substrate that need to be studied in further detail.

We have shown that the reduction of nitrate in *L. plantarum* is an actively regulated process. Nitrate induces transcription of genes that encode the nitrate-reductase complex (*moeA*, *moaA*, *narG*) and *narK*. In *Escherichia coli* Nark enhances nitrate transport and nitrite extrusion. As may prevents nitrite accumulating to toxic levels it plays an important role in driving nitrate reduction (18). In any case, although the electron transport chain requires heme and menaquinone, neither of these compounds had a strong inducing effect on transcription of the selected genes.

The nitrate-reductase cluster contains all genes required to form a functional nitrate-reductase A, except for heme synthesis. The complete absence or presence of this entire gene-set in other strains of *L. plantarum* suggests an island-like topography (Tseneva V. personal communication). Furthermore, since these are co-conserved we have shown that they are likely to play a role in nitrate reduction, *sensu lato*. This compact island-like structure, and the

fact that it has been only observed in *Lactobacilli*, makes horizontal gene-transfer a plausible mechanism for acquisition of these genes. Moreover the acquisition of large genome regions by horizontal gene transfer, such as the pseudovitamin B_{12} synthesis gene cluster, was recently suggested for *Lactobacillus reuteri* F275 (19).

1. **Brooijmans, R. J. W., B. Poolman, G. K. Schuurman-Wolters, W. M. de Vos, and J. Hugenholtz.** 2007. Generation of a membrane potential by *Lactococcus lactis* through aerobic electron transport. J Bacteriol **189:**5203-9.
2. **Brooijmans, R. J. W., J. Hoolwerf, W. M. de Vos, and J. Hugenholtz.** The electron transport chains of *Lactobacillus plantarum* WCFS1 Chapter 4.
3. **Brooijmans, R. J. W., S. Noordermeer, W. M. de Vos, and J. Hugenholtz.** Heme and menaquinone induced aerobic response in *Lactobacillus plantarum* WCFS1. Chapter 5.
4. **Collins, M. D., and D. Jones.** 1981. Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implication. Microbiol Rev **45:**316-54.
5. **Ferain, T., A. N. Schanck, and J. Delcour.** 1996. 13C nuclear magnetic resonance analysis of glucose and citrate end products in an *ldhL-ldhD* double-knockout strain of *Lactobacillus plantarum*. J Bacteriol **178:**7311-5.
6. **Garland, P. B., J. A. Downie, and B. A. Haddock.** 1975. Proton translocation and the respiratory nitrate reductase of Escherichia coli. Biochem J **152:**547-59.
7. **Jones, R. W., A. Lamont, and P. B. Garland.** 1980. The mechanism of proton translocation driven by the respiratory nitrate reductase complex of *Escherichia coli*. Biochem J **190:**79-94.
8. **Jünemann, S.** 1997. Cytochrome *bd* terminal oxidase. Biochimica et Biophysica acta **1321:**107-127.
9. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc Natl Acad Sci U S A **100:**1990-5.
10. **Kolesnikow, T., I. Schroder, and R. P. Gunsalus.** 1992. Regulation of *narK* gene expression in *Escherichia coli* in response to anaerobiosis, nitrate, iron, and molybdenum. J Bacteriol **174:**7104-11.
11. **Magalon, A., M. Asso, B. Guigliarelli, R. A. Rothery, P. Bertrand, G. Giordano, and F. Blasco.** 1998. Molybdenum cofactor properties and [Fe-S] cluster coordination in *Escherichia coli* nitrate reductase A: investigation by site-directed mutagenesis of the conserved his-50 residue in the NarG subunit. Biochemistry **37:**7363-70.
12. **Makarova, K. S., and E. V. Koonin.** 2007. Evolutionary genomics of lactic acid bacteria. J Bacteriol **189:**1199-208.
13. **McFeeters, R. F., and K. Chen.** 1986. Utilization of electron acceptors for anaerobic mannitol metabolism by *Lactobacillus plantarum*. Compounds which serve as electron acceptors. Food Microbiol. **3:**73-81.
14. **Miller, M. J., and R. B. Gennis.** 1985. The cytochrome *d* complex is a coupling site in the aerobic respiratory chain of *Escherichia coli* J. Biol. Chem. **260:**14003-14008.
15. **Moreno-Vivian, C., P. Cabello, M. Martinez-Luque, R. Blasco, and F. Castillo.** 1999. Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. J Bacteriol **181:**6573-84.
16. **Puustinen, A., M. Finel, T. Haltia, R. B. Gennis, and M. Wikstrom.** 1991. Properties of the two terminal oxidases of *Escherichia coli*. Biochemistry **30:**3936-42.
17. **Ross, R. P., S. Morgan, and C. Hill.** 2002. Preservation and fermentation: past, present and future. Int J Food Microbiol **79:**3-16.

18. **Rowe, J. J., T. Ubbink-Kok, D. Molenaar, W. N. Konings, and A. J. Driessen.** 1994. *NarK* is a nitrite-extrusion system involved in anaerobic nitrate respiration by *Escherichia coli*. Mol Microbiol **12:**579-86.
19. **Santos, F., J. L. Vera, R. van der Heijden, G. Valdez, W. M. de Vos, F. Sesma, and J. Hugenholtz.** 2008. The complete coenzyme B12 biosynthesis gene cluster of *Lactobacillus reuteri* CRL1098. Microbiology **154:**81-93.
20. **Sarantinopoulos, P., G. Kalantzopoulos, and E. Tsakalidou.** 2001. Citrate metabolism by *Enterococcus faecalis* FAIR-E 229. Appl Environ Microbiol **67:**5482-7.
21. **Serrano, L. M., D. Molenaar, M. Wels, B. Teusink, P. A. Bron, W. M. de Vos, and E. J. Smid.** 2007. Thioredoxin reductase is a key factor in the oxidative stress response of *Lactobacillus plantarum* WCFS1. Microb Cell Fact **6:**29.
22. **Teusink, B., F. H. van Enckevort, C. Francke, A. Wiersma, A. Wegkamp, E. J. Smid, and R. J. Siezen.** 2005. In silico reconstruction of the metabolic pathways of *Lactobacillus plantarum*: comparing predictions of nutrient requirements with those from growth experiments. Appl Environ Microbiol **71:**7253-62.

Chapter 7

The electron transport chains of anaerobic prokaryotic bacteria

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Abstract

The discovery of respiration in lactic acid bacteria is remarkable as they have long been considered non-respiring. A functional aerobic electron chain was formed, terminating in a *bd*-type cytochrome, when certain cofactors were supplied (heme and menaquinone). This *bd*-type aerobic cytochrome has also been found in other (strictly) anaerobic prokaryotic bacteria. These include propionibacteria, acetogens and the sulfate reducing bacteria which have highly divergent primary metabolisms. These last three groups of bacteria are able to grow anaerobically on relatively poor carbon sources such as lactate and CO_2 which requires conservation of energy via an electron transport chain. Other terminal electron acceptors, such as oxygen, nitrate and nitrite form additional redox sinks, but compete for electron donors. Their effect on growth is not always straightforward and requires examination of the carbon source used, primary metabolism and effiency of energy conservation. In this review we will explore the presence of alternative (and even aerobic) electron transport chains in these organisms and how these impact their physiology.

General introduction

There are groups of prokaryotic bacteria that are considered facultative or even strict anaerobes. Interestingly for bacteria that are known for their anaerobic life-styles, *cydABCD* genes that encode for an aerobic *bd*-type cytochrome are found in several of these groups. For example, these genes are found in members of lactic acid bacteria, propionibacteria, sulphate reducing bacteria and even acetogens.

These four groups of anaerobes are of considerable economic importance. Lactic acid bacteria are widely used to produce many kinds of fermented foods with improved taste, shelf-life and economic value. Propionibacteria are commercially exploited for vitamin-B_{12} and propionic acid production. Acetogens are, as the name suggests, used to produce acetate and are also important gut inhabitants. Sulphate reducing bacteria play an important role in maintaining the global sulfur cycle and as inhabitants of the gut as well. These bacteria are also a causative agent of metal corrosion and can negatively affect methane-yield of microbial wastes treatment plants (82, 152). In fact (not-surprising for anaerobes) members from all these four groups can be isolated form gut environments, although not all are as prominent. These four groups of prokaryotes (lactic acid bacteria, propionibacteria, sulphate reducing bacteria and acetogens) have very different strategies to generate energy in anaerobic conditions. This is also the reasons why the discovery of *cyd* genes in all these groups is so intruiging. While lactic acid bacteria specialise in the quick fermentation of sugars, propionibacteria are able to grown on their fermentation product, lactate. Sulphate reducing bacteria share the ability for growth on lactate but can employ a unique sulphate reducing system. This use of sulphate allows them to extract more energy out of the available lactate than propionibacteria can. Acetogens are able to grown anaerobically with little more than a combination of hydrogen and carbon dioxide.

The ability to grow anaerobically on lactate or CO_2 relies on electron transport chains that conserves energy and maintains redox-balance. Therefore aerobic respiration does not per sé have to lead to increases in growth efficiency. The discovery of *cyd*-genes in members of these groups of anaerobic bacteria is therefore (and also of alternative respiratory systems that use nitrate or nitrite as terminal electron acceptor) warrants some careful deliberations. As anaerobic growth on lactate or CO_2 requires electron transport activity it would be interesting

to assess the impact of a competing (aerobic) respiratory chain. The lactic acid bacteria are somewhat unique in this regard since in normal growth conditions they have no active electron transport chain at all. For them electron transport chain activity should have a dramatic impact.

The goal of this review is to compare and understand the impact of respiration on the characteristic metabolism of these anaerobic prokaryotes. The evidence for the existence of alternative electron transport chain systems and the impact on these types of primary metabolism is reviewed. In order to do this, for each of these groups, first the characteristic primary metabolism is described and how this is intertwined with anaerobic electron transport. Then the current knowledge of the existence of additional electron transport chains systems in these bacteria, such as aerobic or nitrate respiratory chains is reviewed, and how activity of these stimulate (or not stimulate) growth.

Lactic acid bacteria

This group consists of gram-positive, non-spore forming bacteria which are extensively used in the production of fermented (food) products. Lactic acid bacteria form a evolutionary-related group that are found, not only in fermented food products, but also on plants and in the (human) GI-tract (138). They can be considered specialists that are able to quickly ferment available sugars to acids with complex nutritional requirements (amino acids and vitamins). As principal fermentors in an immense array of both industrial-scale and traditional (local) food products, they have a significant impact on world economy and health. As a small example of fermentation processes in which they are used see figure 1. Fermentation not only enhances taste but more importantly lengthens shelf-life. As early as the first human settlements, these bacteria have been fermenting raw-foods (milk, meat and vegetables) that despite this bacterial growth remained edible.

The fermentation of food-stuff usually occurs in mixed cultures, sometimes in conjunction with other non-lactic acid bacteria. In developed societies, many of these fermentations are performed on industrial scales, with (defined) mixed starter cultures. The fermentation of (locally available) foodstuff in third world countries remains a rich source for new isolates, as these are performed under less controlled conditions. In third-world countries the enhancement of shelf-life by fermentation with lactic acid bacteria remains especially

important, for human helath. Lactic acid bacteria prevent growth of other (spoilage) bacteria by the fast conversion of sugars to acids, which acidifies the food-matrix. In addition many are known to produce anti-microbial compounds such as nisin (137).

Type of fermented product	Lactic acid bacteria[a]
Dairy products	
-Hard cheeses without eyes	*L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*
-Cheeses with small eyes	*L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis var. diacetylactis*, *L. lactis* subsp. *cremoris*, *Leuc. mesenteroides* subsp. *cremoris*
-Swiss- and Italian-type cheeses	*Lb. delbrueckii* subsp. *lactis*, *Lb. helveticus*, *Lb. casei*, *Lb. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*
-Butter and buttermilk	*L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis var. diacetylactis*, *L. lactis* subsp. *cremoris*, *Leuc. mesenteroides* subsp. *cremoris*
-Yoghurt	*Lb. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*
-Fermented, probiotic milk	*Lb. casei*, *Lb. acidophilus*, *Lb. rhamnosus*, *Lb. johnsanii*, *B. lactis*, *B. bifidum*, *B. breve*
-Kefir	*Lb. kefir*, *Lb. kefiranofacies*, *Lb. brevis*
Fermented meats	
-Fermented sausage (Europe)	*Lb. sakei*, *Lb. curvatus*
-Fermented sausage (USA)	*P. acidilactici*, *P. pentosaceus*
Fermented fish products	*Lb. alimentarius*, *C. piscicola*
Fermented vegetables	
-Sauerkraut	*Leuc. mesenteroides*, *Lb. plantarum*, *P. acidilactici*
-Pickles	*Leuc. mesenteroides*, *P. cerevisiae*, *Lb. brevis*, *Lb. plantarum*
-Fermented olives	*Leuc. mesenteroides*, *Lb. pentosus*, *Lb. plantarum*,
-Fermented vegetables	*P. acidilactici*, *P. pentosaceus*, *Lb. plantarum*, *Lb. fermentum*
Soy sauce	*T. halophilus*
Fermented cereals	
-Sourdough	*Lb. sanfransiscensis*, *Lb. farciminis*, *Lb. fermentum*, *Lb. brevis*, *Lb. plantarum*, *Lb. amylovorus*, *Lb. reuteri*, *Lb. pontis*, *Lb. panis*, *Lb. alimentarius*, *W. cibaria*
Alcoholic beverages	
-Wine (malolactic fermentation)	*O. oeni*
-Rice wine	*Lb. sakei*

Figure 1 taken from Leroy *et. al.* (83). Lactic acid bacteria are used to ferment a wide-array of (raw) foods.

Important in this respect is that the lactic acid bacteria themselves, at least those that are present in fermented foods, are food-grade since neither do they produce toxins nor are they pathogenic. Furthermore fermentation alters the organo-leptic (taste) profiles and increases the economic value of the product. Recently the actual beneficial effect of consuming specific (live) lactic acid bacteria themselves has been the subject of much investigation (probiotics). Consumption of probiotics in some cases has been shown to reduce allergy- and disease-symptoms (33, 95). Being generally considered non-respiring obligate fermentors most studies have focused on their fermenting capacities. Recently however in a few species there

is evidence of actual respiration (see below).

General metabolism

A characteristic of all lactic acid bacteria is the absence of a complete citric acid cycle. Therefore, they convert sugars (inefficiently but quickly) in (lactic) acids, via the Embden-Meyerhof pathway. The best studied model organisms for metabolism of lactic acid bacteria are *Lactococcus lactis* and *Lactobacillus plantarum*. Glucose is take up and phorphorylated in the process. Glucose-6-phosphate can be directed towards fomation of biomass, via the pentosephosphate pathway or toward lactate, via glycolysis to generate energy (ATP). The conversion of glucose to lactate, results in ATP generation by substrate level phosphorylation. In normal growth conditions (without heme and menaquinone supplementation), anaerobic glycolysis has to maintain a precise NAD^+/NADH balance. NAD^+ that is reduced by glyceraldehydes-3-phosphate dehydrogenase activity is re-oxidised in latter glycolytic steps, as by the Lactate-dehydrogenase (Fig 2).

Conversion of pyruvate (into acetate yields additional ATP but depletes the NAD^+-pool. Extracellular citrate and shunting of pyruvate towards ethanol can be used as redox sink and reastablish redox-balance. *Lactococcus lactis* produces almost exclusively L-lactate in this way.

At a pH of 7 the change in Gibbs free energy when converting glucose to lactate is approximately 198.3 kJ/mol reaction: $\longrightarrow C_6H_{12}O_6 \qquad 2C_3H_5O_3^- + 2H^+$

Despite the huge amount of energy released that could be capitalized for useful work only 2 ATP is generated by substrate level phosphorylation. If we assume that the phophorylation of ATP requires ~+31.8kJ/mol, 2 ATP out of a maximum of 6 are formed, an efficiency of about 33%. Even the end–product lactate still contains a lot of chemical energy which further demonstrates the apparent inefficiency of homo-lactic fermentation.

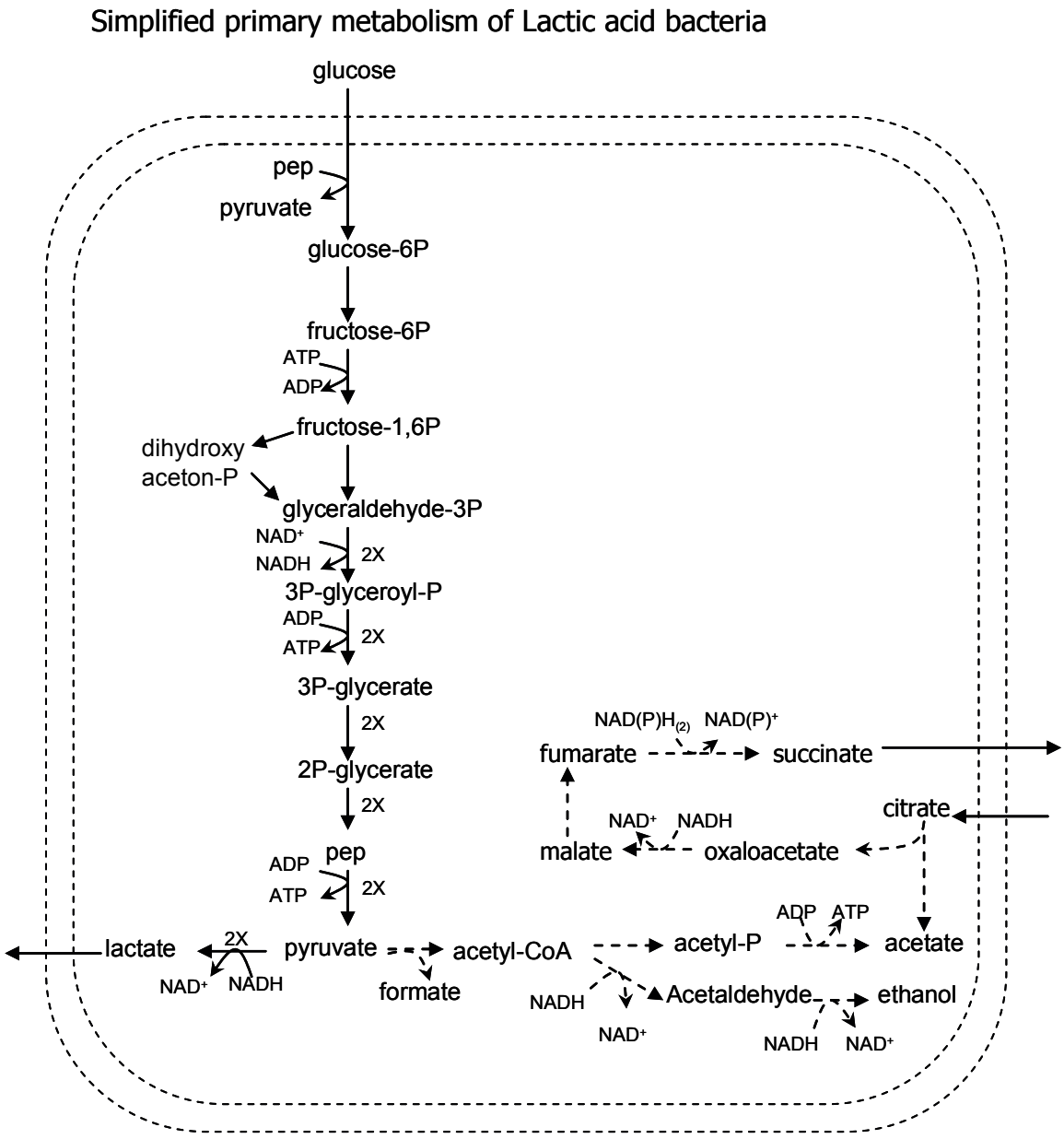


Figure 2. Simplified primary metabolism of lactic acid bacteria. Abbreviations: phosphate (-P), pyruvate (pyr), phosphoenolpyruvate (pep). Dotted double lines symbolise the celmembrane. Solid lines represent homolatic fermentation. 2X designates molar duplication at the point of glyceraldehyde-3P. Single dotted lines represent the alternative catabolism of pyruvate into acetate (yields ATP), ethanol (regenerates NAD^+) and citrate to succinate conversion (regenerates NAD^+).

A shift of homo-lactic to more mixed-acid production can be induced by altered (slower) growth conditions. During anaerobic mixed-acid fermentation, pyruvate is partially (hence mixed acid) converted to acetate (and CO_2 or formate), which generates additional ATP (74). The anaerobic conversion of glucose to acetate net consumes NAD^+, and requires formation of ethanol, conversion of citrate to succinate or any other additional redox-sink (69). The portrayed metabolic conversions in figure 2 are not exclusive. For example *Lactobacillus plantarum* can also be convert oxaloacetate to phospoenolpyruvate and pyruvate, and malate into lactate.

Electron transport chain of Lactic acid bacteria

Lactic acid bacteria are generally considered non-respiring bacteria. In fact they do not produce heme that are essential co-factors of cytochromes, and in many cases, fail to produce menaquinones. Menaquinones are essential components of respiratory chains as they transport electrons between dehydrogenases and the cytochromes in the membrane. Despite being seemingly underequipped for respiration, there is a long history of scattered publications on the improvement of growth yields by some lactic acid bacteria when cultivated aerobically in the presence of heme (also known as hemantin, hemin or haem). Among these are some strains of *Streptococcus* (*Lactococcus*), *Leuconostoc* and *Enterococcus* (112, 118, 129). Several observations suggested that respiration was the underlying mechanism. For example addition of heme induced formation of cytochromes and stimulated growth only in aerobic conditions (3) (146). Furthermore cyanide and mutation of *cydA* suppressed these heme-dependent phenotypes. Recently it was convincingly shown that *Lactococcus lactis* mg1363 has an active (heme-dependent) electron transport chain that can generate proton motive force (16). A *bd*-type cytochrome was shown to be involved encoded by the *cydABCD* genes. This operon is present in many lactic acid bacteria. Of the 45 completely sequenced genomes of lactic acid bacteria the *cyd*-genes were found in about half the species. They are found in *Streptococcus agalactiae*, *Oenococcus oeni*, *Lactococcus lactis, Lactobacillus plantarum*, *Lb. salivarius*, *Lb. gasseri*, *Lb johnsonii*, *Lb. casei*, *Lb. brevis*, *Lb. reuteri*, *Leuconostoc mesenteroides* and *Enterococcus faecalis*. The presence of the *cyd*-genes in many genomes of lactic acid bacteria is remarkable, since many of these species have a long history of selection in (micro)anaerobic food-fermentations. Indeed several species show extensive specialization

with high levels of gene-decay, such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (11, 142).

The idea that many other lactic acid bacteria (besides *Lactococcus lactis*) can respire is intriguing. For some lactic acid bacteria, such as *Lactobacillus plantarum* and *Streptococcus agalactiae* respiration-like phenotypes can be induced by combined addition of both heme and menaquinone (*Brooijmans et. al.* in prep.)(149, 150).

Lactococcus lactis MG1363 has become the model organism for research on the aerobic respiration in lactic acid bacteria. Respiring *Lactococcus lactis* cells dramatically alter their phenotype. The growth yield is increased and the resistance to oxidative-, acid-stress, and long-term cold storage much improved (116). The proposed electron transport chain of *Lactococcus lactis* however, is rather simple and inefficient in conserving energy. It consists of a non-redundant electron transport chain with a NADH-dehydrogenase, menaquinol pool and a *bd*-type cytochrome (9, 16, 51) (Fig 3).

The apparent large impact of respiration in (non-pH controlled) batch fermentations by this inefficient respiratory chain therefore warrants some explaining. (This example also shows why knowledge of primary metabolism is essential to describe the impact of respiration). Firstly, the oxidation of NADH by the electron transport chain affects the redox-balance, and results in a shift from homo-lactic to (more) mixed-acid fermentation. When acetate is produced (in favour of L-lactate) more substrate-level ATP may be generated. Secondly, when acetate is produced in favour of lactate the environment is acidified more slowly, which will enable a more complete fermentation of the available sugars (in non-pH controlled batch cultivations). Thirdly, respiration conserves energy by generating proton motive force (16, 78). Therefore, although the efficiency of the lactic acid bacterial electron transport chain, with the combination of a NDH-II and *bd*-type cytochrome is low (1 H^+/e^-), the additional effects on redox-balance and acidification rate result in a dramatic growth improvement in lactic acid bacteria (16, 43, 51).

Anaerobic electron transport chains in lactic acid bacteria

A range of prokaryotes and Archaea are able to respire using nitrate (22). The *narGHJI* genes that encode this function in *Escherichia coli* are also present in a wide range of other respiring bacteria such as *Bacillus* sp., *Mycobacterium* sp., *Pseudomonas* sp. and surprisingly in the lactic acid bacteria *Lactobacillus plantarum* WCFS1*, Lb. reuteri* 100-23 *and Lb. fermentum* IFO 3956 (96, 136, 151) (Fig. 3). Nitrate respiration generates proton motive force with the reduction of nitrate to nitrite. The *narGHJI* genes of *E. coli* are reported to function in an electron transport chain context and generate proton motive force (21). The respiratory membrane-bound nitrate reductase complex consists of three subunits (α, β and γ) that are encoded by *narG*, *narH* and *narI* respectively.

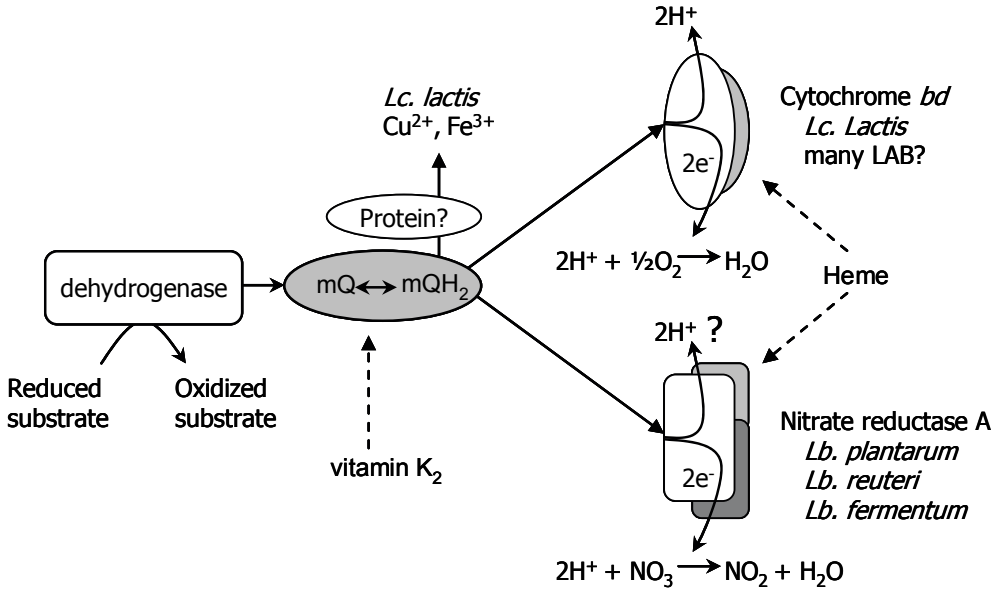


Figure 3. The putative electron transport chain present in lactic acid bacteria. A non-proton motive force generating electron donor (dehydrogenase) reduces the quinone pool. Dashed arrows indicate supplementation of heme and, in some lactic acid bacteria, menaquinones (vitamin K_2). Menaquinol oxidases transfer electrons to oxygen (via a *bd*-type cytochrome) or nitrate (via a nitrate-reductase A). The names indicate for which lactic acid bacteria experimental evidence is available. Abbreviations: mQ (menaquinone), mQH_2 (menaquinol), *Lc.* (*Lactococcus*), *Lb*. (*Lactobacillus*).

The α and β subunits form a complex that contain a molybdenum-pterin cofactor and 4 iron-sulfur clusters (4, 31). The *narI* subunit, containing two heme molecules, provides binding to

the membrane and interaction with the quinone pool in the membrane (40, 53). *NarJ* is required for full activity membrane-bound nitrate reductase activity (18). Similar to the *bd*-type cytochrome menaquinone-oxidase, the proton motive force generating capacity of the nitrate reductase A is low. Scalar chemistry (the spatial separation of the chemical half-reactions on both sides of the cell membrane) alone could account for the observed proton translocation efficiencies in both cases.

The reduction of nitrate by *Lactobacillus* sp. is not new and was already observed in 1955 (27). Nitrate reduction by the nitrate reductase A is probably constitutes a different mechanism since, as a menaquinol-oxidase requires, not only heme, but also menaquinones as cofactors. Recently nitrate-reduction by *Lactobacillus plantarum* WCFS1 has been described that indeed requires supplementation of these cofactors (manuscript in preparation). Nitrate reduction in *Lactobacillus plantarum* differs from aerobic respiration in one important aspect: (high levels of) glucose represses activity.

Recently reduction of metal-ions (Cu^{2+}, Fe^{3+}) by the quinone-pool was reported in *Lactococcus lactis* (Fig 3) (117). It is not clear whether proteins are involved in this transfer process, but it indicates that these compounds can function as redox sink.

_bd_-type cytochrome

The aerobic *bd*-type cytochrome is uniquely found in (strict) anaerobic prokaryotes. Therefore we will discuss this cytochrome in a bit more detail. The *bd*-type respiratory cytochrome functions in aerobic electron transport chains where it oxidises menaquinol with the concomitant reduction of oxygen (72). It is completely unrelated to the heme-Cu super-family or "mitochondrial" terminal oxidases containing three heme prosthetic groups: heme b558, heme b595, and heme d (64, 100, 107). It is a membrane-bound, proton motive force generating enzyme complex composed of 2 subunits (cydA and cydB); cydC and cydD are required for assembly of the oxidase (28). The observed proton motive force generating efficiency of 1 H^+/e^- indicates that no actual proton pumping takes place. Rather, scalar chemistry, with a release of protons on the outside of the cell membrane and the transferral of electrons towards the cytosol where oxygen reduction takes place, is proposed to generate the proton motive force (8, 91, 114).

The cytochrome *bd* oxidase, or at least *cydA* sequences were identified in virtually all

classes of prokaryotes (*Bacteroides*, *Proteobacteria I*, *II*, *Actinobacteria*, *Cyanobacteria*, *Euryarchaeta*, *Firmicutes I*, *II*), suggesting an ancient origin of this complex (6). The bacterial electron transport chains of well studied species such as *Escherichia coli*, *Bacillus subtilis* and *Gluconobacterer oxydans*, have many branches that can couple the dehydrogenation of substrates to the reduction of various terminal electron acceptors (O_2, NO_2, NO_3, fumarate, DMSO). For the aerobic electron transport chain redundancies exist on both sides in multiple NADH-dehydrogenase (type I and II) and terminal cytochrome oxidases (*bó* and *bd*) (23, 107). Specific combinations of the (NADH-) dehydrogenases and cytochromes will generate proton motive force with specific efficiencies from 1 H^+/e^- (NDH-II & oxidase *bd*) to 4 H^+/e^- (NDH-I & oxidase *bó*).

In *E. coli* activity of a *bd*-type, in favour of the more efficient *bó*-type cytochrome would result in decreased energy conservation. The expression of both cytochromes however, is differentially regulated. Cytochrome *bó* is expressed at high-, while the cytochrome *bd* is expressed at low oxygen tensions, which is regulated by Fnr and ArcA/B (86, 102, 141).

The cellular function of cytochrome *bd* goes beyond conservation of energy. In line with activity at low oxygen tensions, cytochrome *bd* has a high affinity for oxygen, making it an effective oxygen scavenger (7, 12, 106). The ability of the *bd*-cytochrome to maintain low-cytosolic oxygen levels is classically illustrated in *Azotobacter vinelandii*. It enables *Azotobacter vinelandii* to fix nitrogen using a oxygen sensitive nitrogenase in aerobic conditions (108). The *bd*-type offers protection against oxygen stress, since it prevents formation of reactive oxygen species and H_2O_2 (53, 130). The presence of cytochrome *bd* in strict anaerobes, such as *Bacteroides fragilis*, *Moorella thermoacetica* and *Desulfovibrio gigas* may desensitize such bacteria to certain levels of oxygen, although its impact on energy metabolism is uncertain, as will be discussed below (6, 32, 81).

Propionibacterium - introduction

Propionibacteria are characteristically found in the gastro-intestinal tracts of ruminants and rarely free-living in soil or water (123). They are used in the production of the hard-type Swiss cheese. During cheese ripening they play a critical role in flavour formation as they produce CO_2, propionate and acetate (105). Since they are rarely found in milk, the traditionally added calve-stomach derived rennin forms the likely source of propionibateria found in the cheese. . Members of the genus *Propionibacterium* are in fact cultivated on industrial scale, not only for the production of vitamin B_{12}, but also of propionate (13, 103). Several strains, such as *P. freudenreichii*, are marketed as probiotic food supplements. More specifically several dairy-derived propionibacteria have been used, usually in combination with other lactic acid bacteria, to promote growth in animals and treat human intestinal disorders (87, 128). A more recent development is the considered use of *P. freudenreichii* in cancer therapy (80). Lan *et. al.* induced colon carcinogenesis in rats with DMH (1,2-dimethylhydrazine). Administration of *P. freudenreichii* TL133 exerted beneficial effects in DMH treated rats on apoptosis and on the proliferation of colonic mucosa (79). In contrast to these beneficial properties of *P. freudenreichii*, other species are known as causative agents of acne (*P. acnes*) (80, 119, 120).

In all these environments, Propionibacteria play an important role in the conversion of lactate to acetate and propionic acid. Lactate itself is the product of various other fermentation processes in the rumen. So growth on lactate is known as secondary fermentation. Similar to several lactic acid bacteria most members of the genus propionibacteria are sensitive to oxygen as they do not grow on agar plates when exposed to air. In contrast, in the last vitamin B_{12} production step, when propionibacterium cultures are aerated at the end of growth, in the presence of DMB, growth is stimulated (ref jeroen).

General metabolism and fumarate respiration

The ability to anaerobically use lactate as energy source is an important ecological trait, since lactate is the main (fermentative) end-product of lactic acid bacteria. The conversion of lactate to propionate by Propionibacteria proceeds via the methylmalonyl-CoA pathway and has a stoichiometry of (44, 125):

$$3\ C_3H_5O_3^- \longrightarrow 2\ C_3H_5O_2^- + C_2H_3O_2^- + CO_2 + H_2O + (2\text{-}3\ ATP)$$

The Gibbs free energy change of this conversion is -164 kJ. These ratio's, in more complex matrices, for example in the production of Swiss-type cheese, are rarely found, since other carbon sources are used as well. This overall reaction is the overall result of two separate metabolic pathways that are closely linked to maintain redox-balance. Of the three lactate molecules, one is converted to acetate and the other two to propionate (Fig 4). The conversion of lactate to acetate generates substrate-level ATP, but requires additional reducing power. The reducing power is provided by the conversion of lactate to propionate which also generates proton motive force.

Lactate catabolism of *Propionibacterium sp*.

The bacteria that convert lactate via this pathway produce one ATP via substrate level phosphorylation, and a further 2x2/3 ATP by oxidative phosphorylation (125). If we assume that the Gibbs free energy associated with the generation of ATP is about ~+31.8kJ/mol a theoretical maximum of 5 ATP (164kJ / 31.8 kJ) could be formed. Thus this process harnesses roughly 50% (2 1/3 ATP) of the liberated energy, which is more efficient than the homolactic fermentation of glucose in lactic acid bacteria.

The two metabolic processes (conversion of lactate to either acetate or propionate) are interdependent in anaerobic conditions, without the presence of extracellular electron acceptors or the ability to produce H_2, as they respectively reduce or oxidise a finite NAD^+-pool. The conversion of one molecule of lactate into acetate results in the reduction of two molecules of NADH. The conversion of one molecule of lactate to propionate however consumes one molecule NADH. It follows that, during anaerobic growth on lactate the observed ratio of acetate/propionate must be 1:2, as is also observed. These two conversion-

routes are separated when lactate is oxidised, via a membrane associated NAD^+ dependent or menaquinone-dependent reaction (111, 124).

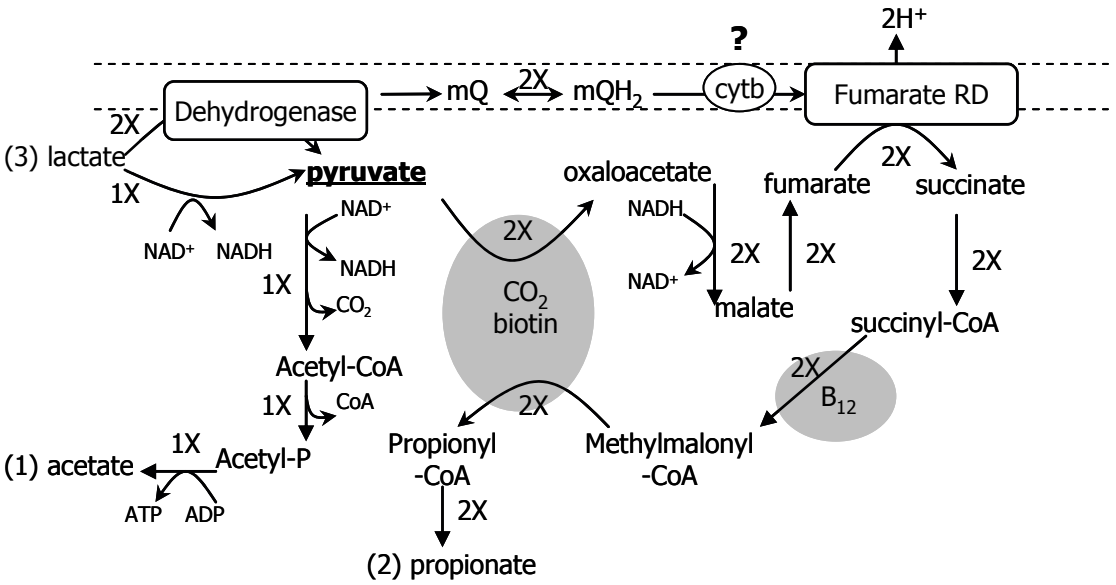


Figure 4. A simplified version of the anaerobic lactate catabolism of propionibacteria with the methylmalonyl-CoA pathway. The conversion of lactate to acetate yields substrate level ATP and reduces the NAD^+ pool. Lactate conversion to propionate via the methylmalonyl-CoA pathway conserves energy by generating proton motive force and oxidises the NAD^+-pool. The conversion of 3 molecules of lactate into 1 molecule of acetate and 2 molecules of propionate is redox-balanced. A *b*-type cytochrome may be involved in this anaerobic electron transport chain. Abbreviations: mQ (menaquinone), mQH_2 (menaquinol), -p (phosphate), -DH (dehydrogenase), -RD (reductase), cytb (*b*-type cytochrome).

Pyruvate that is converted to acetate is first activated by CoA, a reaction that produces NADH. Acetyl-CoA is then converted via acetyl-phosphate to acetate. This last step yields substrate level ATP. Pyruvate (that is converted to propionate) is first carboxylated by methylmalonyl-CoA pyruvate transcarboxylase (hence the name) to oxaloacetate. This transcarboxylase is a biotin-dependent enzyme. Oxaloacetate is reduced to malate, which is then dehydrated to fumarate. At this point an anaerobic electron transport chain becomes

involved that uses the fumarate as a terminal electron acceptor. In effect fumarate respiration yields succinate (122). Succinate is finally converted to propionate in enzymatic steps that involve the CoA cofactor. Although the anaerobic utilization of lactate is referred to as a secondary fermentation, it is a combined of fermentation and fumarate-respiration. Lactate serves as electron donor, and fumarate as terminal electron acceptor.

Alternative electron transport chains

Propionibacteria are sensitive to high levels of oxygen, as seen by their inability to grown on agar plates that are exposed to air. Despite this sensitivity to exposure to high concentrations of oxygen, under controlled (micro)aerated conditions an improved biomass formation is observed (18, 55). *Propionibacteria* should in fact be considered microaerophilic (123). *Propionibacteria* are in fact genetically well equipped to deal with fluctuating oxygen conditions.

The sequenced genome *of Propionibacterium acnes* KPA171202 contains all the genes to form a complete TCA-cycle, as is reported for other *Propionibacteria* (19, 36). As explained earlier, the electron transport chain strongly reduces the redox-carrier pools (notably NAD^+), and usually operates in conjunction with an efficient electron sink, such as respiration. All the components seem to be encoded that could form an aerobic electron transport chain (Fig. 5). A type-I NADH-dehydrogenase is found in the genome of *P. acnes* that, in contrast to the lactic acid bacterial NADH-dehydrogease type II, is capable of forming proton motive force. Furthermore, $_{D,L}$lactate, $_{L}$glycerol-3-phoshate and succinate can be oxidized via membrane associated enzyme complexes in *P. shermanii* (Fig. 4) (124). Menaquinones, components of electron transport chains, are produced by *Propionibacteria*, as were observed in *P. acnes* (www.KEGG.com) *P. shermanii* and *P. arabinosum* (65, 131, 132).

Branched electron transport chain of *Propionibacterium sp.*

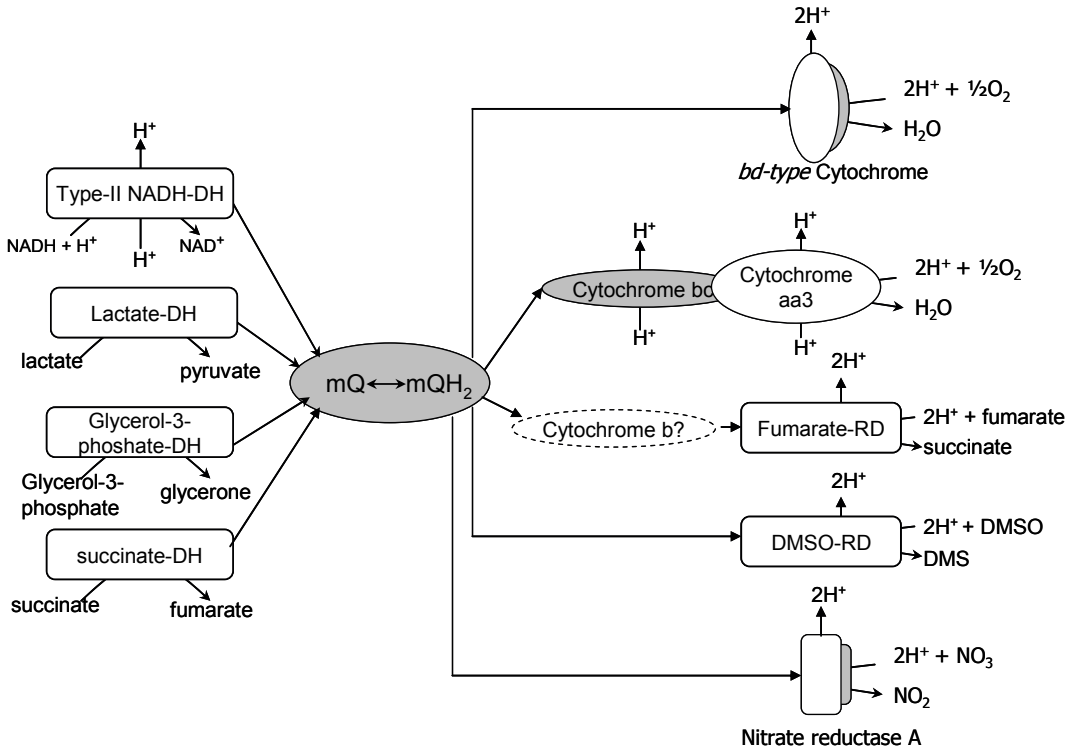


Figure 5. The brached electron transport chain as it may exist in many propionibacteria. At least for *Propionibacterium acnes*, as the only fully sequenced, exists clear genetic evidence for the existence of these components. Participation of a *b*-type cytochrome in anaerobic metabolism is suggested. Abbreviations: mQ (menaquinone), mQH_2 (menaquinol), -DH (dehydrogenase), -RD (reductase).

In contrast to lactic acid bacteria the genome sequence of *P. acnes* contains a complete heme biosynthesis gene-set (www.kegg.com). The biosynthesis of cobalamin B12 and heme share a common pathway up to the uroporphyrin III intermediate. From there the synthesis of heme requires "only" 3 additional enzymes. Heme production by other species of propionibacteria is evidenced by the observation of cytochrome-structures in many species (see below).

D-,*b*-, o- a- and c- containing cytochromes have been detected in *P. shermanii* (19, 124). The genome of *P. acnes* encodes a cytochrome c-reductase, cytochrome c-oxidase (aa_3-type cytochrome) and a- *bd*-and type cytochrome. The aa_3- and *bd*-type cytochromes have a

high and low affinity for oxygen respectively (19). This combination of terminal oxidases is also found in the electron transport chains of other well-studied (and aerophilic) gram positives, such as *Bacillus subtilis* and *Corynebacterium glutamaticum* (12, 147).

An electron transport chain that consist of a NADH type-I dehydrogenase, a menaquinone pool, cytochrome c-reductase and that terminates in the aa3-type cytochrome is much more efficient than the respiratory chain of lactic acid bacteria. Proton motive force can be generated at the type-I NADH-dehydrogenase site (1 H^+/e-) and the aa3-cytochrome oxidase (2 H^+/e-) with a total efficiency of 2 ATP/NADH generated (6H^+ translocated). *Propionibacteria* seem, despite of their best known anaerobic lifestyles, equipped to thrive in (micro) aerobic conditions.

Anaerobic electron transport chains

There are indications that cytochromes are involved in the anaerobic metabolism. Activity of a *b*-type cytochrome has been linked to succinate dehydrogenase activity (and fumarate reduction) (34, 35, 124). The genome of *P. acnes* harbours several genes of a succinate dehydrogenase. Homologue of this type of succinate dehydrogenase in *E.coli* is known to contain a *b*-type heme (104).

Actual dissimilatory nitrate-reduction with a positive effect on growth has been observed for *Propionibacteria acnes* and *P. pentosaceum* (1, 29, 52, 143). The genome of *Proprionibacterium acnes* encodes a nitrate reductase A complex that also contains (two) heme b-types. The addition of nitrate increases growth rate and growth yield in anaerobic batch cultures while the nitrate is actively reduced to nitrite (1, 143). The anaerobic electron transport chain that uses nitrate as electron acceptor is able to uncouple the production of acetate and propionate, by its ability to re-oxidise NADH. *Propionibaterium pentosaceum* grown in the presence of nitrate produce less propionic acid, with no drastic effect on acetate production. Although acetate production is not significantly effected the molar growth yield is increased, suggesting that the nitrate requiring electron transport chain conserves additional energy, most likely by formation of a proton motive force (143). Dissimilatory nitrate reduction has also been observed for *P. freudenreichii*, *P. jensii*, *P. shermanii* and *P. thoenii* and should probably be considered a general trait for Propionibacteria (76). The reduction of nitrite has been observed for several species and is considered only a detoxification

mechanism (76).

Finally, the completely sequenced genome of *Propionibacterium acnes* KPA171202 revealed the presence of genes that encode a dimethylsulfoxide reductase (19).

Sulfate reducing bacteria

Sulfate reducing bacteria are a heterogeneous group of (strict) anaerobes that can conduct dissimilatory sulfate reduction. Only a relatively small amount of sulfur is assimilated in the biomass, most is released in the form of H_2S. Sulfate reducing bacteria play an important role in the global sulfur and carbon cycle. These bacteria are found in a wide variety of environments, such as the intestinal tract (*Desulfotomaculum ruminis* and *D. acetoxidans*), marine sediment en fresh water (sediments) systems (*Desulfobacter*, *Desulfolobus* and *Desulfovibrio*) (4, 37, 66, 109). In fact they can be isolated from almost any natural environment that is sufficiently reduced (and therefore anaerobic). In environments with low concentrations of sulfate the production of hydrogen by these bacteria allow for syntrophy with hydrogen consuming methanogens (20, 89). More unique is the interaction between sulfate-reducing bacteria and various photo-trophic green (not purple) sulfur bacteria, constituting a miniature sulfur-cycle (and carbon-cycle) in what is called a sulfuretum (4). Many genera (14+) of sulfate-reducing bacteria are described of which the best studied is *Desulfovibrio*. In particular the completely sequenced *Desulfovibrio vulgaris* has been well studied (62).

Primary metabolism

As a heterogeneous group sulfate-reducing bacteria are known to oxidize a wide array of (over a hundred) organic substrates, many of which are typical products of fermentative bacteria. The utilization of biopolymers however, such as starch, is rare (57, 58). *Desulfovibrio* is known to convert many acids (such as lactate, pyruvate, and many acids that are typical intermediates of the tricarboxylic acid cycle), glycerol and ethanol and to acetate and CO_2 (56). We will limit ourselves and only describe the interplay between the catabolism of lactate with the concomitant reduction of sulfate. Lactate is an excellent growth substrate for many sulfate-reducing bacteria, in some cases even in the absence of sulfate (101, 134). The Gibbs free energy change shows that the conversion of lactate to acetate with production

of hydrogen can generate ATP only at low H_2-levels (Table 1). Sulfate reduction provides an efficient redox sink, as four H_2 are oxidized for every H_2S. The combined process of lactate catabolism and sulfate reduction releases a considerable amount of energy.

overall reaction	Gibbs free energies in kJ/mol			
	ΔG°	ΔG° pH[a]	ΔG° H_2[b]	ΔG°_e[c]
$C_3H_5O_3^- + H_2O = C_2H_3O_2^- + CO_2 + 2\ H_2$	-8.8	n.a.	-43	-43
$4\ H_2 + SO_4^{2-} + 2\ H^+ = 4\ H_2O + H_2S$	-237.8	-158.1	-169.3	-89.6
$2\ C_3H_5O_3^- + SO_4^{2-} + 2\ H^+ =$				
$2\ C_2H_3O_2^- + 2\ CO_2 + H_2S + 2\ H_2O$	-255.5	-175.7	n.a.	-175.7

[a]modification of standard Gibbs free energy for environmental pH of 7.0

[b]modification of standard Gibbs free energy change at 1.10^{-3} atm H_2 (g)

[c]ΔG_e modified Gibbs free energy that combines environmental pH and H_2 conditions

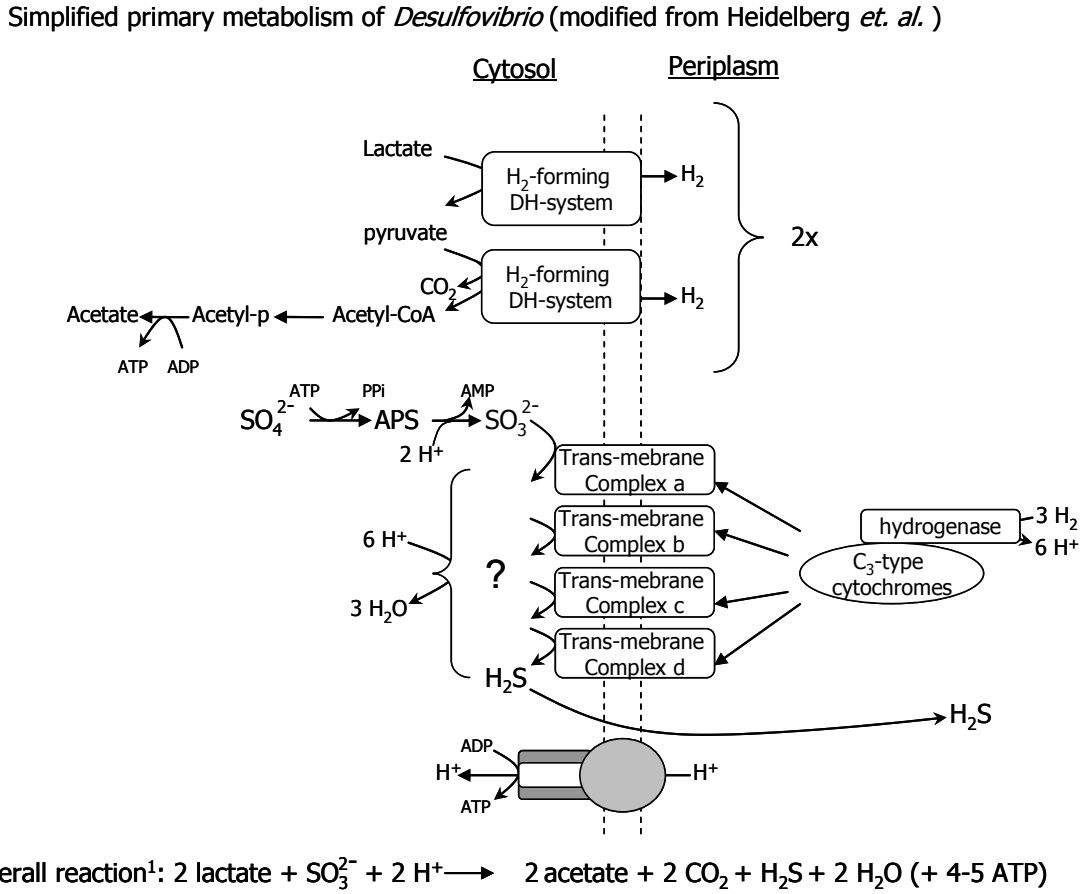
n.a. not applicable modification for this reaction

Table 1. Gibbs free energy changes of selected reactions in sulphate reducing bacteria. The Gibbs free energy changes were largely calaculated based on Gibbs free energies of formation, using data from Thauer *et. al.* (140).

Lactate is converted to pyruvate by lactate-dehydrogenases on the internal aspect of the cytoplasmic membrane or cytoplasm (98). The pyruvate is converted to acetyl-phosphate, with the release of CO_2. Finally acetyl-phosphate is converted to acetate, at which point ADP is phosphorylated and ATP formed. The ability to generate ATP by conversion of lactate to acetate is quite common among bacteria. However the proposed role of hydrogen, formed during the cytosolic oxidation of lactate and pyruvate, and that is used by periplamsic hydrogenases is unique. Hydrogen, generated by cytosolic oxidation processes, diffuses across the membrane, and is used as periplasmic electron donor for the reduction of sulfate to (in a series of steps) H_2S (Fig. 6). In essence, in stead of proton pumping to generate proton motive force, sulfate reducing bacteria form and oxidize hydrogen on different sides of the membrane.

Soluble periplasmic [NiFe] hydrogenases are present in most, if not all, *Desulfovibrio* species (145). This type of hydrogenase is found in other bacteria such as *Escherichia*, *Azotobacter* and *Rhizobium* anchored however, to the cytoplasmic side. The periplasmic localization of the [NiFe] hydrogenase in *Desulfovibrio* enables it to interact with the C_3 cytochrome that is also located in the periplasm (144). Hydrogen is thus a periplamic electron donor that, via activity of the hydrogenase, C_3 –type cytochromes and transmembrane spanning complexes, drives cytosolic sulfate reduction. The proton motive force that is formed by periplasmic hydrogen oxidation can subsequently be used by the F_1F_0 ATPase to generate ATP. Besides hydrogen that is derived from lactate metabolism extra-cellular hydrogen can also serve as electron donor. The production or consumption of hydrogen, by lactate-grown *Desulfovibrio* depends on the presence of the electron-sink sulfate. Lactate catabolism and sulfate reduction are therefore not strictly interdependent.

The reduction of sulfate to sulfide is a complex multi-step process of which the exact nature of all intermediate metabolites is not know, and not of particular importance to evaluate the overall energetics (Fig. 6). The complete reduction of sulfate to sulfide requires a total of eight electrons (four hydrogen). These are provided by the conversion of two lactate to acetate. The generation of four hydrogen molecules requires catabolism of two lactate molecules to acetate.



[1]For simplicity, the overall reaction of lactate catabolism with the concomitant reduction of sulfite is given. Reduction of sulfate requires an initial activation with ATP that produces AMP.

Figure 6. Schematic representation of lactate catabolism and sulfate reduction. Lactate and pyruvate are dehydrogenated on the cytosolic side of the membrane in a process that generates H_2. H_2 as neutral, non-polar compound can diffuse across the cell membrane. Interaction of periplasmic hydrogenases and C-type cytochromes with specific heme-containing transmembrane complexes enables H_2 to be used for the reduction of sulfate to H_2S (S^{2-}) in the cytosol. These transmembrane complexes are represented schematically by the "Trans-membrane complexes" a-d. The reduction of sulfite to H_2S is a complex process that involves partial recycling of metabolic intermediates. The exact nature of all the metabolic intermediates of sulfite catabolism is unsure. This scalar chemistry generates a proton motive force that can be used to generate ATP by the F_1F_0 ATPase. Abbreviations: -p (phosphate), PPi (pyrophosphate), DH (dehydrogenase).

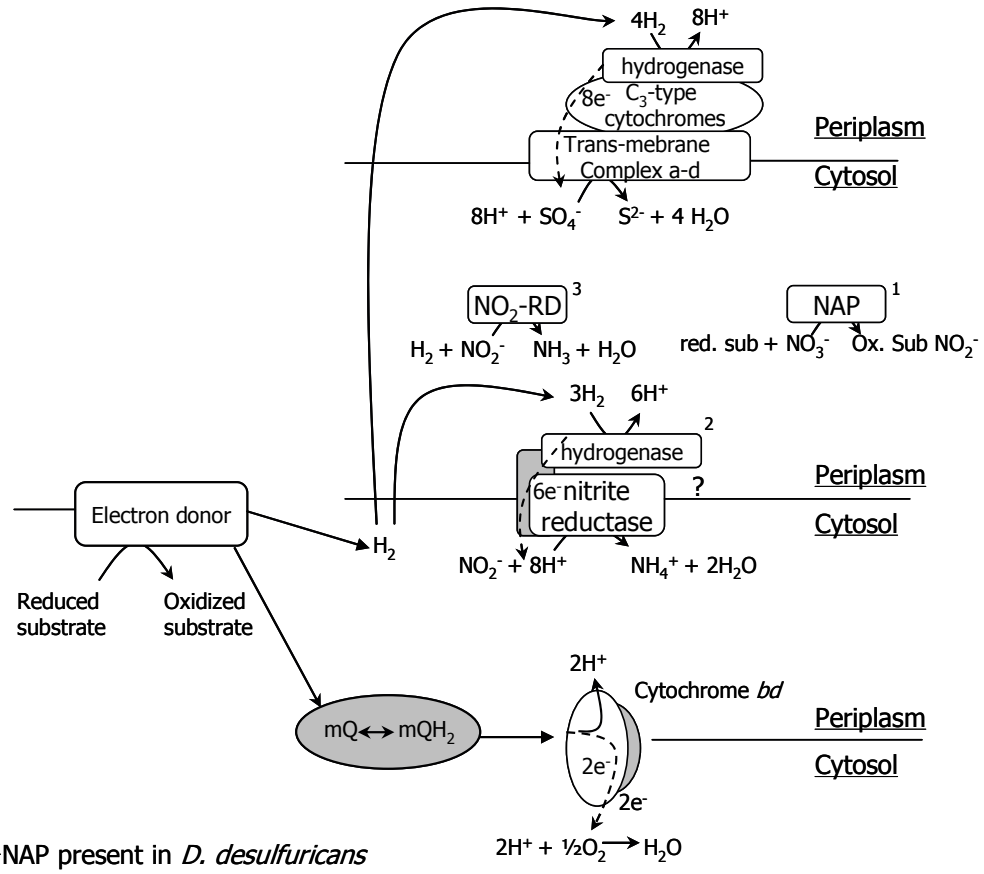
During this conversion two ATP are generated by substrate level phosphorylation (one per acetate that is formed). Two ATP are overall required for the initial activation of sulfate to

produce APS, since AMP is formed. In addition proton motive force is used (dissipated) to drive sulfate uptake (30). The periplasmic oxidation of four hydrogen moles with concomitant use of cytosolic protons for sulfate reduction creates a proton motive force of "8" protons. If we assume that the efficiency of the F_1F_0 ATPase is 3 H^+/ ATP, then (in total) 8 protons generate roughly 2 ATP (127). Since sulfite does not require to be activated by ATP, the use of sulfite as redox sink is energetically more favorable and will yield more (roughly 4 ATP).

Alternative electron transport chain components

Electron transport chains consist of dehydrogenases, a (mena)quinone pool and terminal oxidases. The way in which proton motive force is generated, in sulfate reducing bacteria, in this sense, does not constitute an electron transport chain, since no (mena)quinone pool is involved. (Mena)quinones however have been detected in many *Desulfovibrio* sp. and other sulfate reducing bacteria (38, 99, 121). It is even used as a distinguishing feature since different groups of sulfate reducing bacteria often show a difference in major menaquinone species. *Desulfovibiro* sp. can reduce other inorganic substrate (besides sulfate), such as nitrite and nitrate.

Nitrite reduction has been reported for many *Desulfovibrio* sp. (92). It appears that nitrite reduction generates a proton motive force in some sulfate-reducing bacteria, while it inhibits growth in others. Haveman *et. al.*, reported that the reduction of nitrite however did stimulate growth in *D. vulgaris* (59). Nitrite negatively affected sulfate reduction and therefore nitrite-reduction was considered to be a detoxifying process (54). A periplasmic non-membrane bound orientation of the nitrite reductase that uses hydrogen to remove nitrite will in fact inhibit proton motive force generation (60).



[1]NAP present in *D. desulfuricans*

[2]membrane-bound nitrite-reductase complex present in *D. desulfuricans* and *D. gigas*

[3]detoxifying nitrite reductase as found in *D. vulgaris.*

Figure 7. Energy conservation and electron transport in *Desulfovibrio* sp. Sulfate is a highly effective redox sink when fully converted to H_2S and is depicted as single process for simplicity. The *bd*-type cytochrome and menaquinol production has been observed in many species. While the nitrite reductase of *D. desulfuricans* and *D. gigas* generates proton motive force that of *D. vulgaris* does not and in fact likely competes with the sulfate reduction for H_2. In this way it reduces the amount of proton motive force generated. Abbreviations: mQ (menaquinone), mQH_2 (menaquinol), -DH (dehydrogenase), -RD (reductase), NAP (periplasmic nitrate-reductase).

Nitrite-reductase activity may have beneficial effects in other sulfate reducing bacteria, such as *D. desulfuricans* and *D. gigas*. A cytochrome-c nitrite reductase complex that is membrane-bound and can use hydrogen as electron donor for the reduction of nitrite was found in *D. desulfuricans* (26, 84). Steenkamp *et. al.* provided evidence that this nitrite reductase complex may have a cytosolic nitrite accepting side and the hydrogen consuming

side periplasmically orientated (Fig 7) (135). It should therefore create a proton motive force, which in fact has been shown for both *D. desulfuricans* and *D. gigas* (5, 135). In the absence of sulfate nitrite may (in these species) function as alternative hydrogen electron sink, leading to a proton motive force generation and growth.

Nitrate-reduction has been reported specifically for *D. desulfuricans* where nitrate appears to be as effective an electron sink as sulfate in stimulating growth on lactate (77, 88). The nitrate-reductase complex involved however is soluble and periplasmically located (NAP) and does not generate a proton motive force. The removal of hydrogen may stimulate lactate catabolism, although the importance of NAP activity could be the formation of nitrite, since nitrite reduction can generate proton motive force. In this regard it is important to note that nitrate-reductase activity was observed to be lower then the nitrite-reductase activity. Thus nitrite that is formed by the periplasmic nitrate-reductase will not accumulate to toxic levels (21, 93). Nitrate is in fact an energetically more favorable redox sink than sulfate, when proton motive force is generated via a nitrite-reductase, since it does not have to be activated at the cost of ATP.

Although considered a (strict) anaerobe, oxygen reducing *bd*-type cytochromes are found in the membranes of *Desulfovibrio* sp. (at least for *D. vulgaris*, *D. gigas*. *D. desulfuricans* and probably *D. africanus*) (62, 71, 81). This *bd*-type cytochrome is characterized as a membrane-bound menaquinol-oxidase that is capable of generating proton motive force by scalar chemistry (17, 72, 114). It can effectively scavenge oxygen and offer some protection against oxidative stress. Oxygen consumption by membrane preparations of *D. desulfuricans* and *D. gigas* was stimulated by NADH and succinate, supporting the membrane localization of this cytochrome (81). Various key enzymes in the pathways for substrate oxidation of *Desulfovibrio* sp. are oxygen-labile (47, 63, 133). The high-oxygen affinity of the *bd*-type cytochrome offers a level of protection and would sustain proton motive force generation while sulfate reduction is inhibited. Furthermore it explains the presence of menaquinol in many species. The oxygen-liability of hydrogen producing lactate-dehydrogenase, may allow NADH-producing lactate dehydrogenases to reduce menaquinol in aerobic conditions (133). This difference in oxygen-liability is a simple mechanism to prevent the loss of reducing power, in the form of hydrogen, during aerobic conditions, and use it for the aerobic electron transport chain. Even though energy may be conserved in aerobic

conditions with the *bd*-type cytochrome, true aerobic growth even at micro-aerobic conditions has never been shown and indicates aerobic-liability of key-enzymes involved in biomass formation (45).

Homoacetogenic bacteria

Homoacetogens (further reffered to as acetogenic bacteria) are defined as anaerobic prokaryotes that are able to produce acetate using H_2 and CO_2 (or to catabolise a hexose to three molecules of acetate) (97). The acetogenic bacteria comprise members from various related genera, such as *Clostridium*, *Acetobacterium*, *Sporomusa*, *Eubacterium* and *Thermoanaebacter*. Except for *Acetobacterium* and *Sporomusa*, these genera contain also non-acetogenic members. Acetogenic bacteria colonize a diverse range of habitats. They heavily colonize mammalian gastro-intestinal tracts (110, 148). Acetogenic bacteria inhabit the guts of wood-eating termites, that use the acetate that is produced as an oxidizable energy source (14). Furthermore these bacteria colonize the rhizosphere of various plant species and are recovered form a range of other ecological habitats. In essence they participate in complex microbial networks that degrade organic matter, where they growth on fermentation products of other bacteria (42).

The Acetyl-CoA pathway (or Wood-Ljungdahl pathway)

Acetogenesis from H_2 and CO_2 allows the use of CO_2 as sole carbon source. Acetogens with this metabolism are therefore autotrophs. The process is initiated by entry of CO_2 in two separate pathways: the Methyl branch and the Carbonyl branch. The Methyl branch converts CO_2 via a series of steps to methyl-CoA, while the Carbonyl branch converts CO_2 to CO using Acetyl-CoA synthase. Methyl and carbonyl are combined into acetyl-CoA. Acetyl-CoA is converted either to acetate (an energy producing reaction) or shunt into biomass producing pathways. It is interesting to note that the production of acetate from CO_2 does not yield net ATP by substrate-level phosphorylation. ATP-evolution plays a catalytic role in driving the primary metabolism. All energy that is formed with CO_2 as sole carbon source must be generated by linking the oxidation of hydrogen to formation of proton (or sodium) motive force (Fig. 8). Growth on H_2 and CO_2 demands efficient energy conservation since for every acetyl-CoA that is shunt into biomass there is a net loss of 1 ATP.

The dependency, of growth on CO_2, on H_2-respiration is made apparent by energetic considerations. The standard overall free energy change for acetogenesis from CO_2 and H_2 was calculated to be in the order of -55.1kJ/mol, under standard conditions (49). However a more realistic estimation of the Gibbs free energy under natural conditions with a pH of 7 (H^+ of 1.10^{-7}) and a H_2 concentration of (1uM or 1.10^{-3} atm) is -81.5 kJ/mol (25, 49).

$$4H_2 + 2CO_2 \longrightarrow CH3COOH + 2H_2O + H^+$$

The production of 1 mol acetate requires 4 moles of hydrogen, which are consumed at 4 different intermediate conversion reactions. The involvement of at least 4 reactions dictates that the total energy released per reaction (in a most direct conversion series) is roughly a quarter of the total Gibbs free energy change. The $\Delta G/H_2$ then is -20.4kJ/H_2 for acetogenesis. Since the ΔG for ATP formation is +31.8kJ/mol it is clear that growth on CO_2 and H_2 requires involvement of an electron transport chain. The electron transport chain can use lower free energy changes to translocate H^+ from the cytosol to the external environment, and in this way store and accumulate these to the extent that it can drive ATP formation. If we take a membrane potential of for example of -70mV we can calculate that the energy required to translocate one proton is in the order of +6.7kJ/mol H^+. For acetogens the combination of a low overall free energy change (from CO_2 and H_2 to acetogen) and the involvement of many enzymatic conversions thus necessitate energy conservation by an electron transport chain.

Besides autotrophic growth on CO_2 and hydrogen acetogens are also able to use sugars as carbon source. Typicall for acetogens is the complete conversion of sugars to acetate (for example 1 mole of glucose to 3 moles of acetate) (85). Furthermore, *Peptostreptococcus productus*, and probably many other acetogens have a classic anaerobic pyruvate metabolism (41). Pyruvate for example can be converted to lactate or ethanol to regenerate NAD^+.

Two methods of energy conservation that use either proton or sodium driven ATP generation, separates the acetogenic bacteria in two groups. These are represented by *Moorella thermoacetica* (formerly known as *Clostridium thermoaceticum*) and *Acetobacter woodii* respectively. The members of the "*Clostridium* group" contain cytochromes (often two distinct *b*-type cytochromes are observed) and a menaquinone pool. The "*Acetobacter* group"

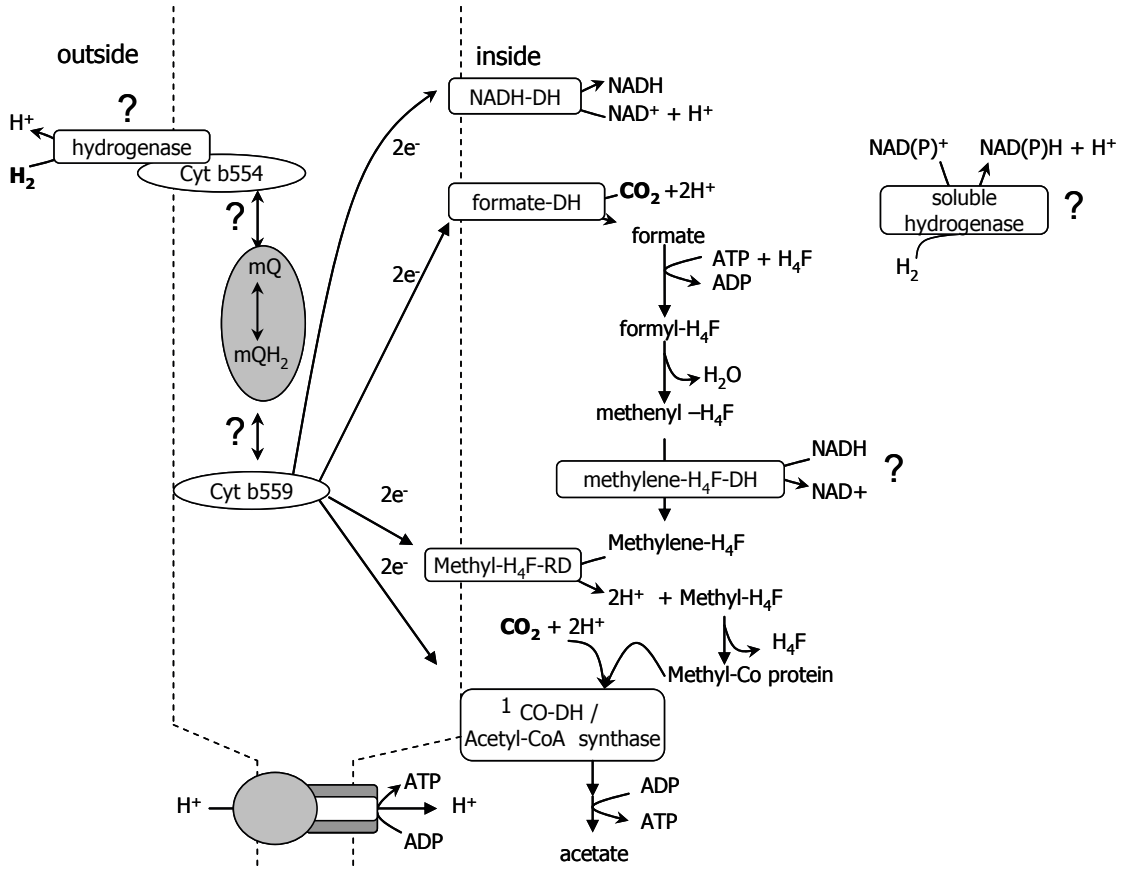
however lack detectable cytochromes and use membrane bound corrinoids to translocate sodium ions (94). For both groups the exact characteristics of the electron transport chains, efficiencies and points of energy conservation remain speculative and lack strong experimental support.

The electron transport chain

Growth on CO_2 and H_2 requires that the flow of electrons from H_2 is coupled to the various reductive steps of acetate formation. In order to convert this flow of electrons to usable proton (sodium) motive force, membrane-linked dehydrogenation and oxidation reactions must be involved. For acetogenic *Clostridium* sp model is proposed that combines the production of acetate from H_2 and CO_2 with energy conservation (Fig 8).

The orientation of the hydrogenase, accepting hydrogen and releasing protons on the inside or outside of the cell is unsure (41). In terms of energy conservation, a model in which the electrons that are derived from the periplasmic oxidation of hydrogen, are used to reduce $NAD(P)^+$ and methylene-H_4F in the cytosol, would generate proton motive force.

The proposed electron transport chain of *Moorella thermoacetica* contains two *b*-type cytochromes (554 and 559) in which *b*-type 554 is oxidized by the menaquinole pool, while *b*-type 559 is possibly reduced by it (68). In any case the *b*-type cytochromes, together with menaquinone may facility transport of electrons from hydrogen to the cytosolic reduction steps. There is at least evidence for the membrane-bound location of a hydrogenase, CO-dehydrogenase and NADH-dehydrogenase activity (94). In fact in the case of CO-dehydrogenase there is actual experimental evidence that this activity can be associated with formation of proton motive force, and in the case of *Moorella thermoacetica*, is associated with *b*-type cytochrome oxidation (67).



[1] Moorella thermoacetica has a bifunctional carbonmonoxide dehydrogenase/ acetyl-CoA synthase

Figure 8. Proposed model that combines primary metabolism of the H^+-dependent acetogens with energy conservation. Modified from Fuchs *et. al.* and Müller (49, 94). Tetrahydrofolate plays an important role as single-carbon carrier in this metabolism. Membrane-bound locations of NADH, formate and methyl-H_4F is proposed. The dotted lines represent the boundaries of the cell membrane. Abbreviations: mQ (menaquinone), mQH_2 (menaquinol), -DH (dehydrogenase), -RD (reductase), -THF (tetrahydrofolate), H_4F (tetrahydrofolate).

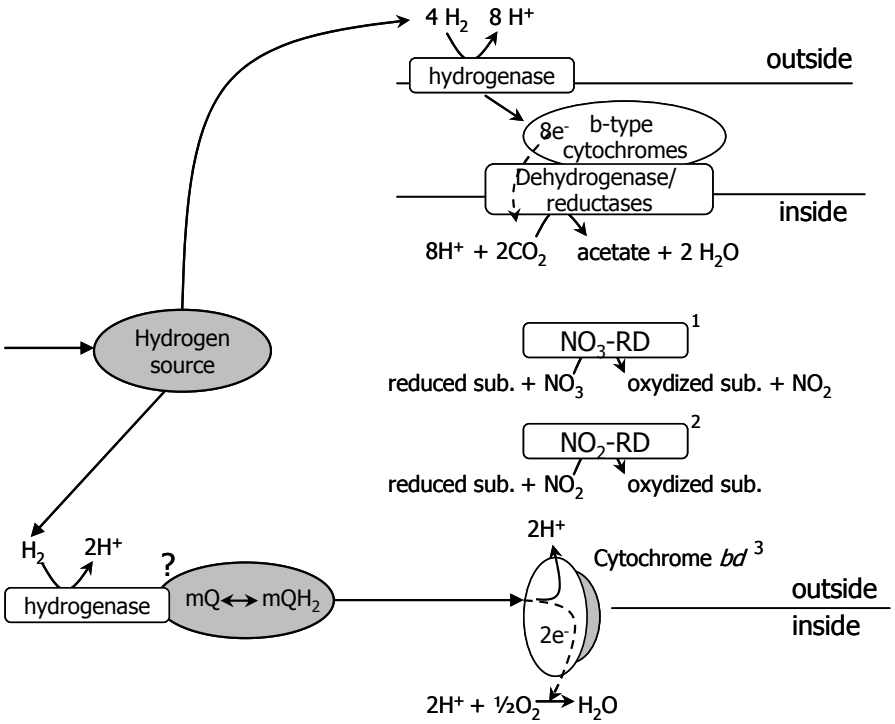
Moorella thermoacetica has a bifunctional carbonmonoxide dehydrogenase/ acetyl-CoA synthase, thus this enzyme may be membrane-bound or activity of a different membrane-bound CO-dehydrogenase was measured (40). The presence of *b*-type cytochromes are ubiquitously found in the proton-motive force (as opposed to sodium-motive force) generating acetogens (73, 94) .

An assumed electron acceptor for the anaerobic electron transport chain when cells are

grown using CO_2 as carbon source and H_2 to provide reducing power, is the membrane bound methylene-H_4F-reductase (94). Alternatively it has been suggested that the transfer of electrons to the carbon monoxide dehydrogenase is also membrane associated, thus possible allowing the creation of proton motive force (68). Various carbon monoxide dehydrogenase enzymes have the ability to reduce protons/oxidize hydrogen directly, which is not the case for *Moorella thermoacetica* , which makes sense in the light of energy conservation (90, 115). Energy conservation via the flow of electrons from hydrogen towards carbon dioxide has been reported in literature, catalysed by certain members of the [NiFe]-hydrogenases (61). To conclude, although the enzyme machinery that allow acetogenic bacteria to grow solely on H_2 and CO_2 has been characterized in detail, the underlying energetics, and electron-flows, of these processes are still poorly experimentally elucidated.

Aerobic electron transport chain

Exposure to air (oxygen) inhibits growth of most, if not all, acetogenic bacteria. Despite the lack of growth, consumption of oxygen in cell-suspensions has been observed for the acetogens *Sporomusa termitida* , *Clostridium glycolicum*, *Acetonema longum* and *Acetobacterium woodii* (10). Hydrogen stimulated depletion of oxygen in sealed environments with the reoccurrence of growth. *bd*-type cytochromes were detected in *Moorella thermoacetica*, where they accounted for ~70% of the oxygen consumption (Fig 9) (32, 147). Oxygen consumption by acetogens therefore plays an important role in oxidative stress protection and the generation of anoxic environments. It had however little positive effects on biomass production. In fact when growing with CO_2 or methanol as carbon source, oxygen consumes the hydrogen that is required for CO_2 reduction. The growth-inhibitory effect of oxygen can therefore be explained. The ability of *Moorella thermoacetica* to conserve energy with a *bd*-type cytochrome, while attempting to make the environment anoxic, may prolong the survival-period, during oxygen exposure.



[1]Nitrate-dissimilation observed for *Clostridium thermoaceticum* (*Moorella thermoacetica*) and *C. thermoautotrophicum*

[2]Nitrit-dissimilation observed *Clostridium thermoaceticum* (*Moorella thermoacetica*)

[3]*bd*-type cytochrome observed for *Clostridium thermoaceticum* (*Moorella thermoacetica*)

Figure 9. Electron transport chains of acetogens. The best studied acetogen *Moorella thermoacetica* can utilize nitrate, nitrite and oxygen as redox sink. The location and proton motive force generating capabilities of the nitrate and nitrite reductases are not known. Abbreviations: mQ (menaquinone), mQH_2 (menaquinol), -DH (dehydrogenase), -RD (reductase).

Alternative anaerobic electron transport chain

Moorella thermoacetica (*Clostridium thermoaceticum*), *C. thermoautotrophicum* and can actively reduce nitrate (2, 126). These acetogenic bacteria use nitrate as redox-sink, in preference over CO_2, when provided with an alternative utilizable carbon-source. However autotrophic growth on CO_2 and H_2 is inhibited in the presence of nitrate (48). This is again not surprising, since in these conditions nitrate competes with CO_2 for electrons and thus prevents the reduction of CO_2 that is required for growth. Nitrate-reduction by these *Clostridium* species shows their metabolic versatility. In fact the addition of nitrate increases the substrate

range of *C. thermoacetica* (41). At least for *C. thermoacetica* even nitrite can also serve as efficient electron source and enhance energy conservation on more complex carbon sources (126). Whether nitrate or nitrite reduction actually leads to generation of proton motive force, or that they merely positively affect the redox balance is not known. The *NarGHJI*-genes that encode the proton motive force generating nitrate reductase A were not found in the genome of *C. thermoaceticum*. Since nitrate inhibits growth on CO_2 and H_2 it suggests that in their natural habitats nitrate is usually present with more complex organic substrates. Other (perhaps more specialised) acetogens, such as *Acetobacterium kiviu*, *A. woodii*, *Clostridium aceticum*, *C. formicoaceticum* and *Peptostreptococcus productus* did not reduce nitrate (48).

Concluding remarks

We have reviewed the types of electron transport chain systems that are present in lactic acid bacteria, propionibacteria, sulfate reducing bacteria and acetogens. We have focussed on current knowledge of several key-species in each of these groups, and shown a remarkable degree of versatility in the use of extra-cellular electron acceptors to enhance growth efficiency. It is important to realize that not all bacteria strive to optimize growth efficiency in all conditions.

A case in point is the metabolic strategy of lactic acid bacteria, such as *Lactococcus lactis* and *Lactobacillus plantarum*. When these bacteria are grown on glucose they initially produce only lactate, even when the presence of heme, menaquinone and oxygen makes respiration possible. These bacteria specialise in growth speed rather then growth efficiency. Only when their growth speed severely decreases do they use available redox sinks and become more efficient mixed-acid producers (24, 139). In batch cultivation for example aerobic respiration is only apparent at the late-growth phases (50). At this point aerobic respiration starts to have a noticeable impact on metabolism. The fact that no member of this group has retained the ability to produce heme (or porphyrins) is testament to their specialization for growth speed. Respiration in lactic acid bacteria is opportunistic, and employed as boosts for energy production only in certain conditions. It is safe to say however, that in more natural environments long periods of slow growth are the norm. In addition respiration conveys significant advantages besides enhancement of growth efficiency. Respiration of *Latococcus lactis* leads to more robust cells that can better withstand stress

conditions. Thus the added robustness and ability to use oxygen to conserve energy could be a more relevant for survival in nature. Still, when bacteria are (in theory) able to enhance growth by respiration, this does not necessarily mean that they will do so.

To asses the activity of aerobic and other alternative electron transport chains in propionibacteria, sulphate-reducing bacteria and acetogens, it is important to realize they must compete with the "normal anaerobic" electron transport chain. For example, (anaerobic) growth of propionibacteria on lactate as carbon source employs an electron transport chain that already conserves additional energy. The reduction of fumarate by the electron transport chain creates proton motive force by scalar chemistry, just as the *bd*-type cytochrome (70). Activity of an aerobic electron transport chain with a *bd*-type cytochrome is therefore not more efficient in conserving energy. Other considerations must be taken into account to understand consequences of (aerobic) respiration. For example when bacteria are exposed to air, oxygen is a limitless redox sink.

When oxygen is used as terminal electron acceptor, and not fumarate, all pyruvate can thus be converted to acetate. This by itself will increase ATP yield. (The ATP-yield per pyruvate converted to acetate is one, whereas per pyruvate converted to propionate is two-thirds (~0.67) ATP). Furthermore ATP is also generated by proton motive force formation by the aerobic electron transport chain. The switch from mixed acetate/propionate production to almost completely acetate has in fact been reported for *Propionibacterium shermanii* (113). An active aerobic transport chain that uses a cytochrome c reductase with an aa_3-type cytochrome c oxidase would conserve even more ATP, as it is more efficient in generating proton motive force. Propionibacteria seem to be almost too well adapted, as oxygen-sensitive secondary fermentors, to cope with fluctuating oxygen concentrations. These oxygen sensitive anaerobes have in fact far superior aerobic respiratory systems then the facultative anaerobic lactic acid bacteria.

In contrast to acetic acid bacteria and propionibacteria, sulfate reducing bacteria and acetogens are severely sensitive to oxygen and strict anaerobes (15). Several key-enzymes in the primary metabolism of sulfate reducing bacteria appear to be oxygen-labile, such as Lactate-, ethanol- and even hydrogen dehydrogenases (46, 63, 75, 133). The high-affinity of a *bd*-type cytochrome is well suited to maintain low intracellular oxygen levels. Aerobic respiration with a *bd*-type terminal oxidase have would yield slightly more ATP, as ATP is

consumed in the initial activation of sulfate to APS. Possibly the toxic effects of oxygen on other key enzymes outweigh it positive effects on energy generation. In any case, aerobic respiration that was coupled to ATP generation, but not with growth, was observed for various sulfate reducing bacteria, although is not clear why (39). The increase in growth yield of some sulfate reducers that can use nitrate as electron acceptor supports the idea of oxygen-liability of key biomass producing enzymes (31). Thus aerobic respiration in sulfate reducing bacteria, in (laboratory) practise, full fills a protective role only.

A clear example of a purely protective role of aerobic respiration is seen in acetogens that grow on CO_2 as sole carbon source. Firstly, several enzymes in the acetyl coenzyme A pathway, are extremely sensitive to oxygen. Therefore the oxygen scavenging activity *bd*-type cytochrome is important for promoting anaerobosis after (transient) exposure to oxygen. The termite gut is a known natural habitat for acetogens with steep oxygen gradients where the *bd*-type cytochrome could thus play an important role (10). As hydrogen stimulates the reduction of oxygen, in acetogens, it indicates that hydrogen is the source of reducing power.

Secondly, for acetogens to grow on CO_2, it is essential that the reducing power of hydrogen is used to reduce CO_2. The reduced CO_2 will eventually be transformed in acetatyl-CoA that is used as C-source for actual biomass. Therefore although many acetogens are able to reduce oxygen, and in the case of *Clostridium thermoacetica*, conserve energy in the process, it will directly prevent growth. In the same way nitrate-reduction, by *Moorella thermoacetica* and *C. thermoautotrophicum* will also prevent biomass formation, when growing on CO_2 as carbon course and using hydrogen as reducing power (48). Thus, the use of oxygen, nitrate or nitrite as redox sinks can only enhance growth efficiency when more complex organic substrates are used as carbon source.

1. **Allison, C., and G. T. Macfarlane.** 1989. Dissimilatory nitrate reduction by *Propionibacterium acnes*. Appl. Environ. Microbiol. **55:**2899-2903.
2. **Arendsen, A. F., M. Q. Soliman, and S. W. Ragsdale.** 1999. Nitrate-dependent regulation of acetate biosynthesis and nitrate respiration by *Clostridium thermoaceticum*. J Bacteriol **181:**1489-95.
3. **Barron, E. S., and H. R. Jacobs.** 1938. Oxidations Produced by Hemolytic Streptococci. J Bacteriol **36:**433-49.
4. **Barton, L. L. (ed.).** 1995. Sulfate-reducing bacteria. Plenum Press, New York.
5. **Barton, L. L., J. LeGall, J. M. Odom, and H. D. Peck, Jr.** 1983. Energy coupling to nitrite respiration in the sulfate-reducing bacterium *Desulfovibrio gigas*. J Bacteriol **153:**867-71.
6. **Baughn, A. D., and M. H. Malamy.** 2004. The strict anaerobe *Bacteroides fragilis* grows in and benefits from nanomolar concentrations of oxygen. Nature **427:**441-4.
7. **Belevich, I., V. B. Borisov, D. A. Bloch, A. A. Konstantinov, and M. I. Verkhovsky.** 2007. Cytochrome *bd* from *Azotobacter vinelandii*: Evidence for High-Affinity Oxygen Binding. Biochemistry.
8. **Bertsova, Y. V., A. V. Bogachev, and V. P. Skulachev.** 1997. Generation of protonic potential by the *bd*-type quinol oxidase of *Azotobacter vinelandii*. FEBS Lett **414:**369-72.
9. **Blank, L. M., B. J. Koebmann, O. Michelsen, L. K. Nielsen, and P. R. Jensen.** 2001. Hemin reconstitutes proton extrusion in an H^+-ATPase-negative mutant of *Lactococcus lactis*. J Bacteriol **183:**6707-9.
10. **Boga, H. I., and A. Brune.** 2003. Hydrogen-dependent oxygen reduction by homoacetogenic bacteria isolated from termite guts. Appl Environ Microbiol **69:**779-86.
11. **Bolotin, A., B. Quinquis, P. Renault, A. Sorokin, S. D. Ehrlich, S. Kulakauskas, A. Lapidus, E. Goltsman, M. Mazur, G. D. Pusch, M. Fonstein, R. Overbeek, N. Kyprides, B. Purnelle, D. Prozzi, K. Ngui, D. Masuy, F. Hancy, S. Burteau, M. Boutry, J. Delcour, A. Goffeau, and P. Hols.** 2004. Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. Nat Biotechnol **22:**1554-8.

12. **Bott, M., and A. Niebisch.** 2003. The respiratory chain of *Corynebacterium glutamicum*. J Biotechnol **104:**129-53.
13. **Boyaval, P.** 1987. Continuous fermentation of sweet whey permeate for propionic acid production in a CSTR with a UF recycle. Biotechnol. Lett. **9:**801-806.
14. **Breznak, J. A., and J. M. Switzer.** 1986. Acetate Synthesis from H_2 plus CO_2 by Termite Gut Microbes. Appl Environ Microbiol **52:**623-630.
15. **Briukhanov, A. L., and A. I. Netrusov.** 2007. Aerotolerance of strictly anaerobic microorganisms and factors of defense against oxidative stress: a review. Prikl Biokhim Mikrobiol **43:**635-52.
16. **Brooijmans, R. J., B. Poolman, G. K. Schuurman-Wolters, W. M. de Vos, and J. Hugenholtz.** 2007. Generation of a membrane potential by *Lactococcus lactis* through aerobic electron transport. J Bacteriol.
17. **Brooijmans, R. J., B. Poolman, G. K. Schuurman-Wolters, W. M. de Vos, and J. Hugenholtz.** 2007. Generation of a membrane potential by *Lactococcus lactis* through aerobic electron transport. J Bacteriol **189:**5203-9.
18. **Bruggemann, H.** 2005. Insights in the pathogenic potential of *Propionibacterium acnes* from its complete genome. Semin Cutan Med Surg **24:**67-72.
19. **Bruggemann, H., A. Henne, F. Hoster, H. Liesegang, A. Wiezer, A. Strittmatter, S. Hujer, P. Durre, and G. Gottschalk.** 2004. The complete genome sequence of *Propionibacterium acnes*, a commensal of human skin. Science **305:**671-3.
20. **Bryant, M. P., L. L. Campbell, C. A. Reddy, and M. R. Crabill.** 1977. Growth of *Desulfovibrio* in lactate or ethanol media low in sulfate in association with H_2-utilizing methanogenic bacteria. Appl Environ Microbiol **33:**1162-9.
21. **Bursakov, S. A., C. Carneiro, M. J. Almendra, R. O. Duarte, J. Caldeira, I. Moura, and J. J. Moura.** 1997. Enzymatic properties and effect of ionic strength on periplasmic nitrate reductase (NAP) from *Desulfovibrio desulfuricans* ATCC 27774. Biochem Biophys Res Commun **239:**816-22.
22. **Cabello, P., M. D. Roldan, and C. Moreno-Vivian.** 2004. Nitrate reduction and the nitrogen cycle in archaea. Microbiology **150:**3527-46.

23. **Calhoun, M. W., K. L. Oden, R. B. Gennis, M. J. de Mattos, and O. M. Neijssel.** 1993. Energetic efficiency of *Escherichia coli*: effects of mutations in components of the aerobic respiratory chain. J Bacteriol **175:**3020-5.

24. **Cocaign-Bousquet, M., C. Garrigues, P. Loubiere, and N. D. Lindley.** 1996. Physiology of pyruvate metabolism in *Lactococcus lactis*. Antonie Van Leeuwenhoek **70:**253-67.

25. **Conrad, R., and W. Wetter.** 1990. Influence of temperature on energetics of hydrogen metabolism in homoacetogenic, methanogenic and other anaerobic bacteria. Arch Microbiol **155:**94-98.

26. **Costa, C., J. J. Moura, I. Moura, Y. Wang, and B. H. Huynh.** 1996. Redox properties of cytochrome *c* nitrite reductase from *Desulfovibrio desulfuricans* ATCC 27774. J Biol Chem **271:**23191-6.

27. **Costilow, R. N., and T. W. Humphreys.** 1955. Nitrate reduction by certain strains of *Lactobacillus plantarum*. Science **121:**168.

28. **Cruz-Ramos, H., G. M. Cook, G. Wu, M. W. Cleeter, and R. K. Poole.** 2004. Membrane topology and mutational analysis of *Escherichia coli* CydDC, an ABC-type cysteine exporter required for cytochrome assembly. Microbiology **150:**3415-27.

29. **Cummins, C. S., and J. L. Johnson.** 1980. The genus *Propionibacterium*. Springer-Verlag, New York.

30. **Cypionka, H.** 1989. Characterization of sulfate transport in *Desulfovibrio desulfuricans*. Arch Microbiol **152:**237-43.

31. **Cypionka, H.** 2000. Oxygen respiration by *Desulfovibrio* species. Annu Rev Microbiol **54:**827-48.

32. **Das, A., R. Silaghi-Dumitrescu, L. G. Ljungdahl, and D. M. Kurtz, Jr.** 2005. Cytochrome *bd* oxidase, oxidative stress, and dioxygen tolerance of the strictly anaerobic bacterium *Moorella thermoacetica*. J Bacteriol **187:**2020-9.

33. **de Roos, N. M., and M. B. Katan.** 2000. Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998. Am J Clin Nutr **71:**405-11.

34. **De Vries, W., M. I. Aleem, A. Hemrika-Wagner, and A. H. Stouthamer.** 1977. The functioning of cytochrome *b* in the electron transport to furmarate in

Propionibacterium freudenreichii and *Propionibacterium pentosaceum*. Arch Microbiol **112:**271-6.

35. **de Vries, W., W. M. Wijck-Kapteijn, and A. H. Stouthamer.** 1972. Influence of oxygen on growth, cytochrome synthesis and fermentation pattern in propionic acid bacteria. J Gen Microbiol **71:**515-24.
36. **Delwiche, E. A., and S. F. Carson.** 1953. A citric acid cycle in *Propionibacterium pentosaceum*. J Bacteriol **65:**318-21.
37. **Deplancke, B., K. R. Hristova, H. A. Oakley, V. J. McCracken, R. Aminov, R. I. Mackie, and H. R. Gaskins.** 2000. Molecular ecological analysis of the succession and diversity of sulfate-reducing bacteria in the mouse gastrointestinal tract. Appl Environ Microbiol **66:**2166-74.
38. **Devereux, R., M. Delaney, F. Widdel, and D. A. Stahl.** 1989. Natural relationships among sulfate-reducing eubacteria. J Bacteriol **171:**6689-95.
39. **Dilling, W., and H. Cypionka.** 1990. Aerobic respiration in sulfate-reducing bacteria. FEMS Microbiol Lett **71:**123-128.
40. **Doukov, T. I., T. M. Iverson, J. Seravalli, S. W. Ragsdale, and C. L. Drennan.** 2002. A Ni-Fe-Cu center in a bifunctional carbon monoxide dehydrogenase/acetyl-CoA synthase. Science **298:**567-72.
41. **Drake, H. L., S. L. Daniel, K. Kusel, C. Matthies, C. Kuhner, and S. Braus-Stromeyer.** 1997. Acetogenic bacteria: what are the in situ consequences of their diverse metabolic versatilities? Biofactors **6:**13-24.
42. **Drake, H. L., K. Kusel, and C. Matthies.** 2002. Ecological consequences of the phylogenetic and physiological diversities of acetogens. Antonie Van Leeuwenhoek **81:**203-13.
43. **Duwat, P., S. Sourice, B. Cesselin, G. Lamberet, K. Vido, P. Gaudu, Y. Le Loir, F. Violet, P. Loubiere, and A. Gruss.** 2001. Respiration capacity of the fermenting bacterium *Lactococcus lactis* and its positive effects on growth and survival. J Bacteriol **183:**4509-16.
44. **Emde, R., and B. Schink.** 1990. Oxidation of glycerol, lactate, and propionate by *Propionibacterium freudenreichii* in a poised-potential amperometric culture system. Arch. Microbiol. **153:**506-512.

45. **Fareleira, P., B. S. Santos, C. Antonio, P. Moradas-Ferreira, J. LeGall, A. V. Xavier, and H. Santos.** 2003. Response of a strict anaerobe to oxygen: survival strategies in *Desulfovibrio gigas*. Microbiology **149:**1513-22.

46. **Fitz, R. M., and H. Cypionka.** 1990. Generation of a proton gadient in *Desulfovibrio vulgaris*. Arch Microbiol **155:**444-448.

47. **Fournier, M., C. Aubert, Z. Dermoun, M. C. Durand, D. Moinier, and A. Dolla.** 2006. Response of the anaerobe *Desulfovibrio vulgaris Hildenborough* to oxidative conditions: proteome and transcript analysis. Biochimie **88:**85-94.

48. **Frostl, J. M., C. Seifritz, and H. L. Drake.** 1996. Effect of nitrate on the autotrophic metabolism of the acetogens *Clostridium thermoautotrophicum* and *Clostridium thermoaceticum*. J Bacteriol **178:**4597-603.

49. **Fuchs, G.** 1986. CO_2 fixation in acetogenic bacteria: variations on a theme. FEMS **39:**181-213.

50. **Gaudu, P., G. Lamberet, S. Poncet, and A. Gruss.** 2003. CcpA regulation of aerobic and respiration growth in *Lactococcus lactis*. Mol Microbiol **50:**183-92.

51. **Gaudu, P., K. Vido, B. Cesselin, S. Kulakauskas, J. Tremblay, L. Rezaiki, G. Lamberret, S. Sourice, P. Duwat, and A. Gruss.** 2002. Respiration capacity and consequences in *Lactococcus lactis*. Antonie Van Leeuwenhoek **82:**263-9.

52. **Gent-Ruijters, M. L., F. A. Meijere, W. Vries, and A. H. Stouthamer.** 1976. Lactate metabolism in *Propionibacterium pentosaceum* growing with nitrate or oxygen as hydrogen acceptor. Antonie Van Leeuwenhoek **42:**217-28.

53. **Goldman, B. S., K. K. Gabbert, and R. G. Kranz.** 1996. The temperature-sensitive growth and survival phenotypes of *Escherichia coli cydDC* and *cydAB* strains are due to deficiencies in cytochrome *bd* and are corrected by exogenous catalase and reducing agents. J Bacteriol **178:**6348-51.

54. **Greene, E. A., C. Hubert, M. Nemati, G. E. Jenneman, and G. Voordouw.** 2003. Nitrite reductase activity of sulphate-reducing bacteria prevents their inhibition by nitrate-reducing, sulphide-oxidizing bacteria. Environ Microbiol **5:**607-17.

55. **Gribbon, E. M., J. G. Shoesmith, W. J. Cunliffe, and K. T. Holland.** 1994. The microaerophily and photosensitivity of *Propionibacterium acnes*. J Appl Bacteriol. **77:**583-590.

56. **Grossman, J. P., and J. R. Postgate.** 1953. Cultivation of sulphate-reducing bacteria. Nature **171:**600-2.

57. **Hansen, T. A. (ed.).** 1993. Carbon metabolism of sulfate reducing bacteria. Springer-Verlag, New York.

58. **Hansen, T. A.** 1994. Metabolism of sulfate-reducing prokaryotes. Antonie Van Leeuwenhoek **66:**165-85.

59. **Haveman, S. A., E. A. Greene, C. P. Stilwell, J. K. Voordouw, and G. Voordouw.** 2004. Physiological and gene expression analysis of inhibition of *Desulfovibrio vulgaris hildenborough* by nitrite. J Bacteriol **186:**7944-50.

60. **He, Q., K. H. Huang, Z. He, E. J. Alm, M. W. Fields, T. C. Hazen, A. P. Arkin, J. D. Wall, and J. Zhou.** 2006. Energetic consequences of nitrite stress in *Desulfovibrio vulgaris Hildenborough*, inferred from global transcriptional analysis. Appl Environ Microbiol **72:**4370-81.

61. **Hedderich, R., and L. Forzi.** 2005. Energy-converting [NiFe] hydrogenases: more than just H_2 activation. J Mol Microbiol Biotechnol **10:**92-104.

62. **Heidelberg, J. F., R. Seshadri, S. A. Haveman, C. L. Hemme, I. T. Paulsen, J. F. Kolonay, J. A. Eisen, N. Ward, B. Methe, L. M. Brinkac, S. C. Daugherty, R. T. Deboy, R. J. Dodson, A. S. Durkin, R. Madupu, W. C. Nelson, S. A. Sullivan, D. Fouts, D. H. Haft, J. Selengut, J. D. Peterson, T. M. Davidsen, N. Zafar, L. Zhou, D. Radune, G. Dimitrov, M. Hance, K. Tran, H. Khouri, J. Gill, T. R. Utterback, T. V. Feldblyum, J. D. Wall, G. Voordouw, and C. M. Fraser.** 2004. The genome sequence of the anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris Hildenborough*. Nat Biotechnol **22:**554-9.

63. **Hensgens, C. M., J. Vonck, J. Van Beeumen, E. F. van Bruggen, and T. A. Hansen.** 1993. Purification and characterization of an oxygen-labile, NAD-dependent alcohol dehydrogenase from *Desulfovibrio gigas*. J Bacteriol **175:**2859-63.

64. **Hill, J. J., J. O. Alben, and R. B. Gennis.** 1993. Spectroscopic evidence for a heme-heme binuclear center in the cytochrome *bd* ubiquinol oxidase from *Escherichia coli*. Proc Natl Acad Sci U S A **90:**5863-7.

65. **Hojo, K., R. Watanabe, T. Mori, and N. Taketomo.** 2007. Quantitative measurement of tetrahydromenaquinone-9 in cheese fermented by propionibacteria. J Dairy Sci **90:**4078-83.

66. **Howard, B. H., and R. E. Hungate.** 1976. *Desulfovibrio* of the sheep rumen. Appl Environ Microbiol **32:**598-602.

67. **Hugenholtz, J., D. M. Ivey, and L. G. Ljungdahl.** 1987. Carbon monoxide-driven electron transport in *Clostridium thermoautotrophicum* membranes. J Bacteriol **169:**5845-7.

68. **Hugenholtz, J., and L. G. Ljungdahl.** 1989. Electron transport and electrochemical proton gradient in membrane vesicles of *Clostridium thermoautotrophicum*. J Bacteriol **171:**2873-5.

69. **Hugenholtz, J., L. Perdon, and T. Abee.** 1993. Growth and energy generation by *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* during citrate metabolism. Appl Environ Microbiol **59:**4216-4222.

70. **Iverson, T. M., C. Luna-Chavez, G. Cecchini, and D. C. Rees.** 1999. Structure of the *Escherichia coli* fumarate reductase respiratory complex. Science **284:**1961-6.

71. **Jones, H. E.** 1971. A re-examination of Desulfovibrio africanus. Arch Mikrobiol **80:**78-86.

72. **Junemann, S.** 1997. Cytochrome *bd* terminal oxidase. Biochim Biophys Acta **1321:**107-27.

73. **Kamlage, B., A. Boelter, and M. Blaut.** 1993. Spectroscopic and potentiometric characterization of cytochromes in two *Sporomusa* species and their expression during growth on selected substrated. Arch Microbiol **159:**189-196.

74. **Kandler, O.** 1983. Carbohydrate metabolism in lactic acid bacteria. Antonie Van Leeuwenhoek **49:**209-24.

75. **Karnholz, A., K. Kusel, A. Gossner, A. Schramm, and H. L. Drake.** 2002. Tolerance and metabolic response of acetogenic bacteria toward oxygen. Appl Environ Microbiol **68:**1005-9.

76. **Kaspar, H. F.** 1982. Nitrite Reduction to Nitrous Oxide by Propionibacteria: Detoxication Mechanism. Arch Microbiol **133:**126-130.

77. **Keith, S. M., and R. A. Herbert.** 1983. Dissimilatory nitrate reduction by a strain of *Desulfovibrio desulfuricans*. FEMS Microbiol Lett **18:**55-59.

78. **Koebmann, B. J., L. M. Blank, C. Solem, D. Petranovic, L. K. Nielsen, and P. R. Jensen.** 2007. Increased biomass yield of *Lactococcus lactis* during energetically limited growth and respiratory conditions. Biotechnol Appl Biochem.

79. **Lan, A., A. Bruneau, M. Bensaada, C. Philippe, P. Bellaud, S. Rabot, and G. Jan.** 2008. Increased induction of apoptosis by *Propionibacterium freudenreichii* TL133 in colonic mucosal crypts of human microbiota-associated rats treated with 1,2-dimethylhydrazine. Br J Nutr**:**1-9.

80. **Lan, A., D. Lagadic-Gossmann, C. Lemaire, C. Brenner, and G. Jan.** 2007. Acidic extracellular pH shifts colorectal cancer cell death from apoptosis to necrosis upon exposure to propionate and acetate, major end-products of the human probiotic propionibacteria. Apoptosis **12:**573-91.

81. **Lemos, R. S., C. M. Gomes, M. Santana, J. LeGall, A. V. Xavier, and M. Teixeira.** 2001. The 'strict' anaerobe *Desulfovibrio gigas* contains a membrane-bound oxygen-reducing respiratory chain. FEBS Lett **496:**40-3.

82. **Lens, P. N. L., A. Visser, A. J. H. Janssen, L. W. Hulshoff Pol, and G. Lettinga.** 1998. Biotechnological treatment of sulfate-rich wastewaters. Crit Rev Env Sci Tec **28:**41-88.

83. **Leroy, F., and L. De Vuyst.** 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry Trends Food Sci Technol **15:**67-78.

84. **Liu, M. C., and H. D. Peck, Jr.** 1981. The isolation of a hexaheme cytochrome from *Desulfovibrio desulfuricans* and its identification as a new type of nitrite reductase. J Biol Chem **256:**13159-64.

85. **Ljungdahl, L. G.** 1986. The autotrophic pathway of acetate synthesis in acetogenic bacteria. Annu Rev Microbiol **40:**415-50.

86. **Lynch, A. S., and E. C. Lin.** 1996. Transcriptional control mediated by the ArcA two-component response regulator protein of *Escherichia coli*: characterization of DNA binding at target promoters. J Bacteriol **178:**6238-49.

87. **Mantere-Alhonen, S.** 1995. Propionibacteria used as probiotics - A review. Lait **75:**447-452.

88. **McCready, R. G. L., W. D. Gould, and F. D. Cook.** 1983. Respiratory nitrate reduction by *Desulfovibrio* sp. . Arch Microbiol **135:**182-185.

89. **McInerney, M. J., and M. P. Bryant.** 1981. Anaerobic Degradation of Lactate by Syntrophic Associations of *Methanosarcina barkeri* and *Desulfovibrio Species* and Effect of H_2 on Acetate Degradation. Appl Environ Microbiol **41:**346-354.

90. **Menon, S., and S. W. Ragsdale.** 1996. Unleashing hydrogenase activity in carbon monoxide dehydrogenase/acetyl-CoA synthase and pyruvate:ferredoxin oxidoreductase. Biochemistry **35:**15814-21.

91. **Miller, M. J., and R. B. Gennis.** 1985. The cytochrome *d* complex is a coupling site in the aerobic respiratory chain of *Escherichia coli* J. Biol. Chem. **260:**14003-14008.

92. **Mitchell, G. J., J. G. Jones, and J. A. Cole.** 1986. Distribution and regulation of nitrate and nitrite reduction by *Desulfovibrio* and *Desulfotomaculum* species Arch Microbiol **144:**35-40.

93. **Moura, I., S. Bursakov, C. Costa, and J. J. Moura.** 1997. Nitrate and nitrite utilization in sulfate-reducing bacteria. Anaerobe **3:**279-90.

94. **Muller, V.** 2003. Energy conservation in acetogenic bacteria. Appl Environ Microbiol **69:**6345-53.

95. **Naidu, A. S., W. R. Bidlack, and R. A. Clemens.** 1999. Probiotic spectra of lactic acid bacteria (LAB). Crit Rev Food Sci Nutr **39:**13-126.

96. **Nakano, M. M., and P. Zuber.** 1998. Anaerobic growth of a "strict aerobe" (*Bacillus subtilis*). Annu Rev Microbiol **52:**165-90.

97. **Nakano, M. M., and P. Zuber.** 2004. Strict and Facultative Anaerobes. Medical and Environmental Aspects. Horizon Bioscience, Beaverton, USA.

98. **Odom, J. M., and H. D. Peck, Jr.** 1984. Hydrogenase, electron-transfer proteins, and energy coupling in the sulfate-reducing bacteria *Desulfovibrio*. Annu Rev Microbiol **38:**551-92.

99. **Odom, J. M., and H. D. Peck, Jr.** 1981. Localization of dehydrogenases, reductases, and electron transfer components in the sulfate-reducing bacterium *Desulfovibrio gigas*. J Bacteriol **147:**161-9.

100. **Osborne, J. P., and R. B. Gennis.** 1999. Sequence analysis of cytochrome *bd* oxidase suggests a revised topology for subunit I. Biochim Biophys Acta **1410:**32-50.

101. **Pankhania, I. P., W. Spormann, W. A. Hamilton, and R. K. Thauer.** 1988. Lactate conversion to acetate, CO_2 and H_2 in cell suspensions of *Desulfovibrio vulgaris* (Marburg): indications for the involvement of an energy driven reaction. Arch Microbiol **150:**26-31.

102. **Partridge, J. D., G. Sanguinetti, D. P. Dibden, R. E. Roberts, R. K. Poole, and J. Green.** 2007. Transition of *Escherichia coli* from aerobic to micro-aerobic conditions involves fast and slow reacting regulatory components. J Biol Chem **282:**11230-7.

103. **Pérez Mendoza, J. L.** 1983. Fermentation of a waste product from the industrial processing of the lime for vitamin B_{12} production. Biotechno. Lett. **5:**259-264.

104. **Peterson, J., C. Vibat, and R. B. Gennis.** 1994. Identification of the axial heme ligands of cytochrome b_{556} in succinate: ubiquinone oxidoreductase from *Escherichia coli*. FEBS Lett **355:**155-6.

105. **Piveteau, P.** 1999. Metabolism of lactate and sugars by dairy propionibacteria: A review. Lait **79:**23-41.

106. **Poole, R. K.** 1994. Oxygen reactions with bacterial oxidases and globins: binding, reduction and regulation. Antonie Van Leeuwenhoek **65:**289-310.

107. **Poole, R. K., and G. M. Cook.** 2000. Redundancy of aerobic respiratory chains in bacteria? Routes, reasons and regulation. Adv Microb Physiol **43:**165-224.

108. **Poole, R. K., and S. Hill.** 1997. Respiratory protection of nitrogenase activity in *Azotobacter vinelandii*--roles of the terminal oxidases. Biosci Rep **17:**303-17.

109. **Postgate, J. R.** 1984. The sulphate-reducing bacteria, Second edition ed. Cambridge university press.

110. **Prins, R. A., and A. Lankhorst.** 1977. Synthesis of acetate from CO_2 in the cecum of some rodents. FEMS Microbiol. Lett. **1:**255-258.

111. **Pritchard, G. G., and R. V. Asmundson.** 1980. Aerobic electron transport in *Propionibacterium shermanii*. Effects of cyanide. Arch Microbiol **126:**167-73.

112. **Pritchard, G. G., and J. W. Wimpenny.** 1978. Cytochrome formation, oxygen-induced proton extrusion and respiratory activity in *Streptococcus faecalis* var. *zymogenes* grown in the presence of haematin. J Gen Microbiol **104:**15-22.

113. **Pritchard, G. G., J. W. Wimpenny, H. A. Morris, M. W. Lewis, and D. E. Hughes.** 1977. Effects of oxygen on *Propionibacterium shermanii* grown in continuous culture. J Gen Microbiol **102:**223-33.

114. **Puustinen, A., M. Finel, T. Haltia, R. B. Gennis, and M. Wikstrom.** 1991. Properties of the two terminal oxidases of *Escherichia coli*. Biochemistry **30:**3936-42.

115. **Ragsdale, S. W., J. E. Clark, L. G. Ljungdahl, L. L. Lundie, and H. L. Drake.** 1983. Properties of purified carbon monoxide dehydrogenase from *Clostridium thermoaceticum*, a nickel, iron-sulfur protein. J Biol Chem **258:**2364-9.

116. **Rezaiki, L., B. Cesselin, Y. Yamamoto, K. Vido, E. van West, P. Gaudu, and A. Gruss.** 2004. Respiration metabolism reduces oxidative and acid stress to improve long-term survival of *Lactococcus lactis*. Mol Microbiol **53:**1331-42.

117. **Rezaiki, L., G. Lamberet, A. Derre, A. Gruss, and P. Gaudu.** 2008. *Lactococcus lactis* produces short-chain quinones that cross-feed Group B *Streptococcus* to activate respiration growth. Mol Microbiol **67:**947-57.

118. **Ritchey, T. W., and H. W. Seely, Jr.** 1976. Distribution of cytochrome-like respiration in streptococci. J Gen Microbiol **93:**195-203.

119. **Rolfe, R. D.** 2000. The role of probiotic cultures in the control of gastrointestinal health. J Nutr **130:**396S-402S.

120. **Rosen, T.** 2007. The *Propionibacterium acnes* genome: from the laboratory to the clinic. J Drugs Dermatol **6:**582-6.

121. **Sakaguchi, T., A. Arakaki, and T. Matsunaga.** 2002. *Desulfovibrio magneticus* sp. nov., a novel sulfate-reducing bacterium that produces intracellular single-domain-sized magnetite particles. Int J Syst Evol Microbiol **52:**215-21.

122. **Schink. B.** 1988. Konservierung kleiner Energiebeiträge bei gärenden Bakterien. In Präve P, SchlingmannM, Crueger W, Esser K, Thauer R, Wagner F (eds) Jahrbuch Biotechnologie, vol. 2. Hanser, München Wien.

123. **Schlegel, H. G.** 1993. General Microbiology, 7th ed. University Press, Cambridge.

124. **Schwartz, A. C., and J. Sporkenbach.** 1975. The electron transport system of the anaerobic *Propionibacterium shermanii*: cytochrome and inhibitor studies. Arch Microbiol **102:**261-73.

125. **Seeligera, S., P. H. Janssen, and B. Schink.** 2002. Energetics and kinetics of lactate fermentation to acetate and propionate via methylmalonyl-CoA or acrylyl-CoA. FEMS Microbiol Lett **211:**65-70.

126. **Seifritz, C., H. L. Drake, and S. L. Daniel.** 2003. Nitrite as an energy-conserving electron sink for the acetogenic bacterium *Moorella thermoacetica*. Curr Microbiol **46:**329-33.

127. **Senior, A. E.** 1990. The proton-translocating ATPase of *Escherichia coli*. Annu Rev Biophys Biophys Chem **19:**7-41.

128. **Sidorchuk, II, and V. M. Bondarenko.** 1984. Selection of a biologically active mutant of *Propionibacterium shermanii* and the possibility of its use in complex therapy of enteral dysbacteriosis. J Hyg Epidemiol Microbiol Immunol **28:**331-8.

129. **Sijpesteijn, A. K.** 1970. Induction of cytochrome formation and stimulation of oxidative dissimilation by hemin in *Streptococcus lactis* and *Leuconostoc mesenteroides*. Antonie Van Leeuwenhoek **36:**335-48.

130. **Skulachev, V. P.** 1994. Decrease in the intracellular concentration of O_2 as a special function of the cellular respiratory system. Biokhimiia **59:**1910-2.

131. **Sone, N.** 1974. Isolation of a novel menaquinone with partly hydrogenated side chain from *Propionibacterium arabinosum*. J. Biochem. **76:**133-136.

132. **Sone, N.** 1974. The redox reactions in propionic acid fermentation. IV. Participation of menaquinone in the electron transfer system in *Propionibaterium arabinosum*. J. Biochem. **76:**137-145.

133. **Stams, A. J. M., and T. A. Hansen.** 1982. Oxygen-labile L(+) dehydrogenase activity in *Desulfovibrio desulfuricans*. FEMS Microbiol Lett **13:**389-394.

134. **Stams, A. J. M., D. R. Kremer, K. Nicolay, G. H. Weenk, and T. A. Hansen.** 1984. Pathway of propionate formation in *Desulfobulbus propionicus*. Arch Microbiol **139:**167-173.

135. **Steenkamp, D. J., and H. D. Peck, Jr.** 1981. Proton translocation associated with nitrite respiration in *Desulfovibrio desulfuricans*. J Biol Chem **256:**5450-8.

136. **Stermann, M., L. Sedlacek, S. Maass, and F. C. Bange.** 2004. A promoter mutation causes differential nitrate reductase activity of *Mycobacterium tuberculosis* and *Mycobacterium bovis*. J Bacteriol **186:**2856-61.

137. **Stiles, M. E.** 1996. Biopreservation by lactic acid bacteria. Antonie Van Leeuwenhoek **70:**331-45.

138. **Stiles, M. E., and W. H. Holzapfel.** 1997. Lactic acid bacteria of foods and their current taxonomy. Int J Food Microbiol **36:**1-29.

139. **Teusink, B., A. Wiersma, D. Molenaar, C. Francke, W. M. de Vos, R. J. Siezen, and E. J. Smid.** 2006. Analysis of growth of *Lactobacillus plantarum* WCFS1 on a complex medium using a genome-scale metabolic model. J Biol Chem **281:**40041-8.

140. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev **41:**100-80.

141. **Tseng, C. P., J. Albrecht, and R. P. Gunsalus.** 1996. Effect of microaerophilic cell growth conditions on expression of the aerobic (*cyoABCDE* and *cydAB*) and anaerobic (*narGHJI*, *frdABCD*, and *dmsABC*) respiratory pathway genes in *Escherichia coli*. J Bacteriol **178:**1094-8.

142. **van de Guchte, M., S. Penaud, C. Grimaldi, V. Barbe, K. Bryson, P. Nicolas, C. Robert, S. Oztas, S. Mangenot, A. Couloux, V. Loux, R. Dervyn, R. Bossy, A. Bolotin, J. M. Batto, T. Walunas, J. F. Gibrat, P. Bessieres, J. Weissenbach, S. D. Ehrlich, and E. Maguin.** 2006. The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. Proc Natl Acad Sci U S A **103:**9274-9.

143. **Van Gent-Ruijters, M. L., W. DeVries, and A. H. Southamer.** 1975. Influence of nitrate on fermentation pattern, molar growth yields and synthesis of cytochrome *b* in *Propionibacterium pentosaceum*. J Gen Microbiol **88:**36-48.

144. **Voordouw, G.** 1995. The Genus Desulfovibrio: The Centennial. Appl Environ Microbiol **61:**2813-2819.

145. **Voordouw, G., V. Niviere, F. G. Ferris, P. M. Fedorak, and D. W. Westlake.** 1990. Distribution of hydrogenase genes in *Desulfovibrio* spp. and their use in identification of species from the oil field environment. Appl Environ Microbiol **56:**3748-3754.

146. **Winstedt, L., L. Frankenberg, L. Hederstedt, and C. von Wachenfeldt.** 2000. *Enterococcus faecalis* V583 contains a cytochrome *bd*-type respiratory oxidase. J Bacteriol **182:**3863-6.

147. **Winstedt, L., and C. von Wachenfeldt.** 2000. Terminal oxidases of *Bacillus subtilis* strain 168: one quinol oxidase, cytochrome aa_3 or cytochrome *bd*, is required for aerobic growth. J Bacteriol **182:**6557-64.

148. **Wolin, M. J., and T. L. Miller.** 1994. Acetogenesis from CO_2 in the human colonic ecosystem. Chapman Hall, New York.

149. **Yamamoto, Y., C. Poyart, P. Trieu-Cuot, G. Lamberet, A. Gruss, and P. Gaudu.** 2005. Respiration metabolism of Group B *Streptococcus* is activated by environmental haem and quinone and contributes to virulence. Mol Microbiol **56:**525-34.

150. **Yamamoto, Y., C. Poyart, P. Trieu-Cuot, G. Lamberet, A. Gruss, and P. Gaudu.** 2006. Roles of environmental heme, and menaquinone, in *Streptococcus agalactiae*. Biometals **19:**205-10.

151. **Zumft, W. G.** 1997. Cell biology and molecular basis of denitrification. Microbiol Mol Biol Rev **61:**533-616.

152. **Zuo, R.** 2007. Biofilms: strategies for metal corrosion inhibition employing microorganisms. Appl Microbiol Biotechnol **76:**1245-53.

Chapter 8

General discussion and future perspectives

Introduction

Lactic acid bacteria are best known for their ability to ferment a wide range of foods from raw materials, such as milk, meat and plant-materials. These fermented products have improved shelf-life with altered organoleptic profiles and nutritional properties (17). Lactic acid bacteria cannot make complete respiratory chains, as indicated by the absence of cytochromes, when grown in standard laboratory media or milk. In fact heme an essential co-factor of cytochromes cannot be produced by this group of bacteria. These bacteria even lack a complete citric acid cycle, that is found in most aerobically respiring bacteria and which allows a complete breakdown of carbon sources, such as glucose, to CO_2 and H_2O. Therefore lactic acid bacteria produce mostly incompletely oxidized metabolites such as acids (lactic acid). Lactic acid bacteria are in general classified as (obligate) fermentative, non-respiring bacteria.

The conversion of glucose to incompletely oxidized substrates, which are still rich in high-energy bonds, is inefficient but fast. Thus (homo-fermentative) lactic acid production may result in out-competition of other microorganisms by out-growing them and by acidification of the environment. Indeed the metabolic model of *Lactobacillus plantarum* shows that this bacterium does not optimize pyruvate metabolism for maximal ATP production (31). Lactic acid in fact is the major acid produced by most lactic acid bacteria, in preference of acetic acid (and ethanol co-) production that potentially yields more substrate-level ATP (13).

Despite their specialized fermentative strategy, as mentioned in **Chapter 1**, several observations were made of respiratory-like behavior, in *Lactococcus lactis* and *Enterococcus faecalis* in particular, when these bacteria are supplied with a heme source.

The general idea was put forward that heme-grown *Lactococcus lactis* cells were actively respiring, and were able to exploit oxygen as electron sink and conserve additional energy in the form of proton motive force. The genomes of many lactic acid bacteria do in fact contain *cydABCD* genes that encode a heme-requiring cytochrome. Respiration would explain a number of the observed phenotypes. For example, respiration that consumes oxygen can maintain low intracellular oxygen levels and can, thus, provide improved tolerance to (high) levels of environmental oxygen.

PMF generation by the *Lactococcus lactis* ETC

The link between respiration and higher biomass production is more complex, since respiration influences fermentation patterns. Biomass increases therefore could be a consequence of increased substrate level ATP yields as well as oxidative phosphorylation ATP yields.

Since *Lactococcus lactis* has no complete citric acid cycle, a shift in redox balance, by oxidation of NADH, will lead to a shift from homo-lactic to mixed-acid fermentation (and more acetate) (5). The production of acetate itself leads to more ATP generation via substrate level phosporylation. Energy conservation by an electron transport chain does not have to be involved to explain increases in biomass formation. Furthermore, when acetate is produced in place of lactate, the culture will acidify more slowly, because of the higher pK value of acetic acid. This allows for more complete sugar utilization, reduced acid stress and reduced maintenance energy (ATP) demands. The observation of higher biomass production by heme-grown *Lactococcus lactis* cells may thus well be explained solely by the change in acidification profile.

Thus to prove that *Lactococcus lactis* cells are truly respiring, proton motive force generation by the electron transport chain must be shown, since measuring biomass increases will not be conclusive. The main mechanism by which a proton motive force is generated in (fermenting) lactic acid bacteria is by F_1F_0 ATPase activity at the expense of ATP. Therefore, contrary to most other bacteria (and eukaryotic mitochondria) the lactic acid F_1F_0 ATPase operates in reverse (15). The F_1F_0 ATPase activity has to be inhibited so that the proton motive force generating capability of the ETC can be measured.

In **Chapter 2** of this thesis we demonstrated for the first time that the aerobic electron transport chain of *Lactococcus lactis* can generate a proton motive force (2). The critical observation was the ability of heme-grown cells to generate proton motive force even when their ATPase is inhibited by DCCD. (DCCD is generally used to specifically inhibit ATPase activity in a variety of cell-types.) The insensitivity of heme-grown cells to DCCD-treatment was lost in the *cydA* mutant that is unable to form one of the structural proteins of the *bd*-type cytochrome. We have shown that the electron transport chain is indeed membrane-located in *Lactococcus lactis* by measuring NADH-induced oxygen consumption

in membrane vesicles. Membranes-vesicles that were derived from heme-grown wild-type cells had a dramatically increased oxygen consumption-rate.

Menaquinones are essential for the heme-induced phenotype

Membrane-integral electron carriers are essential components of electron transport chains. There is evidence that menaquinones are involved in the electron transport chains of lactic acid bacteria (10, 12, 26). Many *Lactobacillus* sp. do not produce menaquinones, which can also be deduced from the available complete genome sequences. The respiratory phenotype (increased biomass production) was induced in several *Lactobacillus* species, by a combination of heme and menaquinone (vitamin K_2) supplementation (**Chapter 3**). For *Lactococcus lactis* the menaquinone-requirement for respiration could also be shown. In *Lactooccus lactis* B1157 (**Chapter 3**) mutants in menaquinone biosynthesis genes were respiration-negative.

Many strains of *Lactococcus lactis* produce menaquinones, even when grown anaerobically and no heme has been added (21). We grew *Lactococcus lactis* aerobically and anaerobically with and without heme supplementation, to see which conditions would induce the highest levels of menaquinones. Surprisingly in anaerobic conditions cells produced most menaquinones (**Chapter 3**). Although, the absolute menaquinone concentration was lower, there was a shift towards species with longer tail-lengths in aerated cultures. Short-chain menaquinone species (MK-3, but not MK8-10) acted as respiratory quinones in *Streptococcus agalactiae*. This is in accordance with the ability of vitamin K_2 (MK-4) to function as electron carrier in the aerobic electron transport chain of *Lactobacillus plantarum*. Menaquinones were also shown to be involved in reduction of Cu^{2+} and Fe^{3+}. The metal reduction may facilitate metal assimilation and favorably affect redox-balance (26). The stimulation of growth of *Lactococcus lactis* by copper addition to the medium was in fact published (14). The function of long-tailed menaquinone species remains to be established. Their specific production in aerobic conditions suggests involvement in aerobic metabolism. The fact the long-tailed menaquinone species could not stimulate respiration in *Streptococcus agalactiae* may reflect that they are not taken up as readily as short-chained species. Still in the *Lactococcus lactis* ETC they may have an important function, and are perhaps less easily lost to other bacteria (26). Lactic acid bacteria form a choice group of

bacteria to study the roles of menaquinones in the respiratory system, since they can be easily manipulated without inducing severe growth defects.

High-throughput screening of *Lactococcus lactis* respiration mutants

Chapter 3 describes the aerobic screening of 8000 random insertion mutants of *Lactoccocus lactis* B1157, to find those mutants that are not growth stimulated by heme addition. We developed a 96-wells plate screening method using breath seals and incubation at a 1000rpm rotation, to ensure adequate aeration. Of the 8000 mutants that were screened 1% (~70) were respiration-negative mutants, of which 13 were further analyzed. 5 of these had insertions in genetic regions containing genes involved in menaquinone-biosynthesis and 3 in regions containing the *cyd*-genes. A respiratory negative mutant with an insertion in the *noxA* gene region was also found. The high number of verifiable respiratory mutants validates the high throughput screening method. Thus, screening collections of lactic acid bacteria to select heme- (and menaquinone-) induced respirators is possible. The increased biomass production and robustness that are associated with the respiratory-like phenotype (as observed for *Lactococcus lactis* and *Lactobacillus plantarum*) are certainly industrially desirable traits.

NADH-dehydrogenases involved in the aerobic ETC

The *noxA*-gene neighbours *noxB* on the genome of *Lactococcus lactis* MG1363 and, possibly these genes, together, encode the NADH-dehydrogenase. A model for the *Lactococcus lactis* ETC takes shape that consists of at least three components (Fig 1).

A type II NADH dehydrogenase (DH), encoded by *NoxA(B)* transfers electrons from NADH to the menaquinol poo*Lactobacillus* The menaquinone pool in turn can donate electrons to the *bd*-type cytochrome (*bd*-cyt) or to metal-ions such as Cu2+ and Fe3+ (ferric iron). It is not clear if intermediate proteins are involved in the metal reduction by the quinone pool or if this is a direct transfer of electrons. Since the type-II NADH-dehydrogenase does not translocate protons the cytochrome is the only component that generates PMF. The overall PMF generating efficiency therefore is $2H^+$/NADH. Interestingly a knock-out of a single NADH-dehydrogenase of *Lactobacillus plantarum* WCFS1 (Δ*ndh1*) also makes these cells unresponsive to heme (and menaquinone) addition (**Chapter 4**).

A NADH-dehydrogenase, a menaquinone pool that can be supplemented and a heme-dependent *bd*-type cytochrome form a simple, non-redundant chain. This chain composition is not very efficient in the generation of proton motive force. The type of NADH-dehydrogenase (type-II) involved does not translocate protons. Furthermore the *bd*-type cytochrome itself does not actually "pump" protons but operates by scalar chemistry at an efficiency of $1H^+$/e- (or $2H^+$/NADH).

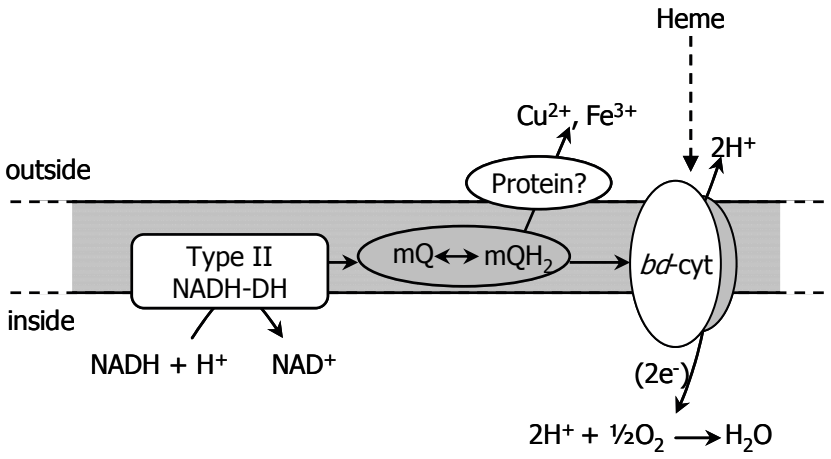


Figure 1. Proposed aerobic electron transport chain of *Lactococcus lactis*. The shaded bar represent the lipid bi-layer of the cell membrane. An extracellular heme-source must be supplied (dashed arrow) to activate the cytochrome.

The simple electron transport chain may lend itself for transfer to other (non-respiring) lactic acid bacteria. In fact we have already cloned a functional *cyd*-operon on a plasmid and used it to complement a *Lactococcus lactis cydA* mutant (**Chapter 2**). As shown by *Lactobacillus plantarum*, menaquinones, in the form of vitamin K_2, can be taken up from the extracellular environment. Heme- and menaquinone induced respiration can be transferred to other (lactic acid) bacteria by expressing the *cyd*-genes and the *ndh1*-gene (or *noxAB* genes) on a transferable plasmid. As respiration leads to higher biomass production and increased robustness, achieving this in a food-grade way would, be especially interesting for many industrial applications. Respiratory growth is for example already used for the industrial-scale production of starter cultures of *Lactococcus lactis*. Furthermore improved robustness probably will result in higher survival rates of probiotic bacteria through the gastro-intestinal tract and thus possibly enhance the probiotic effects.

Distribution of *cyd*-genes and respiratory-like phenotypes in lactic acid bacteria

Heme-induced respiration has been observed, or suggested for, several lactic acid bacteria. *Leuconostoc mesenteroides,* several *Streptococcus* sp. and *Enterococcus faecalis* form cytochromes in the presence of heme, while extensive phenotypic characterization is available only for *Lactococcus lactis* MG1363. As mentioned above and in **Chapter 4**, *Lactobacillus plantarum* requires both heme and menaquinone supplementation, and this was also observed for *Streptococcus agalactiae* strains (37). We have grown 20 species of LAB aerobically with heme and discovered several additional species of respiring lactic acid bacteria (**Chapter 3**). *Enterococcus casseliflavus, Streptococcus uberis*, *Lactobacillus paralimentarius* and *Carnobacterium divergens* almost doubled their final biomass. In the case of *Steroptococcis uberis* and *Carnobacterium divergens* aerobic growth without heme supplementation was very poor.

In a combined heme and menaquinone supplemented aerobic screening, several *Lactobacillus* sp. (*brevis*, *garviae* and *rhamnosus*) responded in the form of higher biomass production without further acidification (**Chapter 3**). The screening of roughly 20 species revealed only 8 new potential respirators. Although cofactor-induced respiratory-like behavior is not a general trait for lactic acid bacteria, screening of multiple strains of *Lactococus lactis* and *Lactobacillus plantarum* demonstrates that respiration, at least for these two species, can be considered characteristic. 84 out of 88 *Lactococcus lactis* strains and 16 out of 20 *Lactobacillus plantarum* strains were heme (and menaquinone) stimulated.

The observation that respiration may be specific for particular species is confirmed by the distribution of *cydABCD* genes among the sequenced genomes. The *cyd*-genes are uniformly present or absent in all strains of a species (either all strains that belong to the same species have them or all strains do not). The consistency of the *cyd*-gene presence thus correlates well with the consistency of the heme (and menaquinone) induced response of the *Lactobacillus plantarum* and *Lactococcus lactis* strains.

When we assayed the available genomes for the presence of the *cyd*-genes they are found among the whole diversity-range of LAB (*Lactobacillus* sp., *Streptococcus* sp., *Enterococcus* sp., *Lactococcus* sp. and *Leuconostoc* sp.), see also figure 1 of the introduction (**Chapter 1**). Furthermore the *cyd*-genes are uniformly present or absent in all strains that belong to the same species. For example in the genus *Streptococci cyd*-genes are found in all

8 strains of *Streptoccocus agalactiae*, but not in any of the 11 strains of *Streptococcus pneumoniae* or the 13 strains of *Streptococcus pyogenes*. This suggests ancient origins of the *cyd*-genes in the group of lactic acid bacteria, or many horizontal gene transfers which is less probable (**Chapter 2**).

Electron transport chain using NO_3 as terminal electron acceptor

The analysis of the genomes for genes that code for components of the electron transport chain revealed that the *narGHJI* genes, encoding a nitrate-reductase A system, are present in the genomes of *Lactobacillus plantarum* WCFS1, *Lactobacillus reuteri* 100-23 and *Lactobacillus fermentum*. IFO3956 (ERGO bioinformatics suite). This nitrate-reductase A has been thoroughly studied in *Escherichia coli* and *Bacillus subtilis* and is similar in some respects to the *bd*-type cytochrome. Like the latter, the nitrate reductase A catalyses the transfer of electrons from the menaquinol-pool to the terminal electron acceptor, with a concomitant generation of PMF via scalar chemistry. As a cytochrome complex, in lactic acid bacteria, it requires supplementation with heme as a cofactor (11, 12, 20). However, there are significant differences with the *bd*-type cytochrome. Most importantly it uses nitrate as electron acceptor and can thus function in anaerobic conditions. Furthermore, besides a heme cofactor the mature complex uses molybdopterin cofactors as well (30). The complete gene-set that is required to synthesis the molybdo-pterin cofactor lies in close proximity to the *narGHJI* genes on the genome of *Lactobacillus plantarum* (Fig 2).

In **Chapter 4** we demonstrate that *Lactobacillus plantarum* is able to reduce nitrate to nitrite only when both heme and menaquinone (vitamin K_2) are supplied, which confirms its electron transport chain context. Furthermore, *Lactobacillus plantarum* reduced nitrite with a concomitant increase of ammonia levels. This nitrite-reduction was not dependent of the presence of heme, menaquinone or a functional *narG*. Nitrate reductase activity in pH-controlled batch fermentations on complex medium (MRS) lead to a significant increase in biomass production, when grown on low concentrations of glucose. High glucose-levels in fact inhibit the reduction of nitrate. In contrast, high concentrations of the reduced sugar mannitol do not. In **Chapter 6**, anaerobic growth was compared of *Lactobacillus plantarum* on chemically defined medium with mannitol as carbon source. Citrate, nitrate-reductase

activity, or any suitable redox sink is required to sustain anaerobic growth on mannitol (19). In these particular pH-controlled conditions no higher biomass levels were achieved by the nitrate-reducing culture. These experiments did demonstrate that nitrate can function as effective redox-sink as high levels ~20mM of nitrate (and nitrite) were completely reduced overnight.

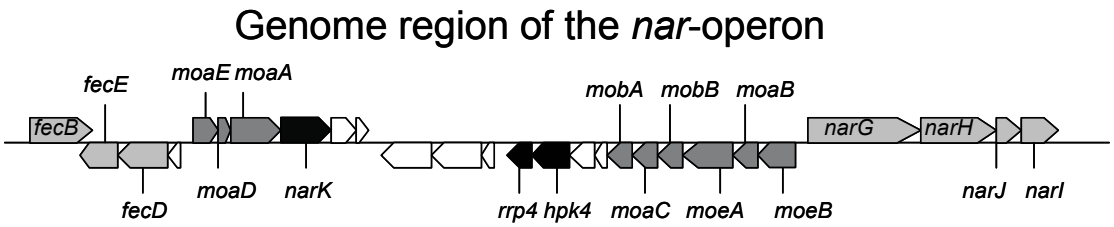


Figure 2 The genomic region upstream of the *nar*-operon (*narGHJI*) in *Lactobacillus plantarum* WCFS1, taken from **Chapter 4**. Genes coding for molybdo-pterin cofactor biosynthesis (*moaABEDA*,*mobAB*,*moeAB*), iron transport (*fecBED*), nitrite extrusion (*nark*) and a kinase/response regulator system (*rrp4*, *hpk4*) are found in this region. Putative genes are indicated by white arrows.

Nitrate and nitrite reductase activity

During growth under these conditions, only a transient levels (~<3mM) of nitrite were detected although 20mM of nitrate was reduced. It suggests that the nitrite reductase activity is about equal or faster then the nitrate reductase activity. This difference in reductase activity may prevent nitrite from accumulating and prevent the toxic effects of nitrite from manifesting (4, 25). The reduction of 20mM nitrate (and nitrite) coincides with an increase in ammonia of about 9mM. This suggests that some of the nitrogen ends up in other compounds that are still unidentified. However there is no direct evidence that the nitrogen of nitrate ends up in ammonia. Ammonia could be a by-product of altered amino acid metabolism. The use of nitrogen-15 labeled nitrate may allow identification of the metabolic routes followed by the nitrate-derived nitrogen.

In **Chapter 5** we demonstrated that a mutation in the *Lactobacillus plantarum ndh1* gene was unable to respond to the presence of heme and menaquinone in aerobic conditions. However, this mutation did not abolish heme and menaquinone nitrate reductase activity.

The *ndh1* mutant reduces nitrate to nitrite similarly to the wild type cells. This suggests that the branched electron transport chain of *Lactobacillus plantarum* that is able to use either oxygen or nitrate as terminal electron acceptor is also branched at the dehydrogenase-end (Fig 3).

The aerobic branch of the ETC resembles that of *Lactococcus lactis* and probably generates proton motive force. The NADH-dehydrogenase involved requires expression of the *ndh1* gene. PMF generation by the nitrate reductase A has not been established. Other unknowns include the identity of the dehydrogenase of the anaerobic branch, the substrate that acts as electron donor for the reduction of nitrate and of the protein system that reduces nitrite to ammonia. In fact, the complete reduction of nitrate to ammonia has not been unequivocally proven. The labeling of nitrite (or nitrate) may allow for the identification of compounds that will ultimately contain these nitrogen-atoms.

Respiration and homo-lactic fermentation

Lactic acid bacteria are though to be specialized for growth rate (homo-lactic fermention) rather than growth efficiency. Respiration, however, stimulates growth efficiency by generating PMF, at least in *Lactococcus lactis*. Furthermore, in **Chapter 5** we observed that mixed-acid production (and energy-efficient growth) is highly stimulated in *Lactobacillus plantarum* when grown aerobically with heme and menaquinone. Indeed within 48 hours in non-pH controlled batch cultivation *Lactobacillus plantarum* cultures, that were supplemented with these compound, became homo-acetic. These two metabolic strategies seem to oppose each other and in fact, when we followed organic acid production in time, we observed two distinct phases. Initially, despite supplementation with heme and menaquinone *Lactobacillus plantarum* cells do catabolize glucose to mainly lactate. Then about half-way in exponential growth lactate production decreases in favor for acetate, and finally the produced lactate was actively converted to acetate. It is not clear what induces this metabolic switch, since for example glucose was not yet depleted at this half-way point.

Future perspectives

In this work we have artificially supplemented cultures with heme and menaquinone sourced. There are however ecological niches where these compounds could be readily available, such as in meat fermentations and the gastro-intestinal tract (GI-tract).

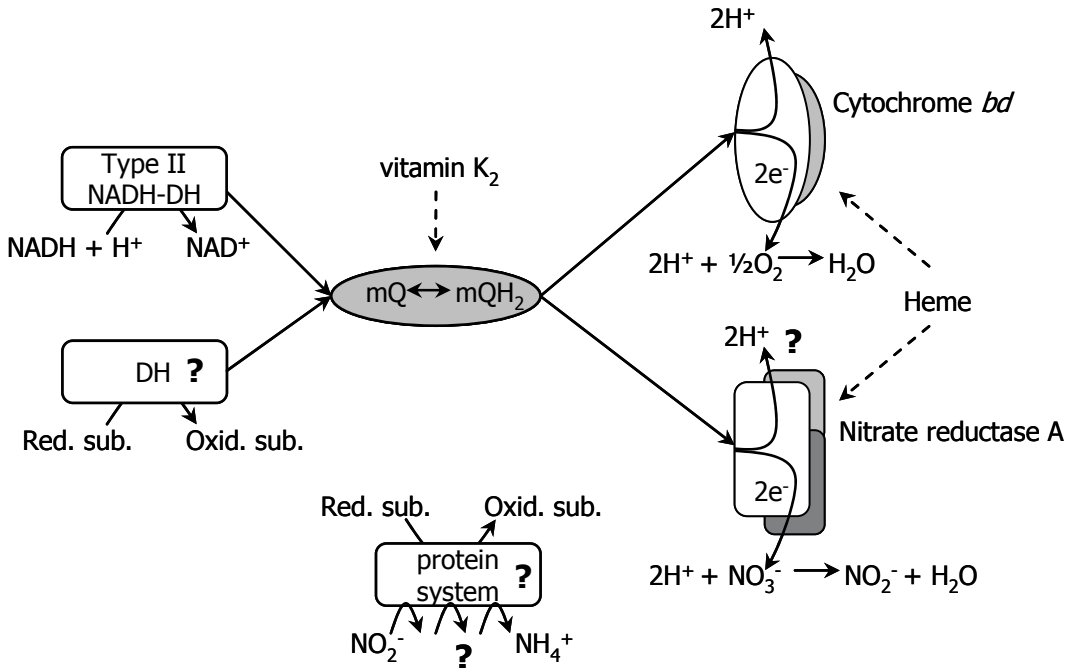


Figure 3. Electron transport chain system of *Lactobacillus plantarum*. Dashed arrows signify cofactor supplementation. Question marks signify uncertainties in the proposed model of *Lactobacillus* Abbreviations: Red. sub. (uncharacterized reduced substrate), Oxid. sub. (uncharacterized oxidized substrate), DH (dehydrogenase), mQ (menaquinone), mQH_2 (menaquinol).

Nitrate-reduction in the GI-tract

Heme is found in all eukaryotic mitochondria, and is thus present both in animal and plant tissues. Intake of heme, most obvious in red-meat, has been associated with increased risk of colonic cancer (24, 27). Lactic acid bacteria do not produce hemes, but these are taken up from the surrounding medium for use in cytochromes or heme dependent catalases (18, 35). An interesting future research topic would investigate the heme-sequestering ability

of lactic acid bacteria, and the possible alleviating effects of the consumption of lactic acid bacteria on the heme-induced carcinogenesis of red meats.

Menaquinones are produced by many prokaryotic bacteria in the GI-tract. In fact most of the human nutritional vitamin K_2 requirements are supplied by the gut microflora (6, 7). Nitrate and nitrite are also part of the normal human diet (8, 23, 38). In some food products, in particular all kinds of meats, nitrate and nitrite are actively added as preservative, although a large part of the dietary nitrate and nitrite intake comes from consumption of green-leafed vegetables (33).

Furthermore *Lactobacillus plantarum* is a major colonizer of the human gastrointestinal mucosa and is studied for probiotic activity (1, 22, 36). The GI-tract is therefore an ecological niche that contains nitrate (and nitrite), heme, menaquinones and *Lactobacillus plantarum*. Nitrate reduction therefore is a likely metabilic activity this bacterium in human GI-tract. Especially for future studies that focus on unraveling mechanisms of probiotic activity in in-vitro settings, they should consider the impact of nitrate (and nitrite) reduction on the studied metabolic activities.

Nitric oxide production by *Lactobacilli*

Nitric oxide is produced by many *Lactobacillus* sp. and *Streptococcus thermophilus* that are marketed as probiotics or that are commonly found in the human GI-tract. Production of nitric oxide was highly stimulated by the presence of nitrite, and less so by nitrate (29, 32). Thus reduction of nitrate to nitrite in the GI-tract by *Lactobacillus plantarum*, *Lactobacillus fermentum* and *Lactobacillus reuteri*, is likely to increase nitric oxide production. In fact direct increases of the GI-tract nitric oxide levels, by combined dietary supplementation of nitrate and lactobacilli was shown (28). Nitric oxide production has beneficial effect as it ameliorated effects of colonic inflammation (16). Furthermore nitric oxide production has been implicated as host defensive mechanism against pathogens (9). Thus high-level nitrate-reduction by *Lactobacillus* sp. (as stimulated by heme and menaquinone), with the conversion of nitrite (by other commensal bacteria) to nitric oxide species, may have significant disease preventing effects.

Engineering of the LAB electron transport chains

Lactcoccus lactis respiration is used in starter culture industries to increase biomass yields. As mentioned the proposed aerobic electron transport chain of LAB has a relatively poor translocation efficiency of 2 H^+/NADH. To increase biomass yields by respiratory metabolism even further several strategies could be followed. Firstly, (engineering of) a complete citric acid cycle would results in the generation of more NADH, which in turn would stimulate aerobic respiration and oxidative phosphorylation. Secondly, expressing a more efficient, proton motive force generating, type-I NADH and/or *bo*-type cytochrome as they exist in *Escherichia coli* for example would further increase ATP yields via oxidative phosphorylation (3). These metabolic engineering strategies however are quite ambitious as they require transfer and functional expression of operons that contain many genes.

As mentioned, a more realistic proposal is the transfer of the genes that for aerobic respiration to other LAB, which do not have these, to increase their biomass yield. As we understand the *Lactococcus lactis* ETC at this point, this would require transfer and expression of the *cydABCD* and genes that encode the NADH dehydrogenase, encoded by *noxAB* in lactis, and *ndhI* in *Lactobacillus plantarum*. Plasmids that functionally express the *cydABCD* genes in *bd*-type cytochrome mutants of *Bacillus subtilis* and *Lactococcus lactis* have in fact already been constructed (**Chapter 2**) (34).

Final remarks

To conclude, functional electron transport chains are present in several species of lactic acid bacteria. They help these bacteria to survive adverse conditions and allow growth on a greater range of substrates. While aerobic respiration is already used to promote biomass formation, nitrate-reduction by *Lactobacilli* holds promises for enhanced probiotic functionality and gut health.

1. **Ahrne, S., S. Nobaek, B. Jeppsson, I. Adlerberth, A. E. Wold, and G. Molin.** 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J Appl Microbiol **85:**88-94.
2. **Brooijmans, R. J., B. Poolman, G. K. Schuurman-Wolters, W. M. de Vos, and J. Hugenholtz.** 2007. Generation of a membrane potential by *Lactococcus lactis* through aerobic electron transport. J Bacteriol **189:**5203-9.
3. **Calhoun, M. W., K. L. Oden, R. B. Gennis, M. J. de Mattos, and O. M. Neijssel.** 1993. Energetic efficiency of *Escherichia coli*: effects of mutations in components of the aerobic respiratory chain. J Bacteriol **175:**3020-5.
4. **Cammack, R., C. L. Joannou, X. Y. Cui, C. Torres Martinez, S. R. Maraj, and M. N. Hughes.** 1999. Nitrite and nitrosyl compounds in food preservation. Biochim Biophys Acta **1411:**475-88.
5. **Cocaign-Bousquet, M., C. Garrigues, P. Loubiere, and N. D. Lindley.** 1996. Physiology of pyruvate metabolism in *Lactococcus lactis*. Antonie Van Leeuwenhoek **70:**253-67.
6. **Conly, J. M., and K. Stein.** 1992. The production of menaquinones (vitamin K2) by intestinal bacteria and their role in maintaining coagulation homeostasis. Prog Food Nutr Sci **16:**307-43.
7. **Conly, J. M., K. Stein, L. Worobetz, and S. Rutledge-Harding.** 1994. The contribution of vitamin K2 (menaquinones) produced by the intestinal microflora to human nutritional requirements for vitamin K. Am J Gastroenterol **89:**915-23.
8. **Dich, J., R. Jarvinen, P. Knekt, and P. L. Penttila.** 1996. Dietary intakes of nitrate, nitrite and NDMA in the Finnish Mobile Clinic Health Examination Survey. Food Addit Contam **13:**541-52.
9. **Fang, F. C.** 1997. Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. J Clin Invest **99:**2818-25.
10. **Haddock, B. A., and C. W. Jones.** 1977. Bacterial respiration. Bacteriol Rev **41:**47-99.

11. **Hoffmann, T., B. Troup, A. Szabo, C. Hungerer, and D. Jahn.** 1995. The anaerobic life of *Bacillus subtilis*: cloning of the genes encoding the respiratory nitrate reductase system. FEMS Microbiol Lett **131:**219-25.
12. **Ingledew, W. J., and R. K. Poole.** 1984. The respiratory chains of *Escherichia coli*. Microbiol Rev **48:**222-71.
13. **Kandler, O.** 1983. Carbohydrate metabolism in lactic acid bacteria. Antonie Van Leeuwenhoek **49:**209-24.
14. **Kaneko, T., M. Takahashi, and H. Suzuki.** 1990. Acetoin Fermentation by Citrate-Positive *Lactococcus lactis* subsp. *lactis* 3022 Grown Aerobically in the Presence of Hemin or Cu. Appl Environ Microbiol **56:**2644-2649.
15. **Koebmann, B. J., D. Nilsson, O. P. Kuipers, and P. R. Jensen.** 2000. The membrane-bound H(+)-ATPase complex is essential for growth of *Lactococcus lactis*. J Bacteriol **182:**4738-43.
16. **Lamine, F., J. Fioramonti, L. Bueno, F. Nepveu, E. Cauquil, I. Lobysheva, H. Eutamene, and V. Theodorou.** 2004. Nitric oxide released by *Lactobacillus farciminis* improves TNBS-induced colitis in rats. Scand J Gastroenterol **39:**37-45.
17. **Leroy, F., and L. De Vuyst.** 2004. Lactic acid bacteria as functional starters for the fermentation industry. Trends Food Sci Technol **15:**67-78.
18. **Mares, A., K. Neyts, and J. Debevere.** 1994. Influence of pH, salt and nitrite on the heme-dependent catalase activity of lactic acid bacteria. Int J Food Microbiol **24:**191-8.
19. **McFeeters, R. F., and K. Chen.** 1986. Utilization of electron acceptors for anaerobic mannitol metabolism by *Lactobacillus plantarum*. Compounds which serve as electron acceptors. Food Microbiol. **3:**73-81.
20. **Moreno-Vivian, C., P. Cabello, M. Martinez-Luque, R. Blasco, and F. Castillo.** 1999. Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. J Bacteriol **181:**6573-84.
21. **Morishita, T., N. Tamura, T. Makino, and S. Kudo.** 1999. Production of menaquinones by lactic acid bacteria. J Dairy Sci **82:**1897-903.

22. **Niedzielin, K., H. Kordecki, and B. Birkenfeld.** 2001. A controlled, double-blind, randomized study on the efficacy of *Lactobacillus plantarum* 299V in patients with irritable bowel syndrome. Eur J Gastroenterol Hepatol **13:**1143-7.

23. **Penttila, P. L., L. Rasanen, and S. Kimppa.** 1990. Nitrate, nitrite, and N-nitroso compounds in Finnish foods and the estimation of the dietary intakes. Z Lebensm Unters Forsch **190:**336-40.

24. **Pierre, F., G. Peiro, S. Tache, A. J. Cross, S. A. Bingham, N. Gasc, G. Gottardi, D. E. Corpet, and F. Gueraud.** 2006. New marker of colon cancer risk associated with heme intake: 1,4-dihydroxynonane mercapturic acid. Cancer Epidemiol Biomarkers Prev **15:**2274-9.

25. **Radcliffe, C. E., N. C. Akram, F. Hurrell, and D. B. Drucker.** 2002. Effects of nitrite and nitrate on the growth and acidogenicity of *Streptococcus mutans*. J Dent **30:**325-31.

26. **Rezaiki, L., G. Lamberet, A. Derre, A. Gruss, and P. Gaudu.** 2008. *Lactococcus lactis* produces short-chain quinones that cross-feed Group B *Streptococcus* to activate respiration growth. Mol Microbiol **67:**947-57.

27. **Sesink, A. L., D. S. Termont, J. H. Kleibeuker, and R. Van der Meer.** 1999. Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme. Cancer Res **59:**5704-9.

28. **Sobko, T., L. Huang, T. Midtvedt, E. Norin, L. E. Gustafsson, M. Norman, E. A. Jansson, and J. O. Lundberg.** 2006. Generation of NO by probiotic bacteria in the gastrointestinal tract. Free Radic Biol Med **41:**985-91.

29. **Sobko, T., C. I. Reinders, E. Jansson, E. Norin, T. Midtvedt, and J. O. Lundberg.** 2005. Gastrointestinal bacteria generate nitric oxide from nitrate and nitrite. Nitric Oxide **13:**272-8.

30. **Stewart, V., and C. H. MacGregor.** 1982. Nitrate reductase in *Escherichia coli* K-12: involvement of *chlC*, *chlE*, and *chlG* loci. J Bacteriol **151:**788-99.

31. **Teusink, B., A. Wiersma, D. Molenaar, C. Francke, W. M. de Vos, R. J. Siezen, and E. J. Smid.** 2006. Analysis of growth of *Lactobacillus plantarum* WCFS1 on a complex medium using a genome-scale metabolic model. J Biol Chem **281:**40041-8.

32. **Verstraete, W., and J. Xu.** 2001. Evaluation of nitric oxide production by lactobacilli. Appl Microbiol Biotechnol **56:**504-507.
33. **White, J. W., Jr.** 1975. Relative significance of dietary sources of nitrate and nitrite. J Agric Food Chem **23:**886-91.
34. **Winstedt, L., L. Frankenberg, L. Hederstedt, and C. von Wachenfeldt.** 2000. *Enterococcus faecalis* V583 contains a cytochrome *bd*-type respiratory oxidase. J Bacteriol **182:**3863-6.
35. **Wolf, G., and W. P. Hammes.** 1988. Effect of hematin on the activities of nitrite reductase and catalse in lactobacilli. Arch Microbiol **149:**220-224.
36. **Wullt, M., M. L. Hagslatt, and I. Odenholt.** 2003. *Lactobacillus plantarum* 299v for the treatment of recurrent Clostridium difficile-associated diarrhoea: a double-blind, placebo-controlled trial. Scand J Infect Dis **35:**365-7.
37. **Yamamoto, Y., C. Poyart, P. Trieu-Cuot, G. Lamberet, A. Gruss, and P. Gaudu.** 2005. Respiration metabolism of Group B *Streptococcus* is activated by environmental haem and quinone and contributes to virulence. Mol Microbiol **56:**525-34.
38. **Ysart, G., P. Miller, G. Barrett, D. Farrington, P. Lawrance, and N. Harrison.** 1999. Dietary exposures to nitrate in the UK. Food Addit Contam **16:**521-32.

Samenvatting- in algemeen begrijpelijk Nederlands

Deze Nederlandse samenvatting is geschreven, voor eenieder die geïnteresseerd is in de inhoud van dit proefschrift, op een manier die niet (teveel) voorkennis vereist. Voor een diepgaande samenvatting verwijs ik naar de Engelstalige samenvatting.

Dit proefschrift bestudeert het gedrag van melkzuurbacteriën. Deze bacteriën werden al door de vroegste beschavingen gebruikt voor de productie van allerlei voedingsmiddelen. Klassieke voorbeelden zijn de fermentatie van melk, om yoghurt en kaas te maken, of de fermentatie van kool om zuurkool te bereiden. Melkzuurbacteriën zijn in staat om zonder de aanwezigheid van zuurstof te groeien in deze voedsel omgevingen door suikers om te zetten in zuren (waaronder melkzuur). Melkzuurbacteriën helpen door fermentatie voedingstoffen in het voedsel vrij te maken en produceren ook allerlei vitaminen. Maar het belangrijkste aan het door hen geferemteerde voedsel is natuurlijk dat ze zelf eetbaar zijn (enkele uitzonderingen daar gelaten) en dus bijvoorbeeld geen gifstoffen produceren. Ze verrijken dus door fermentatie het voedsel maar hebben tevens een positieve invloed op de houdbaarheid ervan. Vergelijk bijvoorbeeld de houdbaarheid van melk met die van kaas. De betere houdbaarheid van het gefermenteerde voedsel wordt deels veroorzaakt doordat de verzuring (met bijvoorbeeld melkzuur) de groei van andere, mogelijk ziekteverwekkende micro-organismen wordt geremd. Naast de verzurende werking produceren veel melkzuurbacteriën ook nog anti-microbiële stoffen (zoals nisine) die een verdere conserverende werking van het voedsel bewerkstelligen.

Fermentatie staat in contrast met respiratie, dit is een vorm van groei waarbij gebruik wordt gemaakt van zuurstof (of andere, vaak inorganische, oxidatieve stoffen). Respiratie maakt het mogelijk om suikers vollediger te kunnen afbreken waarbij meer CO_2 en water vrijkomen. Deze completere afbraak betekent dat meer energie uit de suikers (of andere voedingstoffen) kan worden vrijgemaakt door bacteriën.

Een vereiste voor respiratie is de productie van een aantal eiwitcomplexen, gepositioneerd in “een rij of keten” in de celmembraan. Gezamenlijk zijn zij in staat om de elektronen die vrijkomen tijdens de afbraak van suikers door te geven aan bijvoorbeeld zuurstof. Deze eiwitcomplexen vormen samen een respiratieketen die de energie van de langskomende elektronen gebruiken om positief geladen deeltjes (protonen) over de

celmembraan heen te pompen. In feite wordt zo de celmembraan opgeladen als een batterij om later nuttige arbeid te verrichten voor cellulaire groei. De respiratieketen bestaat uit ten minste 3 onderdelen: 1. een dehydrogenase-complex dat elektronen aanneemt van stoffen die gevormd worden tijdens de suikerafbraak; 2. menaquinonen die elektronen in de celmembraan kunnen transporteren tussen deze dehydrogenasen en cytochromen; 3. cytochromen. De cytochromen vormen het laatste onderdeel van de bacteriële respiratieketens en geven tenslotten de elektronen door van de menaquinonen aan zuurstof. De energie de vrijkomt tijdens deze serie van electronenoverdracht wordt gebuikt om "de batterij" op te laden.

Melkzuurbacteriën worden traditioneel beschouwd als niet in staat zijnde om te kunnen respireren, ze zijn dus verplichte (of obligate) fermentoren. Ze missen namelijk een aantal genen die nodig zijn om bepaalde onderdelen van deze respiratieketen te maken. Zo kunnen ze geen heem-moleculen maken, die een essentiëel onderdeel vormen van cytochromen, en sommige soorten maken zelfs geen menaquinonen aan.

Er zijn in de afgelopen 40 jaar echter een aantal observaties geweest dat enkele melkzuurbacteriën, wanneer deze gekweekt werden in aanwezigheid van heem en zuurstof, een bijzonder gedrag vertonen dat tóch lijkt op respiratie. Dit gedrag is met name voor de melkzuurbacterie *Lactococcus lactis* uitgebreid beschreven. In deze condities neemt de groei efficiëntie enorm toe (er ontstaat meer biomassa op dezelfde hoeveelheid suiker) en worden cellen minder gevoelig voor allerlei soorten stress. Meer specifiek zijn deze heem-gegroeide cellen beter bestand tegen zuurstof, zuur en langdurige bewaring bij lage temperaturen (4 ˚C). Dit zijn een aantal eigenschappen die erg gewaardeerd worden door de industrie omdat deze het werken met de betreffende organismen makkelijker en goedkoper maakt. Het doel van mijn onderzoek was om te kijken wat er nu precies gebeurt wanneer *Lactococcus lactis* groeit in aanwezigheid van heem en zuurstof, is dit nu écht respiratie of een ander fenomeen?

In hoofdstuk I in staat beschreven hoe wij wetenschappelijk bewijs hebben gevonden dat *Lactococcus lactis* inderdaad écht respireert wanneer deze gekweekt wordt in aanwezigheid van heem en zuurstof. Om dit aan te tonen hebben we één van de genen die coderen voor het cytochroom (*cydA*) uit het genoom verwijderd.. Deze "mutant" reageert niet

meer op de aanwezigheid van heem. Ten tweede, om aan te tonen dat deze respiratieketen in staat is om de membraan op te laden was het noodzakelijk de ATPase activiteit stil te leggen. (*Lactococcus lactis*, en melkzuurbacteriën in het algemeen, kunnen namelijk ook hun membraan-batterij opladen met behulp van dit eiwit complex genaamd ATPase.) Om de activiteit van de respiratieketen te meten hebben we daarom de activiteit van de ATPase moeten stil leggen met een chemische stof: DCCD. We hebben zo kunnen aantonen dat *Latococcus lactis* in staat was om de membraan op te laden, ondanks dat de ATPase was stil gelegd, terwijl de *cydA*-mutant dit niet kon.

De heem geïnduceerde eigenschappen, zoals beschreven voor *Lactococcus lactis* zijn ook geobserveerd voor een klein aantal andere soorten melkzuurbateriën, bijvoorbeeld *Leuconostoc*, *Streptococcus* en *Enterococcus* soorten. Om een idee te krijgen of respiratie nu een veel voorkomende eigenschap is voor de groep melkzuurbacteriën als geheel hebben wij een groot aantal soorten getest op hun groeigedrag in de aanwezigheid van heem. Uit deze observaties kwam naar voren, zoals beschreven in hoofdstuk 2, dat heem maar in een relatief klein aantal soorten "respiratie-achtig" gedrag induceert. Echter, zoals eerder is genoemd, maken sommige melkzuurbacteriën, zoals bijvoorbeeld *Lactobacillus plantarum*, ook geen menaquinone, een essentieel onderdeel van veel bacteriële respiratie-ketens. Een tweede screening-ronde waarbij de groei werd bestudeerd in aanwezigheid van niet alleen heem, maar ook menaquinone toonde aan dat veel meer soorten melkzuurbacteriën, waaronder met name *Lactobacillus* soorten, nu wél een respiratie-achtig gedrag vertoonden.

Tegenwoordig zijn de genomen van 62 soorten melkzuurbacteriën volledig bekend en geanalyseerd. Hierdoor konden we in feite ook direct kijken of in genomen van deze bacteriën de genen aanwezig waren die coderen voor het cytochroom. We zagen dat in ruwweg de helft van de soorten deze genen (*cydABCD*) in het genoom aanwezig zijn. Wat bijzonder was, was dat er een patroon zat in de verdeling van deze genen. De aanwezigheid van cytochroom genen is hoogst specifiek voor bepaalde soorten en weer totaal niet te vinden in die van anderen. Dit is interessant omdat dit iets zegt over hoe deze cytochroom genen gedurende de evolutie van deze melkzuurbacteriën zich heeft verspreidt.

De bestudering van de genoomsequentie van melkzuurbacteriën heeft geleid tot nog een opzienbarende vinding. In het genoom van 2 melkzuurbacteriën (*Lactobacillus plantarum*

en *Lactobacillus reuteri*) zijn genen aangetroffen die coderen voor alternatief cytochroom dat in staat is nitraat (in plaats van zuurstof) te gebruiken als electronen acceptor. Deze genen, de *narGHJI* genen genoemd, coderen voor een tweede cytochroom in deze twee bacteriën. Ook dit "nitraat-gebruikend" cytochroom heeft heem nodig om te kunnen functioneren. De *nar*-genen waren al bekenden in andere (niet melkzuur-bacteriën) zoals de intensief bestudeerde *Eschirichia coli*. In *E. coli* is aangetoond dat dit complex functioneert in een electronen transport keten en protonen over de celmembraan kan "pompen". Het bijzondere aan deze respiratie keten is dat deze dus kan functioneren in groeicondities waar geen zuurstof (anaeroob) aanwezig is. Respiratie dat anaeroob kan functioneren met nitraat is in het bijzonder van belang voor melkzuurbacterien omdat deze met name worden gebruikt voor (anaerobe) voedsel-fermentatie. Nitraat-respiratie kan dus, in theorie, gemakkelijker in allerlei productie processen worden toegepast.

Om te bewijzen dat ook *Lactobacillus plantarum* echt in staat is tot nitraat ademhaling hebben we ten eerste laten zien dat nitraat daadwerkelijk electronen accepteert en wordt omgezet in nitriet in aanwezigheid van heem en menaquinonen. Verder is gedemonstreerd dat in condities van nitraatademhaling, cellen in staat zijn om te groeien op gereduceerde suikers. Groei op zulke "gereduceerde" suikers is alleen mogelijk wanneer de cellen, electronen van afbraak producten van deze suikers, kunnen doorgeven aan nitraat. Dit gebeurt alleen in de aanwezigheid van (heem en) menaquinone, dus is er sprake van een electronen transport keten. De directe betrokkenheid van het nitraat-reductase complex is verder aangetoond door het verwijderen van het *narG*-gen uit het genoom. Deze mutant is niet meer in staat om nitraat te reduceren.

Het laatste stukje bewijs dat nodig is om aan te tonen dat *Lactobacillus plantarum* in staat is tot nitraat-ademhaling is het laten zien dat deze keten in staat is om protonen over de membraan te pompen. Helaas is DCCD in *Lactobacillus plantarum* in tegenstelling tot *Lactococcus lactis* niet in staat om direct in hele cellen de ATPase activiteit stil te leggen en zullen in de toekomst andere technieken gebruikt moeten worden.

Lactobacillus plantarum beschikt dus over een "vertakte" electronen transport keten die electronen aan zowel nitraat als zuurstof kan doneren. In hoofdstuk 4 wordt in meer detail gekeken naar de invloed hiervan op het metabolisme. Een voorbeeld hiervan is dat respiratie

electronen ontrekt aan het afbraak producten van suikers. Normaal gesproken worden suikers omgezet in bepaalde zuren. Wanneer er electronen aan producten ontrokken worden, zullen andere type zuren of alcoholen gevormd worden. Respiratie heeft dus gevolgen voor de chemische reacties in de cel. Zo hebben we gemeten dat gedurende zuurstof respiratie *Lactobacillus plantarum* in staat is om zo goed als al het melkzuur (lactaat) om te zetten naar een andere zuur (acetaat). Respiratie maakt van *Lactobacillus plantarum* een acetaat producerende cel.

Melkzuurbacteriën zijn lange tijd beschouwd als een niet-respirerende groep bacterien. Dit onderzoek heeft aangetoond dat in iedergeval een aantal soorten melkzuurbacterien wel degelijk in staat zijn tot respiratie. Niet alleen melkzuurbacterien dragen het (in sommige gevallen) onterechte stempel van niet-respiratieve bacterien, ook andere groepen zoals Propionibacteriën, acetogenen en sulfaat-reducerende bacterien blijken over dezelfde *cyd*-genen te beschikken. In het laatste hoofdstuk van dit proefschrift wordt een literatuurstudie uitgevoerd naar deze als strict-anaeroob beschouwde gram-positive bacteriën. Hierin beschrijven we het metabolisme waar deze bacteriën bekend om staan, wat er bekend is van het bestaan van mogelijke electronen transport ketens (of respiratie-activiteit) en hoe dit het celgedrag beïnvloed.

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About the Author

Rob Josephina Wilhelmus Brooijmans was born on the 29th of September 1979 in Bergen op Zoom, the Netherlands. After graduating from the Roncalli College in Bergen op Zoom, in September that same year, he moved to Leiden where he studied Biology. After one year he obtained his Bachelor's degree *cum laude*. For his Master's degree, in the ensuing four years, he worked first on Yeast genetics at the University of Leiden under the supervision of Rolf Kooistra, then on the ethology of Scorpion Fish in Hawaii, at the Hawaiian Institute of Marine Biology under the supervision of prof Dr. George Losey, and finally on viral-based systems for the site-specific genome-integration of DNA, at the Gene-therapy group (Leiden University) under supervision of Dr. A. A. de Vries.

In November 2003 he started his PhD studies at NIZO Food Research which focused on the respiration-capacity of lactic acid bacteria and resulted in this thesis. This PhD-project was done in collaboration with Kluyver Centre for Genomics of Industrial Fermentation and Top Institute Food and Nutrition.

Currently, he is working as a PostDoc for the Top Institute Food and Nutrition, on the "Zero-growth" project, concerned with the non-growing, yet metabolically active, state of lactic acid bacteria.

List of publications

R. J. W. Brooijmans, B. Poolman, G. K. Schuurman-Wolters, W. M. de Vos and J. Hugenholtz. Generation of a membrane potential by *Lactococcus lactis* through aerobic electron transport. Journal of Bacteriology, July 2007, p. 5203-5209, Vol. 189, No. 14

R. J. W. Brooijmans, B. A. Smit, F. Santos, W. M. de Vos, J. Hugenholtz. Heme and menaquinone induced electron transport in lactic acid bacteria. Submitted to Applied and Environmental Microbiology

R. J. W. Brooijmans, W. M. de Vos, J. Hugenholtz. The electron transport chains of *Lactobacillus plantarum* WCFS1. Submitted to Applied and Environmental Microbiology

R. J. W. Brooijmans, S. Noordermeer, W. M. de Vos, J. Hugenholtz. Heme and menaquinone induced aerobic response in *Lactobacillus plantarum* WCFS1. Manuscript in preparation

R.J.W. Brooijmans, J. Hoolwerf J., W. M. de Vos, J. Hugenholtz. The anaerobic electron transport chain of *Lactobacillus plantarum* is an efficient redox sink. Manuscript in preparation

R. J. W. Brooijmans, S. Kengen, F. Stams., J. Hugenholtz. The electron transport chains of anaerobic prokaryotic bacteria. Manuscript in preparation

Training and Supervision Plan

Discipline- specific activities

- International advanced course on Food Fermentation, VLAG, Wageningen, 2004
- Protein Engineering, Wageningen, 2004
- 8th International symposium on Lactic Acid Bacteria,
  Egmond aan Zee, (poster presentation), 2005
- 9th International symposium on Lactic Acid Bacteria,
  Egmond aan Zee, (poster presentation), 2008
- Kluyver Centre for Genomics of Industrial Fermentation Symposium
  (oral and poster presentations) 2004-7
- WCFS Food Summit, Wageningen, 2003
- Society of Industrial Microbiology Symposium,
  Denver Colorado, (poster presentation), 2007
- Training at the University of Groningen, measurements of membrane potential, 2005

General courses

- Advance course on Bioethics & public perceptions of biotechnology,
  Delft University of Technology, Oxford, 2004
- Writing Grant Proposals, Wageningen University, 2008

Optional courses and activities

- General meetings and discussion, oral presentation, 2003-7
- WCFS We-days, oral presentation, 2003-7
- Participation PhD Study Excursion Japan, 2004
- Participation and participant organizer PhD Study Excursion California, 2006
- Participant organizer Netherlands Genomics Initiative Day, 2007
- Preparing PhD proposal, 2008

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