Lactic Acid Bacteria: Genetics, Metabolism and Applications

Proceedings of the Seventh Symposium on Lactic Acid Bacteria: Genetics, Metabolism and Applications, September 1-5, 2002, Egmond aan Zee, The Netherlands

Edited by
Roland J. Siezen, Jan Kok, Tjakko Abee and Gertjan Schaafsma

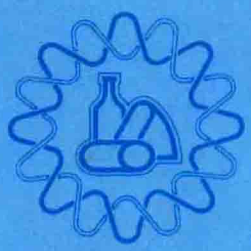

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Table of Contents

Special Issue: **Lactic acid bacteria: genetics, metabolism and applications Proceedings of the seventh symposium on lactic acid bacteria: genetics, metabolism and applications, 1–5 September 2002, Egmond aan Zee, the Netherlands**

Guest Editors: **R.J. Siezen[1], J. Kok[2], T. Abee[3] & G. Schaafsma[4]**
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METABOLISM

METABOLISM SHORT LECTURES

APPLICATIONS

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Antonie van Leeuwenhoek **82:** 1, 2002.

Lactic acid bacteria: genetics, metabolism and applications

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R. J. Siezen[1], J. Kok[2], T. Abee[3] & G. Schaafsma[4]

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Editorial

The Seventh Symposium on Lactic Acid Bacteria

Foods fermented with Lactic Acid Bacteria are an important part of the human diet. Lactic Acid Bacteria play an essential role in the preservation of foods and contribute to the nutritional, sensoric and health properties of food products and animal feed. The importance of Lactic Acid Bacteria in the production of foods throughout the world has resulted in a continued scientific interest over the last two decades in these micro-organisms by academic research groups as well as by industry. This research has resulted in a number of important scientific breakthroughs and has led to new applications. The most recent of these advances is the establishment of the complete genome sequences of a number of different Lactic Acid Bacterial species.

To communicate and stimulate the research on Lactic Acid Bacteria and their applications, a series of tri-annual symposia on Lactic Acid Bacteria was started in 1983 under the auspices of the Netherlands Society for Microbiology (NVVM), which was later also supported by the Federation of European Microbiological Societies (FEMS). The aim of these state-of-the-art symposia, is to offer a unique platform for universities, institutes and industry in this area of biotechnology, to present recent work, to obtain information on new developments and to exchange views with colleagues from all over the world on scientific progress and applications. The growing number of participants to these symposia has been a clear demonstration of the interest of the international industrial and scientific community in this area of research.

The 7th Symposium will be based on a number of plenary lectures that will review the scientific progress of the last years in the different areas of research on Lactic Acid Bacteria, and which are documented in this special issue of Antonie van Leeuwenhoek.

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The cell membrane and the struggle for life of lactic acid bacteria

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Key words: lactic acid bacteria, metabolic energy generation, survival, membrane transport, multidrug resistance

Abstract

The major life-threatening event for lactic acid bacteria (LAB) in their natural environment is the depletion of their energy sources and LAB can survive such conditions only for a short period of time. During periods of starvation LAB can exploit optimally the potential energy sources in their environment usually by applying proton motive force generating membrane transport systems. These systems include in addition to the proton translocating F_oF_1-ATPase: a respiratory chain when hemin is present in the medium, electrogenic solute uptake and excretion systems, electrogenic lactate/proton symport and precursor/ product exchange systems. Most of these metabolic energy-generating systems offer as additional bonus the prevention of a lethal decrease of the internal and external pH. LAB have limited biosynthetic capacities and rely heavily on the presence of essential components such as sources of amino acids in their environment. The uptake of amino acids requires a major fraction of the available metabolic energy of LAB. The metabolic energy cost of amino acid uptake can be reduced drastically by accumulating oligopeptides instead of the individual amino acids and by proton motive force-generating efflux of excessively accumulated amino acids. Other life-threatening conditions that LAB encounter in their environment are rapid changes in the osmolality and the exposure to cytotoxic compounds, including antibiotics. LAB respond to osmotic upshock or downshock by accumulating or releasing rapidly osmolytes such as glycine-betaine. The life-threatening presence of cytotoxic compounds, including antibiotics, is effectively counteracted by powerful drug extruding multidrug resistance systems. The number and variety of defense mechanisms in LAB is surprisingly high. Most defense mechanisms operate in the cytoplasmic membrane to control the internal environment and the energetic status of LAB. Annotation of the functions of the genes in the genomes of LAB will undoubtely reveal additional defense mechanisms.

Introduction

Lactic acid bacteria (LAB) are facultative anaerobic bacteria with a fermentative metabolism (Kandler 1983). The primary source of their metabolic energy is supplied in the form of ATP by substrate level phosphorylation (Kandler 1983; Konings 1985). LAB have a relatively simple metabolism and make high demands on the nutritional composition of their growth media. Usually, in addition to carbon and energy sources, various amino acids, vitamins, nucleic acids and mineral components are required. The environments in which LAB can thrive are restricted by these requirements and are often associated with plants, meat and dairy products (Mundt 1982). The genome sizes of LAB are relatively small, which is consistent with their simple metabolic capabilities. The genome of *Lactococcus lactis* IL1403 contains 2 365 589 base pairs and encodes 2310 proteins (Bolotin et al. 2001). This is about half the size of the genome of *E.coli*. LAB are important bacteria for food fermentations. They play crucial roles in the fermentation of milk products, including cheese, vegetables such as sauerkraut and soy sauce and meat products such as sausages. LAB also contribute significantly to other fermentation processes such as the production of wine. The importance of LAB for food fermentation has strongly stimulated research of these bacteria and *L. lactis* belongs to the best-studied micro-organisms today.

L. lactis might serve as a model organism of physiological minimalism. It is adapted to a hetero-

trophic way of life while it has only rudimentary parts of many of the biosynthetic pathways. Yet, in spite of its simplicity, *L. lactis* has acquired an amazing variety of adaptation and defense mechanisms in order to cope with and to survive in continuously changing and often hostile environments. Many of these adaptation and defense mechanisms rely heavily on energy transducing systems in the cytoplasmic membrane. In particular, transport systems that translocate ions and substrates (*solutes*) from the external medium into the cytoplasm or excrete ions and end products of metabolism into the external medium, are crucial in the survival strategy (Konings et al. 1997). The importance of the cytoplasmic membrane for growth and survival of *L. lactis* is evident from the observation that about 17% of all proteins encoded by its genome are involved in biosynthesis and function of the cytoplasmic membrane (Bolotin et al. 2001).

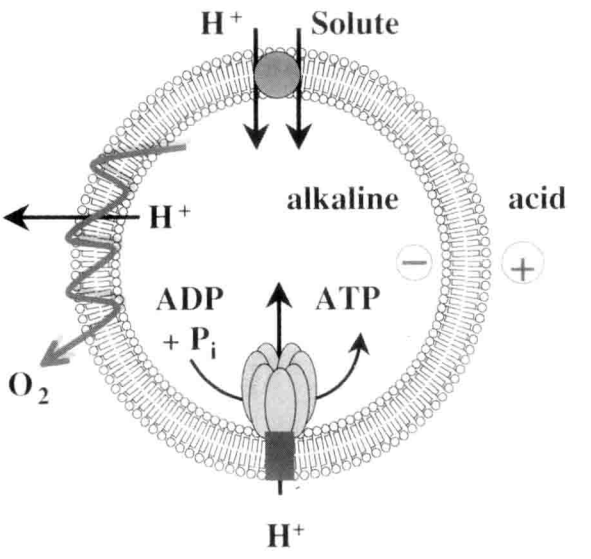

Figure 1. Energy transduction in aerobic bacteria. Protons are pumped by the respiratory chain from the cytoplasm across the membrane into the external medium resulting in the generation of a proton motive force, inside negative and alkaline. This PMF drives energy requiring membrane processes such as ATP-synthesis and solute transport.

The functional role of the cytoplasmic membrane

The cytoplasmic membrane is the only membrane present within the cell wall boundary in most bacteria and archaea (Lindsay et al. 2001). It forms a diffusion barrier and constitutes a physical barrier between the cytoplasm and the external medium. It defines a discrete entity, the cytoplasm that contains molecules belonging only to the organism. The diffusion barrier function of the cytoplasmic membrane restricts not only the diffusion of hydrophilic compounds across the membrane but also of lipophilic compounds (Bolhuis et al. 1996b). The barrier property allows the cytoplasmic membrane to serve two important functions in all living organisms. (i) Conservation of the integrity of the cytoplasm by preventing metabolites and ions from diffusing uncontrolled out of the cells and of external compounds and ions from diffusing in freely; (ii) Chemiosmotic energy transduction.

Inherent to the restricted ion-permeability of the cytoplasmic membrane is the potential to generate electrochemical ion gradients across the membrane by a variety of ion transport systems. The energetically most-important electrochemical ion gradient in most bacteria, including LAB, is that of protons (Mitchell 1966). Specific proton pumps in the membrane form these electrochemical proton gradients. A major proton pump in aerobic bacteria is the respiratory chain (Figure 1). Electron transfer in the respiratory chain can be coupled to the extrusion of protons from the cytoplasm to the external medium. As a result two gradients are formed:

(i) A pH gradient (ΔpH), inside alkaline versus outside, by the removal of protons from the cytoplasm and their accumulation in the external medium (Figure 1). The pH-gradient (expressed in mV) has a value of $-2.3RT/F$ ΔpH (expressed in pH units) in which R is the gas constant, T, the absolute temperature and F, the constant of Faraday. The term 2.3 RT/F is represented by Z and has a value of almost 60 mV. A pH difference of 1 pH unit therefore represents a force of almost –60 mV.

(ii) . A membrane or electrical potential, $\Delta\Psi$, by the translocation of the positive charge of the protons from inside to outside (Figure 1).

The sum of these gradients, the proton motive force or PMF equals: $\Delta\Psi - Z\,\Delta$pH mV. This PMF exerts an inwardly directed force on the protons. Since cytoplasmic membranes have very low proton permeabilities, protons can only cross these membranes via specific transport proteins. The influx of protons, driven by the proton motive force, results in the release of energy, which can be used to drive metabolic energy-requiring membrane-bound processes (Figure 1). Well-known examples are ATP synthesis by the F_oF_1-ATPase, rotation of the flagellar motor and transport of solutes across the membrane (see below).

The function of transport proteins is to couple transport of charge/and or protons to an energy-requiring process, thereby transducing the metabolic energy of the PMF into chemical, mechanical or (electro-)chemical energy of other (ionic)solutes.

In view of the importance of these energy transducing processes for the function of a cell it is not surprising that a PMF of significant magnitude is needed for cell growth and viability. In most bacteria the PMF during logarithmic growth has a value of around –150 mV. When a carbon source is present a PMF of this magnitude can readily be maintained in aerobic organisms by the proton pumping respiratory chain. In fermentative bacteria the situation is drastically different. These organisms very often do not possess proton pumping electron transfer systems and PMF generation relies on the proton pumping F_0F_1-ATPase at the expensive of ATP, formed by substrate level phosphorylation. Even in the presence of carbon and energy sources these fermentative bacteria function under energy-limited conditions and additional supply of metabolic energy will directly be reflected by higher growth rates and growth yields.

This review presents the current knowledge of energy transduction and solute transport systems of LAB and in particular of *L. lactis* with special emphasis on the importance of the systems for energy metabolism and survival of LAB under transient conditions.

Energetic states of LAB

LAB are fermentative organisms and obtain their *metabolic energy* (energy which can be used for energy-requiring processes in the cell) mainly by substrate level phosphorylation. The metabolic energy supplied by fermentation is small. In the metabolism of glucose to lactate, two ATP are formed while an additional ATP can be obtained in further metabolism of pyruvate to acetate. This ATP is needed for the synthesis of cellular macromolecules and other energy requiring processes in the cytoplasm and in the cytoplasmic membrane and for the generation and maintenance of a PMF by the membrane bound F_0F_1-ATP (hydrol-/synthet-)ase (Maloney 1977). The supply of metabolic energy by fermentation is usually not sufficient to allow maximal rates of the biosynthetic machinery and this restricts the growth yield and the growth rate of LAB.

Moreover, in their natural habitats LAB often encounter drastic fluctuations in the composition of their

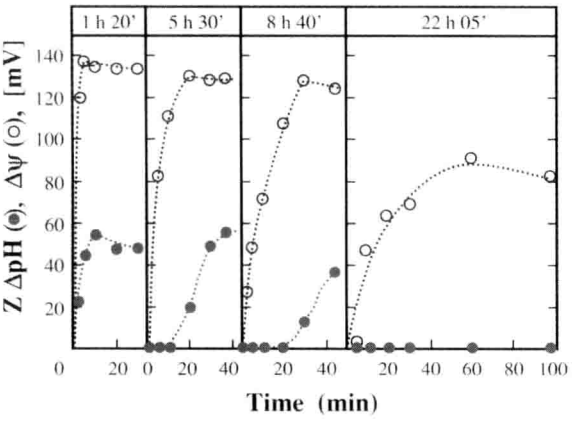

Figure 2. PMF generation in *Lactococcus lactis* Wg2 after various periods of lactose starvation (indicated in the top bar of the figure) (data from Poolman et al. 1987h).

environment. Consequently, LAB are often in a transient state with periods of abundant supply of energy followed by periods of starvation. In the presence of sufficient supply of energy the energetic conditions of the LAB will be optimal. For *L. lactis* these conditions have been found to be: intracellular ATP levels around 2.5 mM (the energy stored in the ATP pool is expressed as the phosphate potential $\Delta G'_p$; the $\Delta G'_p$ in *L. lactis* is $\sim$ –460 mV), PMF $\sim$ –150 mV and the internal pH between pH 7 and pH 7.5 (Konings et al. 1989). When the energy source runs out the rate of glycolysis rapidly decreases. Also the rate of PMF generation decreases rapidly, although the steady state level of the PMF is maintained for several hours (Figure 2) (Poolman et al. 1987g). This loss of activity results in a diminished rate of ATP synthesis and consequently of PMF generation. It also results in a decreased supply of metabolic energy for vital cellular processes and this directly affects viability. When the energy supply is restored within a short period of time glycolysis can start again and the internal ATP-pool and the PMF can be regenerated (Otto 1981; Konings & Otto 1983; Poolman 1987; Poolman et al. 1987 a;). This ability of *L. lactis* to survive periods of starvation is restricted from a few hours up to a few days, depending on the starvation media (Peterkofsky & Gadzar 1979) and the growth conditions preceding the starvation phase (Poolman et al. 1987a). Loss of viability is mainly caused by a loss of glycolytic activity due to the inactivation of glyceraldehyde 3-phosphate dehydrogenase (Poolman et al. 1987a).

From the considerations presented above, it is evident that both during fermentation and transient

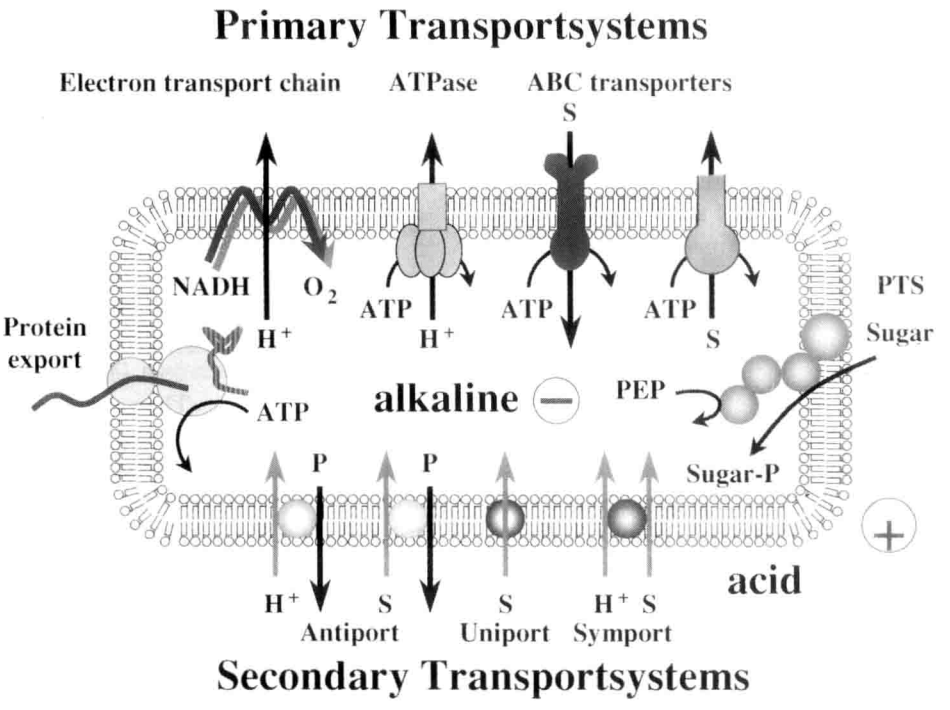

Figure 3. Different classes of transport systems in bacteria.

states the LAB could benefit considerably from a supply of additional usable sources of metabolic energy and such sources will directly contribute to growth and survival of the LAB. This review will describe a number of these additional usable energy sources of LAB.

Diversity of transport systems

Under glycolyzing and growing conditions solutes are taken up and end products of metabolism are excreted at high rates. The restricted permeability of the cytoplasmic membrane prevents high rates of passive fluxes even for solutes, which are significantly membrane permeable. Uptake of solutes and excretion of end products therefore need to be mediated by specific transport systems in the membrane.

The transport systems of LAB belong to four different classes (Figure 3)

(i) Primary transport systems, which use light, redox or chemical energy for the generation of a PMF or electrochemical gradients of other solutes. This class comprises the proton pumping electron transfer systems and F_0F_1-ATPase; the ion pumping P-type ATPases and the ATP-binding cassette (ABC)- transporters. Two categories of ABC-transporters exist: (a) the binding protein-dependent uptake systems and (b) the binding protein-independent extrusion systems.

(ii) Secondary transport systems which use the PMF for the uptake or efflux of solutes. This class comprises the uniport, the proton/solute symport and the solute/ H^+ or solute/solute(product) antiport systems (Figure 3).

(iii) The protein secretion system that uses ATP and PMF to drive proteins across the cytoplasmic membrane.

(iv) The phosphoenol-pyruvate phospho-transferase systems (PTS), which use phospho-enolpyruvate to concomitantly transport and phosphorylate sugars.

The genome of *L. lactis* IL 1403 encodes about 250 polypeptides that are directly involved in solute transport (Table 1). This means that well over 10% of all genes in its genome encode transport proteins, which reflects the important roles of transport processes in this bacterium. The products of these 'transport' genes of *L. lactis* are involved in a variety of transporters (Table 1). The largest class of transporters is secondary transport systems (73) followed by the ABC transporters (41). For comparison, *E.coli* has 194 secondary

Table 1. Transport systems in *Lactococcus lactis* IL1403 identified by genome annotation[a] and experimentally

Classes	Transporters[a]	Number of polypeptides[a]	Identified transporters
Primary transport systems	51	154	
F_0F_1-ATPase	1	7	1
Electron transport	1	12	1
ABC transporters	41	127	12
P-type ATPases	8	8	1
Secondary transport system	73	73	17
Protein secretion systems	1	8	1
PTS transporters	5	18	5
Total	130	253	38

[a] According to Bolotin et al. (2001).

transporters and 74 ABC transporters. The ratio of ABC transporters relative to the secondary transporters is therefore relatively high in *L. lactis*. (Binding protein-dependent) ABC transporters usually have very high affinities for their solutes and catalyze transport at high rates. The possession of such transporters gives *L. lactis* significant competitive advantage by allowing the organism to rapidly scavenge essential or growth-limiting solutes from dilute environments.

The substrates of many of these transporters are by no means known. A list of the transport systems, which have been studied in more or less detail, is given in Table 2. This represents about 30% of all transport systems of *L. lactis*. Clearly, many transporters still need to be characterized. The least studied transporters of *L. lactis* are the P-type ATPases. These transporters catalyze mainly the excretion of (cytotoxic) metals (Silver & Phung 1996).

The translocation of ions and other compounds (*solutes*) across the cytoplasmic membrane is energetically expensive, especially when the solutes have to be transported against a concentration gradient. According to the Nernst equation the energy of a solute (S) concentration gradient equals: Z log $[S]_{in}/[S]_{out}$ mV, in which $[S]_{in}$ is the solute concentration in the cytoplasm and $[S]_{out}$, the solute concentration in the medium. A solute concentration gradient can only be generated and maintained at the expense of metabolic energy, usually ATP or a PMF. The driving forces for a variety of secondary and ATP-driven transporters are given in Figure 4. It should be realized that in all these transport processes the concentration gradients of all solutes or ions transported contribute to the driving force. Thus in all transport processes of solutes the ΔS ($=Z$ log $[S]_{in}/[S]_{out}$) contributes to the driving force. The $\Delta\Psi$ contributes to the driving force only when net charge is translocated in the transport process and the ΔpH is involved only when proton(s) are translocated. When solutes or charges are transported down their gradients their contribution to the driving force has a negative value; in the opposite direction their contribution has a positive value. When the total driving force is zero thermodynamic equilibrium is reached and no net transport will occur.

It has been stated above that ABC-transporters can usually accumulate solutes to high internal concentrations. The $\Delta G'_p$ is an important component of the driving force for solute transport in the F_0F_1-ATPase, the P-type ATPases and the ABC-transporters. The driving force supplied by $\Delta G'_p$ is usually significantly higher than the driving force supplied by the PMF, which explains the strong accumulative power of ABC transporters. The driving force for proton transport by the F_0F_1-ATPase is: $\Delta G'_p$ $-n(\Delta\Psi - Z\Delta\text{pH})$mV, in which n represents the number of protons transported per ATP hydrolyzed or synthesized. When the driving force supplied by $\Delta G'_p$ exceeds the driving force supplied by the PMF [$n(\Delta\Psi - Z\Delta\text{pH})$ mV] ATP-hydrolysis will result in PMF generation; in the reversed situation the PMF will drive ATP-synthesis.

It should be realized that just like the F_0F_1-ATPase, transport processes are in principle reversible and that the direction of the transport processes depends on the direction of the driving force. This means that ATP or a PMF can be used to drive the uptake or excretion of a solute but that under conditions of high solute gradi-

Table 2. Transport systems in *Lactococcus lactis*

Class of transporter	Solute	Reference
PTS-transporters	Glucose and mannose	Thompson (1978)
	Lactose	Thompson (1979)
	Galactose	Thompson (1980)
	Sucrose	Thompson & Chassy (1981)
	Fructose	Thompson et al. (1985)
ABC transporters		
Binding protein-dependent uptake systems	Glutamate and glutamine	Poolman et al. (1987f)
	Asparagine	Truong & Poolman (1987)
	Aspartate	Poolman et al. (1987c)
	Phosphate	Poolman et al. (1987e)
	Glycine-betaine and proline	van der Heide & Poolman (2000a)
	Di- and tri-peptides (Dpp)	Sanz et al. (2001)
	Oligopeptides (Opp)	Kunji et al. (1993)
Extrusion systems		
	Anionic cytotoxic compounds	Molenaar et al. (1992)
	Anionic cytotoxic compounds	Glaasker et al. (1996b)
	Cationic cytotoxic compounds(LmrA)	Bolhuis et al. (1996b)
	Bacteriocins (LmrB)	Gajic et al. (2001)
P-type ATPases		
	Ca^{2+}	Ambudkar et al. (1986)
Secondary transporters		Driessen (1987); Poolman (1987)
Symport systems		
	Leucine, isoleucine, valine and methionine	Driessen et al. (1987a,b)
	Alanine and glycine	Driessen et al. (1987c)
	Serine and threonine	Driessen 1987
	Lysine	Driessen et al. (1989)
	Histidine	Otto et al. (1982)
	Proline	Driessen (1987)
	Cysteine	Driessen (1987)
	Tyrosine and phenylalanine	Poolman & Konings (1988)
	Lactate	Michels et al. (1979)
	α-Ketoglutarate	Marty-Teysset et al. (1996)
	di- and tri-Peptides (DtpT)	Hagting et al. (1994)
Antiport systems		
	Ca^{2+}	Driessen et al. (1985)
	Phosphate/hexose-6-phosphate	Maloney et al. (1984)
	Arginine/ornithine	Driessen et al. (1987d)
	Lysine	Driessen et al. (1989)
	Malate/lactate (MleP)	Poolman et al. (1991)
	Citrate/lactate (CitP)	Blandell et al. (1998)
	Cationic cytotoxic compounds (LmrP)	Bolhuis et al. (1994)

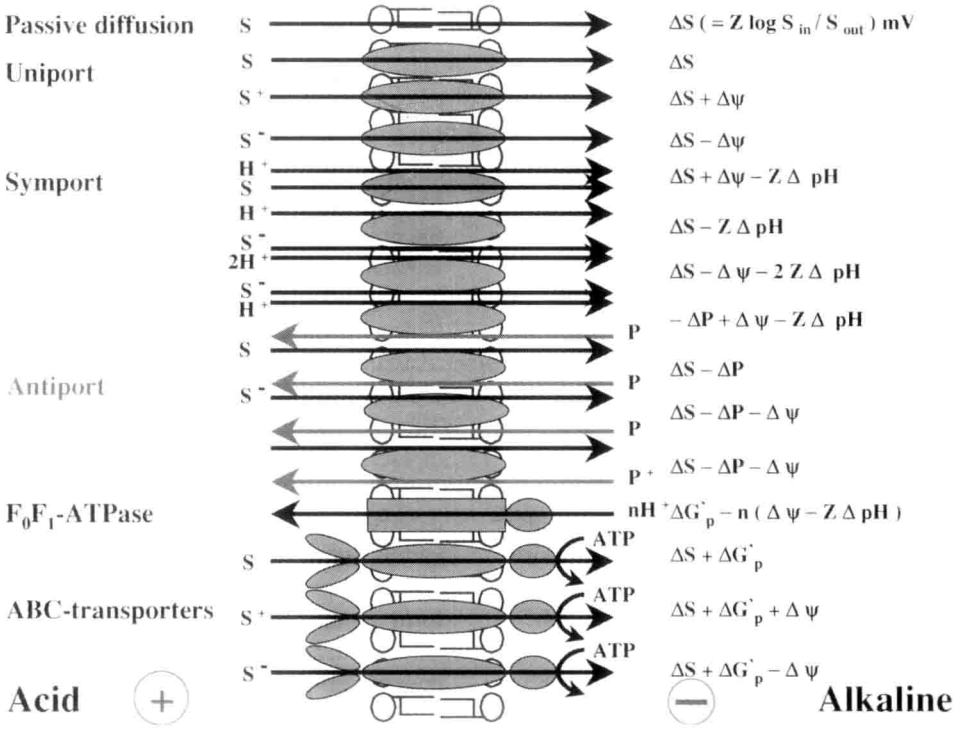

Figure 4. Driving forces for solute transport *via* different transport systems.

ents these gradients can drive the excretion of protons or charges and generate ATP or a PMF. When the total driving force has a negative value the transport process results in the accumulation of the solute at one side of the membrane at the expense of ATP or a PMF. On the other hand, when the driving force is positive ATP or a PMF can be generated at the expense of the solute and/or product gradient.

Metabolic energy supply by respiration

The fermentative nature of LAB has been brought into question by the observation that *L. lactis* can form a respiratory chain in the presence of hemin (Kaars Sijpesteijn 1970; Ritchey & Seeley 1976; Duwatt et al. 2001). These results are consistent with the presence of genes in the genome of *L. lactis* IL1403 encoding respiratory chain proteins (Bolotin et al. 2001). A similar effect of hemin was found for *Streptococcus faecalis* (Clarke & Knowles 1980). When oxygen is present electron transfer in this respiratory chain is coupled to the extrusion of protons and results in the generation of a PMF. Respiration can therefore contribute to the generation of metabolic energy in *L. lactis* (Blank et al. 2001; Duwatt et al. 2001) in a similar way as in obligate and facultative aerobic bacteria (Figure 1). As a result of this respiratory activity *L. lactis* has a greater growth yield and a remarkable improvement of survival, indicating that under fermentative conditions the metabolic energy supply limits growth. With a functional respiratory chain oxygen is beneficial instead of toxic for *L. lactis* (Duwatt et al. 2001).

Metabolic energy supply by secondary transport

In order to grow and to multiply bacteria have to take up and often to accumulate a variety of solutes. Transport processes are energetically expensive and consume a large fraction of the metabolic energy supply. An ABC transporter transports a solute at the expense of at least one ATP per substrate molecule (van Veen et al. 2000). Secondary transporters are energetically less expensive than ABC-transporters. Since the synthesis of one ATP by the F_0F_1-ATPase requires the influx of three to four protons (Jiang et al. 2001) the energy requirement of symport of a neutral solute with one proton represents one-third to one-

quarter ATP-equivalents. Growth requires not only the uptake of solutes (precursors) but also the excretion of end products of metabolism. Since end products are produced inside and excreted in a large volume of medium these excretion processes occur usually down the end product concentration gradients. This suggests that the end product concentration gradient might contain enough energy to drive its own transport out of the cell. If the excretion of end products occurs via secondary transport systems in symport with protons and/or charges, this excretion can lead to the generation of a PMF (see Figure 4). A variety of transport processes in LAB have indeed been found to generate instead of consume the PMF and to supply additional metabolic energy.

The metabolic energy generating transport systems found so far in LAB all belong to the secondary transporters and include uniporters, proton-solute symporters and antiporters (Driessen & Konings 1990; Konings et al. 1995) (Table 3). Theoretically also ABC-transporters and P-type ATPases should be capable to work in the reverse direction and to synthesize ATP in a similar manner as PMF-driven ATP synthesis by the F_0F_1-ATPase. However, since the phosphate potential $\Delta G'_p$ is usually relatively high, energy conversion by the ABC-transporters in the reversed direction is energetically not possible.

Metabolic energy generating Uniport systems

The simplest mechanism by which solute transport can contribute to metabolic energy is via uniport systems in which either a negatively charged solute is taken up and rapidly metabolized internally or a positively charged end product of metabolism is excreted. The driving force for the translocation process is in both cases supplied by the solute gradient ΔS (Z. log $[S]_{in}/[S]_{out}$ mV) or product gradient $-\Delta P$ (Z. log $[P]_{in}/[P]_{out}$) mV and $-n\ \Delta\Psi$ (n represents the number of translocated charges). The charge translocation coupled to solute or product transport will lead to the generation of a $\Delta\Psi$, which at thermodynamic equilibrium will reach a value of: $\Delta\Psi = (\Delta S)/n$ mV. If in addition protons are consumed internally during solute metabolism or product formation the cytoplasm will alkalinize and the overall process will also lead to the generation of a ΔpH. In that case both components of the PMF, a $\Delta\Psi$ and a ΔpH, are generated.

Such PMF-generating uniport systems have been found in *Oenococcus oeni* (previously named *Leuconostoc oenos*, Dicks et al. 1995). This organism is well known for its ability to carry out malolactic fermentation at more acidic pH values and for this reason *O. oeni* is frequently used in the deacidification of wine. Under these acid conditions *O. oeni* maintains a rather constant internal pH of 5.8–6.3 (Salema et al. 1994). Malolactic fermentation in *O. oeni* proceeds through the uptake of the negatively charged mono-protonated $Hmalate^-$, which is the prevalent ionic species of malate under acid conditions (Figure 5A). In the cytoplasm $Hmalate^-$ is rapidly decarboxylated thereby realizing a large inwardly directed $Hmalate^-$ concentration gradient, which drives the uptake of $Hmalate^-$. The decarboxylation of malate leads to the formation of lactate in a proton consuming reaction. This decarboxylation therefore results in the alkalinization of the cytoplasm and the generation of a ΔpH, inside alkaline. Subsequently the end product lactate leaves the cell in the protonated (Hlactate) membrane-permeable form (Salema et al. 1994). Malolactic fermentation by *O. oeni* thus results in the generation of both components of the PMF: the $\Delta\Psi$, inside negative, by the inflow of negative charge with $Hmalate^-$ and a ΔpH , inside alkaline, by the internal consumption of protons in the decarboxylation reaction (Ramos et al. 1994). Another PMF-generating uniport system in *O. oeni* is coupled to the metabolism of citrate. This system involves a more complex metabolism and will be discussed below.

Metabolic energy generating proton-lactate symport systems

During glycolysis LAB continuously produce lactate at a high rate (up to 1 μmol/mg protein $\times$ min) and internal concentrations of lactate can be as high as 200 mM (Konings & Otto 1983). Lactate is a weak acid with a pK of 3.86. For most LAB the internal pH during glycolysis is above pH 7 (Poolman et al. 1987c) and essentially all lactate will be in the dissociated anionic form ($Lactate^-$). Unlike undissociated lactate (Hlactate) $Lactate^-$ is not membrane permeable and can only pass the membrane via a specific transport system (Figure 6). The lactate concentration gradient is directed from in to out and drives the lactate efflux process. When this $Lactate^-$ efflux occurs in symport with at least two protons the efflux process is electrogenic and can result in the generation of a $\Delta\Psi$, inside negative, and a ΔpH, inside alkaline. The energy of the lactate gradient is then converted to a PMF

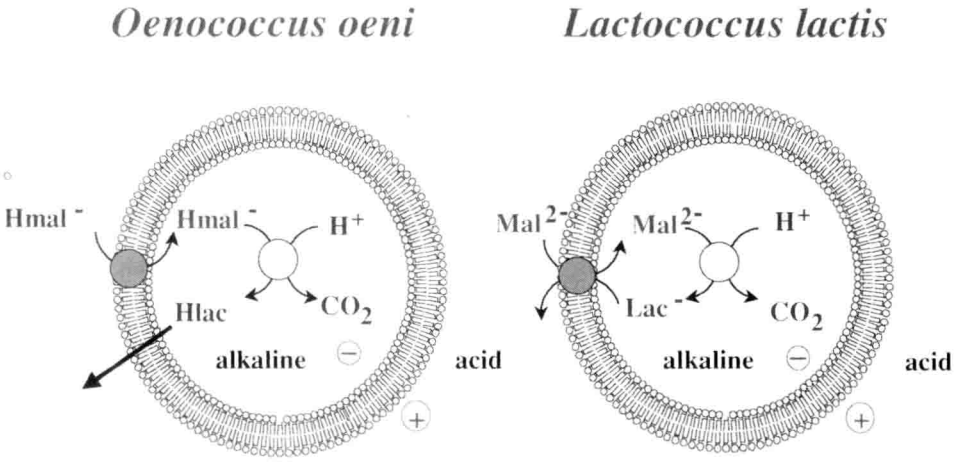

Figure 5. Metabolic energy conservation by malate transport and proton consuming decarboxylation. (A) *Oenococcus oeni*: uniport uptake of mono-anionic malate ($Hmal^-$), proton consumption by malate decarboxylation and passive efflux of undissociated lactate (Hlac). (B) *Lactococcus lactis*: exchange of di-anionic malate (Mal^{2-}) with mono-anionic lactate (Lac^-) and proton consumption by malate decarboxylation.

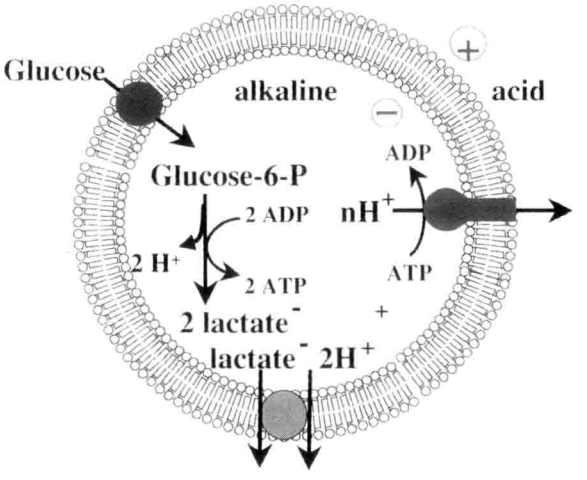

Figure 6. Metabolic energy conservation by electrogenic lactate efflux .

(Michels et al. 1979; Otto et al. 1980a,b, 1982; ten Brink & Konings 1982). This process has been called 'energy recycling' since the energy of the end product gradient is converted into metabolic energy in the form of a PMF. The number of protons excreted with lactate under optimal growth conditions of *L. lactis* has been found to be close to two (ten Brink & Konings 1982).

The metabolic energy gain from $Lactate^-$-proton excretion can be expressed in ATP-equivalents. With a proton/lactate stoichiometry of 2 and a proton/ATP stoichiometry in ATP-synthesis by the F_0F_1-ATPase between 3 and 4 (Jiang et al. 2001) the energy gain per lactate will be equivalent from 0.66 to 0.5 ATP. Glucose fermentation to lactate yields then 2.5 to 2.66 ATP equivalents instead of 2 and the additional energy yield by lactate efflux will be between 25 and 33%.

The beneficial effect of lactate excretion on the growth of *L. lactis* was demonstrated in mixed cultures of *L. lactis* and the lactate consumer *Pseudomonas stutzeri* (Otto et al. 1980a). Lactate consumption by *P. stutzeri* kept the lactate concentration in the medium low and as a result the molar growth yield of *L. lactis* increased significantly. Obviously, the gain of metabolic energy by lactate efflux depends strongly on the conditions inside and outside the cell. When the external pH decreases also the internal pH will decrease (see also Figure 11) (Poolman et al. 1987c) and more lactate will be present in the cytoplasm in the undissociated, membrane permeable Hlactate form. Consequently less $Lactate^-$ will efflux in symport with protons via the transport protein and the metabolic energy gain by lactate efflux will decrease. Furthermore, an increase of the lactate concentration externally as a result of the lactate production will decrease the driving force for the carrier-mediated efflux process and thermodynamic equilibrium will be reached at a lower proton motive force.

Metabolic energy by Precursor/Product exchange

During fermentation solutes (precursors) are taken up, metabolized intracellularly and the end products