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KONINGS, WN; Poolman, Berend; Driessen, Arnold

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BIOENERGETICS AND SOLUTE TRANSPORT IN LACTOCOCCI

Authors: **W. N. Konings**
B. Poolman
A. J. M. Driessen
Department of Microbiology
University of Groningen
Haren, The Netherlands

Referee: Peter C. Maloney
Department of Physiology
The Johns Hopkins University School of Medicine
Baltimore, Maryland

ABBREVIATIONS AND SYMBOLS

ADI	Arginine deiminase
ADP	Adenosine-5′-diphosphate
Agm/Put antiporter	Agmatine/putrescine antiporter
AIB	α-Amino isobutyric acid
AMP	Adenosine-5′-monophosphate
cAMP	Cyclic adenosine-3′,5′-monophosphate
Arg/Orn antiporter	Arginine/ornithine antiporter
ATP	Adenosine-5′-triphosphate
Ca^{2+}	Calcium ion
DCCD	N,N′-dicyclohexylcarbodiimide
2DG	2-Deoxy-D-glucose
2DG6P	2-Deoxyglucose-6-phosphate
DHAP	Dihydroxyacetone-phosphate
FDP	Fructose-1,6-diphosphate
Fru	Fructose
Fru-6P	Fructose-6-phosphate
Gal	Galactose
Gal-1P	Galactose-1-phosphate
Glc	Glucose
Glc-1P	Glucose-1-phosphate
Glc-6P	Glucose-6-phosphate
G3P	Glyceraldehyde 3-phosphate
K^+	Potassium ion
Lac	Lactose
2D-lactose	β-O-D-galactopyranosyl-(1′,4)-2-deoxy-D-glucopyranose
2F-lactose	β-O-D-galactopyranosyl-(1′,4)-2-deoxy-2-fluoro-D-glucopyranose
Man	Mannose
Man-6P	Mannose-6-phosphate
Na^+	Sodium ion
NADH	Reduced nicotinamide adenine dinucleotide
NAD^+	Oxidized nicotinamide adenine dinucleotide
N^6-(1-CE)Lys	N^6-(1-carboxyethyl)lysine
N^5-(1-CE)Orn	N^5-(1-carboxyethyl)ornithine
N^5-AcOrn	N^5-acetylornithine
pCMB	*p*-Chloromercuribenzoate

pCMBS	*p*-Chloromercuribenzene sulfonic acid
PEP	Phosphoenolpyruvate
P-β-galactosidase	Phospho-β-galactosidase
2-PG	2-Phosphoglycerate
3-PG	3-Phosphoglycerate
P_i	Inorganic phosphate
PtdCho	Phosphatidylcholine
PtdEtn	Phosphatidylethanolamine
PtdGro	Phosphatidylglycerol
Ptd_2Gro	Diphosphatidylglycerol
PTS	Phosphoenolpyruvate-dependent sugar transferase system
Suc	Sucrose
Tag-6P	Tagatose-6-phosphate
TMG	Methyl-β-D-thiogalactopyranoside
K_t	Apparent affinity constant for transport
K_i	Apparent affinity constant for competitive inhibition
K_D	Apparent dissociation constant for ligand binding
$\Delta\bar{\mu}H^+$	Transmembrane electrochemical potential difference for protons
$\Delta\bar{\mu}_A$	Transmembrane chemical potential difference for solute A
Δp (or pmf)	Protonmotive force ($0^+ = \Delta\bar{\mu}_H + /F$)
Δψ	Transmembrane electrical potential difference
ΔpH	Transmembrane pH gradient ($= pH_{in} - pH_{out}$)
ZΔpH	2.3(RT/F) ($pH_{in} - pH_{out}$)
$\Delta G'_p$	Phosphate potential
$\Delta G^{o\prime}$	Standard free energy of ATP hydrolysis
~P	Energy-rich phosphate bond
V_{max}	Maximal velocity of transport

I. INTRODUCTION

Streptococci are Gram-positive, catalase-negative, nonsporulating, facultative anaerobic bacteria which ferment carbohydrate to L(+)-lactate as predominant endproduct.[1] Extensive nucleic acid hybridization, physiological, comparative immunological, lipid, and lipoteichoic acid composition studies have provided new insights in the relationships among streptococci, and have been used for a new fundamental taxonomic grouping.[2] The genus *Streptococcus* has now been divided into three genera: (1) the genus *Streptococcus sensu stricto,* comprising the majority of known species, in particular the pyrogenic and oral steptococci, (2) the genus *Enterococcus,* which contains the enterococcal species, such as *S. faecalis,* and (3) the genus *Lactococcus,* encompassing all lactic streptococci (Group N streptococci), e.g., *S. lactis* (and its subspecies), *Lactobacillus hordniae, L. xylosus, S. garvieae, S. plantarum,* and *S. raffinolactis.*[3] Other streptococci including motile *S. lactis* strains which possess the group N antigen are not directly related to these organisms. Also within the *Lactococcus* genus a reclassification has been proposed and the suggestion has been made to rename lactic streptococci of Lancefield serological group N *Streptococcus lactis* (and *Streptococcus diacetylactis*) and *Streptococcus cremoris* in *Lactococcus lactis* and in *Lactococcus lactis* subspecies *cremoris,* respectively. In this review we follow this new taxonomy. Since most readers are familiar only with the old names of the organisms, we have also given these old names in brackets when the new names are given for the first time.

This article mainly reviews the current information about energy transduction in the new genus *Lactococcus* (group N streptococci). Exceptions are made for subjects related to energy

transduction which have not been studied extensively in *Lactococcus* species but received more attention in other "streptococci".

Various properties of lactococci are unstable and have been shown to be plasmid linked. This has been demonstrated for citrate metabolism, the utilization of certain carbohydrates, proteinase activity, modification/restriction systems, inorganic ion resistance, drug resistance, and the production of inhibitory agents like nisin, while for some other properties plasmid DNA has been speculated to be responsible.[4,5]

Lactococci are strictly fermentative and can derive their metabolic energy only from substrate level phosphorylation and chemiosmotic energy-generating processes.[6,7] The relatively simple metabolism of these bacteria makes high demands on the (nutritional) composition of the growth media which usually consist of various amino acids, vitamins, nucleic acids, and others in addition to mineral components and a carbohydrate source. Such requirements limit the natural habitat of these organisms. As a consequence, growth of lactococci is rarely observed in an aqueous environment but often in plant, meat, and dairy products.[8]

II. SUGAR TRANSPORT AND METABOLISM

A. Transport Mechanisms and Sugar Phosphorylation

Lactococci degrade carbohydrates by the glycolytic pathway. The initial steps of carbohydrate fermentation, however, may differ depending on the sugar metabolized and/or the organism investigated. This paragraph will first describe the sugar transport mechanisms which have been found in lactococci and subsequently discuss the information about certain well studied sugar transport systems.

Two mechanisms of energy coupling to sugar transport can be recognized in lactococci (Figure 1): (1) secondary transport via a proton-sugar symport system.[9-11] A specific transport protein facilitates the accumulation of free sugar against a concentration gradient by coupling sugar uptake to the downhill translocation of protons in response to the protonmotive force (pmf) (Figure 1). The accumulated free sugar is phosphorylated intracellularly and (2) phosphoenolpyruvate (PEP)-dependent sugar transferase systems (PTS).[12-16] Sugar uptake by PTS involves chemical modification (phosphorylation) of the solute translocated and release of the reaction product (sugar-phosphate) at the inner side of the membrane (Figures 1 and 2).[15] The driving force for transport is supplied by PEP, of which the phosphoryl group is transferred to the sugar via a number of cytoplasmic phosphoryl carrier proteins. High energy phosphoryl group transfer proceeds by reversible (de)phosphorylation of a specific histidine residue of each of these proteins. The cytoplasmic proteins include sugar specific protein (i.e., Enzyme III) and sugar nonspecific components (i.e, Enzyme I and HPr). The latter can be shared by various PTSs.[15,17] The translocation step of sugar across the cytoplasmic membrane is catalyzed by sugar specific transmembrane proteins (Enzyme II). For PTSs in which the soluble Enzyme III (apparently) has not been found, e.g., the mannose-PTS of *L. lactis*, it has been proposed that this protein is associated with the membrane and/or is part of the corresponding Enzyme II.[16]

The low molecular weight protein HPr and Enzyme I of a number of organisms including *L. lactis* have been purified to homogeneity.[18,19] Although none of the lactococcal Enzymes II have been isolated and purified yet, the nucleotide-sequence for Enzyme II^{lac} of *L. lactis* has recently been resolved.[20] The functional and structural relationships between PTS proteins of various organisms have recently been reviewed.[18]

The major sugar transport systems, which have been identified in lactococci, have been listed in Table 1 according to the mechanism of energy coupling and the substrate specificity.

1. Glucose

The main transport system for glucose in *L. lactis* ML_3 and other lactococci is the

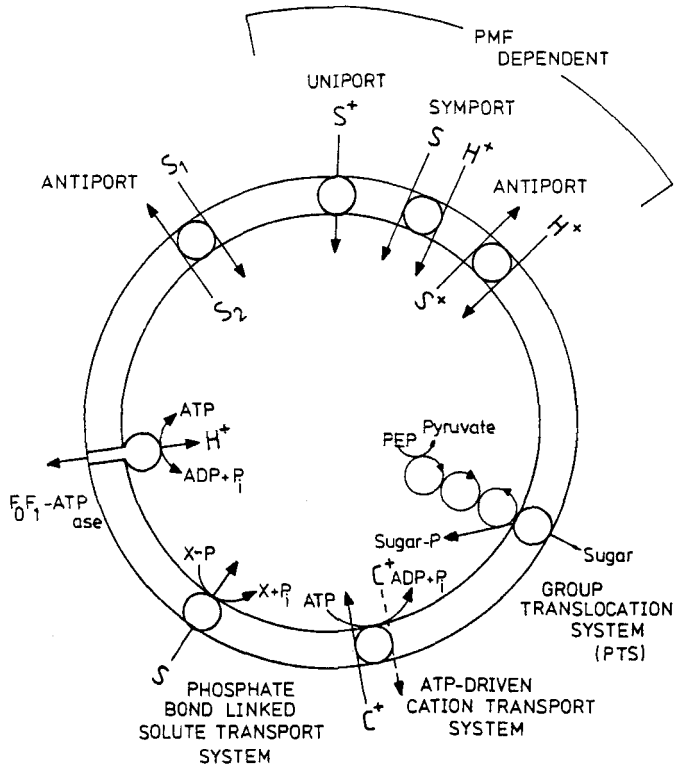


FIGURE 1. Schematic presentation of the mechanisms of carrier-mediated solute transport in lactococci. The solid and dashed arrows of ATP-driven cation (C) transport systems indicate mechanism for uptake and extrusion of cations, respectively. Solutes and protons are indicated by S and H^+, respectively.

constitutive glucose-PTS (glc-PTS).[11,21-25] This system has a high affinity for glucose (K_t = 15 - 23 μM) and 2-deoxy-D-glucose (2DG) (K_t = 80 μM).[22] Mannose and glucosamine are taken up by the glc-PTS, whereas a number of glucose analogs and galactose are not transported. The inhibition constants (K_is) for competitive inhibition of glucose uptake by mannose and glucosamine have been estimated to be 60 and 500 μM, respectively (Table 1). Since the glucose analogue α-methylglucose is not a substrate for this PTS the system is often referred to as mannose-PTS (man-PTS) in analogy to the substrate specificity of one of the glc-PTSs of *Escherichia coli*.[13] The product of the glc-PTS, glucose-6P (Glc-6P), is normally metabolized by the Embden Meyerhof-Parnas (glycolytic) pathway (Figure 2). However, an intracellular hexose-6P:phosphohydrolase has been identified in *L. lactis* by which Glc-6P can be hydrolyzed without the generation of metabolic energy (see Section II.C).[26]

Spontaneous man-PTS defective mutants of *L. lactis* ML_3 and 133 have been isolated which are resistant to 2DG.[25,27] These mutants still grow on glucose, Importantly, mutants which are defective in both the man-PTS and the cytoplasmic ATP-dependent glucokinase, failed to grow in medium containing glucose or glucosamine, suggesting the presence of a second transport system for glucose which could be driven by the pmf.[28]

In *Enterococcus faecalis* (former name *Streptococcus faecalis*)[29] and *S. pyogenes*,[30] a Enzyme II^{man} is present which is very similar to that of *L. lactis*.[22] On the other hand, the oral streptococci *S. salivarius* and *S. mutans* possess a glc-PTS and/or a man-PTS with specificity for glucose, α-methyl-glucoside and 2DG, and glucose, mannose and 2DG,

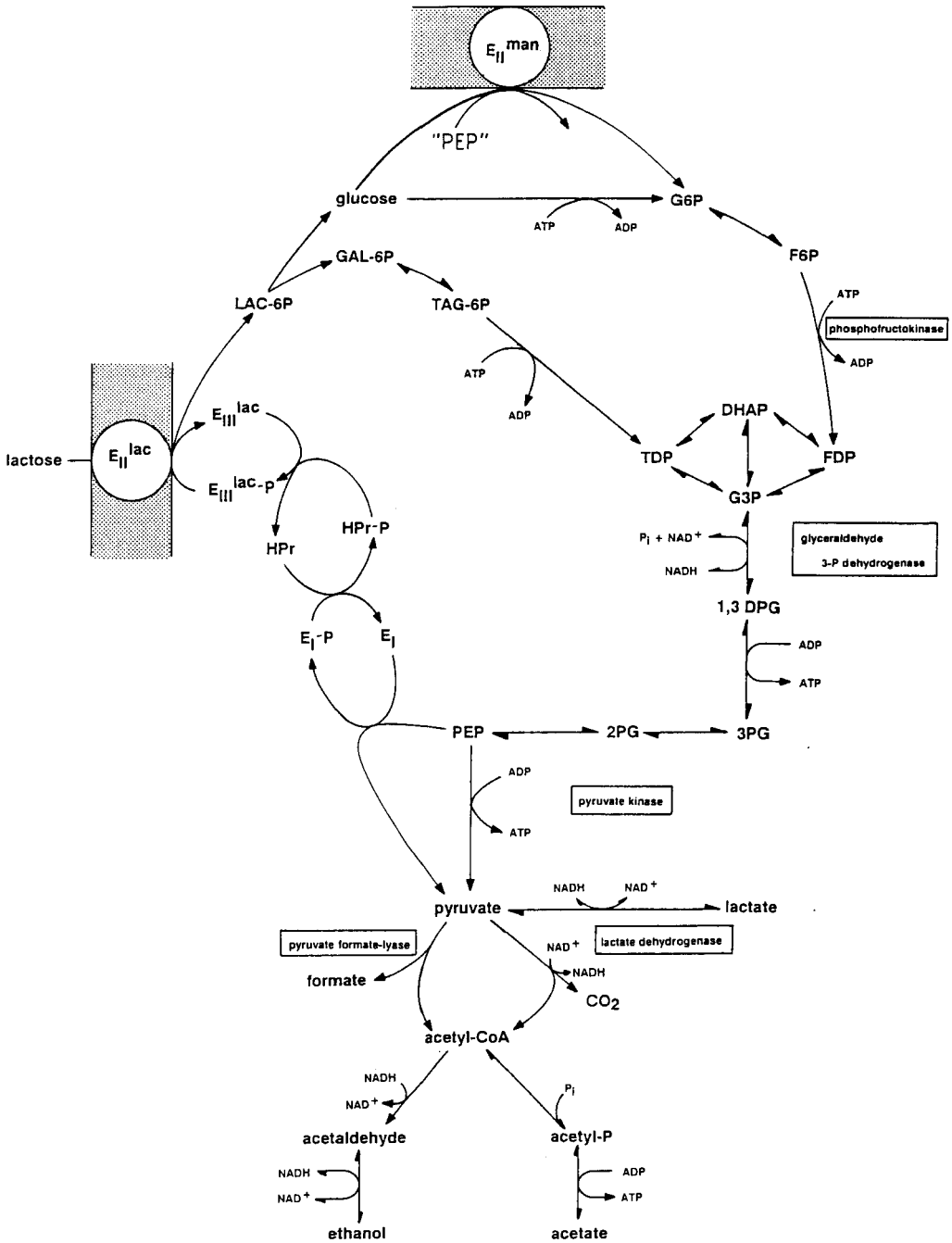


FIGURE 2. Schematic presentation of glycolytic cycle in lactococci.

respectively.[16] These systems resemble the glucose PTSs of *E. coli*, except that Enzyme III seems to be membrane associated.[13]

Some oral streptococci (*S. salivarius*, *S. sanquis*, and *S. mitis*) have the ability to synthesize glycogen for which glucose is phosphorylated at the C_1-position.[31] The phosphoryl donor(s) for the formation of glucose-1-phosphate (Glc-1P) have been identified as carbamoyl phosphate and acetyl-phosphate. Evidence has been presented which indicates that glucose can

Table 1
AFFINITY CONSTANTS OF SUGAR TRANSPORT SYSTEMS IN *LACTOCOCCUS LACTIS* STRAIN ML_3/133 AND STRAIN ATCC 7962

L. lactis strain ML_3/133			*L. lactis* strain ATCC 7962		
Transport system	**Affinity constant**	**(mM)**	**Transport system**	**Affinity Constant**	**(mM)**
Glc/Man-PTS[22]	K_t glucose	0.015	Glc-PTS[38]	K_t glucose	0.023
	K_i mannose	~0.06[a]			
	K_t 2-DG	0.081			
	K_i glucosamine	~0.5[a]			
Lac-PTS[11,37]	K_t TMG	0.7	Lac-PTS[38]	K_t lactose	0.7
	K_t lactose	0.016—0.021			
	K_t 2'D-lactose	0.016			
	K_t 2'F-lactose	0.016			
Gal-PTS[24]	K_t galactose	1.07	Gal-PTS[38]	K_t galactose	21.7
Gal-carrier[24]	K_t galactose	0.13	Gal-carrier[10,45]	High affinity for galactose and TMG	
	K_i TMG	~0.16[b]			
	K_i Methyl-β-galactose	~0.20[b]			
	K_i isopropyl-1-thio-β-D-galactropyranoside	~0.74[b]			
Suc-PTS[48,49]	K_t sucrose	0.022			
			P_i: sugar-6P antiport[53]	K_t glucose-6P	0.017—0.025
				K_t 2DG-6P	0.022—0.026
				K_i mannose-6P	0.023
				K_i fructose-6P	0.150
				K_i glucosamine-6P	0.420

[a] Affinity constant for competitive inhibition (K_i) by analogs on glucose transport by cells of *L. lactis* ML_3.
[b] Affinity constant for competitive inhibition (K_i) by analogs on galactose transport by cells of *L. lactis* ML_3.

be taken up in these organisms by a pmf-dependent system in addition to a PTS.[32] With the former transport system glucose enters the cell as free sugar.

2. Lactose

Lactose transport in lactococci is mediated by an inducible lactose-PTS (lac-PTS),[11] which is plasmid-coded (Lac plasmid).[5,33-35] Lac-PTS activity in "starter" lactococci is maximally expressed upon growth in media containing lactose or galactose; glucose acts as a repressor of lac-PTS activity.[36] By enzyme complementation analysis it has been shown that *L. lactis* C_2 Lac^- variants missing the Lac plasmid were deficient in Enzyme II^{lac}, Enzyme III^{lac}, and also phospho-β-galactosidase (P-β-galactosidase). The lac-PTS in the homolactic *L. lactis* strains exhibits a high affinity for lactose (K_t = 16 to 21 μM) and for lactose analogs in which the glucosyl moiety has been replaced by nonmetabolizable glucose analogues, e.g., β-O-D-galactopyranosyl-(1′,4)-2-deoxy-D-glucopyranose (2D-lactose) and β-O-D-galactopyranosyl-(1′,4)-2-deoxy-2-fluoro-D-glucopyranose (2F-lactose) (Table 1).[11,37] By contrast, the heterofermentative *L. lactis* ATCC 7962 transports lactose via a PTS with very low affinity.[38] The nonmetabolizable lactose analogue methyl-β-D-thiogalactopyranoside (TMG) is often used to assay lac-PTS activity.[21,25,39] It should be realized, however, that the K_t for TMG uptake is 30 to 40 times higher than the K_t for lactose (Table 1).

Lactose-6P[11] is hydrolyzed intracellularly by P-β-galactosidase yielding glucose and Gal-6P (Figure 2). Intracellular glucose can be phosphorylated via two mechanisms: (1) by an ATP-dependent glucokinase or (2) by the man-PTS.[11,28,37] The latter mechanism was demonstrated to function by loading *L. lactis* cells with the nonmetabolizable substrates 2D-lactose or 2F-lactose, synthesized from galactose and 2-deoxy-D-glucose (2DG) or 2-fluoro-D-glucose, respectively. The latter nonmetabolizable compounds are substrates for the man-PTS. Subsequently, upon hydrolysis of 2D-lactose and the generation of PEP by the metabolism of the Gal-6P moiety,[37] phosphorylation of 2DG could be shown intracellularly in a mutant lacking glucokinase and under conditions which preluded re-entry of 2DG via the man-PTS. Since 2DG is not phosphorylated in a double mutant lacking both glucokinase and man-PTS activity, these findings indicate that glucose (analogs) can be phosphorylated intracellularly by the man-PTS.[28,37] Growth of the double mutant on lactose is accompanied by the excretion into the medium of 1 mol of glucose per mole of metabolized lactose.[28] Some evidence has been presented suggesting that the phosphoryl donor for phosphorylation by the man-PTS is PEP and not ATP.[11]

Lactose transport in *L. lactis* ATCC 7962 is facilitated not only by a PTS but (most probably) also by a secondary transport system.[36,38] When lactose is taken up as free sugar, subsequent metabolism involves hydrolysis by β-galactosidase and degradation of glucose and galactose by the glycolytic and Leloir pathway, respectively. In the Leloir pathway galactose is converted to galactose 1-phosphate (Gal-1P) by galactokinase, and the sugar phosphate enters glycolysis after conversion to Glc-6P (via Glc-1P).[6] The slow rate of lactose fermentation by *L. lactis* ATCC 7962 has been attributed to the low activity of phospho-β-galactosidase in these cells.[38] Due to the high activity of the lac-PTS, lactose-6′-phosphate accumulates to concentrations above 100 mM by which non-PTS catalyzed lactose uptake (and the overall metabolism of lactose) may be inhibited.

Lactose transport in *S. thermophilus* is most likely facilitated by a pmf-dependent mechanism.[40] Since most strains of *S. thermophilus* cannot metabolize the galactose moiety of the disaccharide, one mole of galactose is excreted in the medium per mole of lactose consumed.[41-43] While strong evidence is available for lac-PTS activity in oral streptococci, under some conditions (in some strains) non-PTS mediated uptake of lactose followed by hydrolysis by β-galactosidase appears to be the initial steps of lactose metabolism.[16,44]

3. Galactose

Direct evidence for pmf-driven sugar uptake in lactococci has been obtained for β-gal-

actosides in *L. lactis* ATCC 7962.[10,45,46] Upon imposition of an artificial potassium (K^+) diffusion potential in the presence of valinomycin (a specific K^+ ionophore) transient TMG accumulation could be observed.[10] The galactose carrier of *L. lactis* ATCC 7962 is highly specific for galactose, methylgalactoside, TMG, and thio-β-D-digalactopyranoside (TDG), and exhibits a poor affinity for glucose and lactose (Table 1).[45]

Cells of *L. lactis* ML_3 contain a galactose carrier (K_t = 0.13 mM) in addition to a galactose-PTS (gal-PTS) (K_t = 1.1 mM) (Table 1).[16,24] Based on inhibition studies with proton-conducting ionophores, it has been suggested that galactose transport by this carrier is driven by the pmf.[24] The substrate specificity of this system appears to be similar to that of the galactose carrier of *L. lactis* ATCC 7962 (Table 1).

The gal-PTS has a substrate specificity which overlaps with that of the lac-PTS. Enzymatic analysis and genetic studies have provided evidence that both systems are separate entities.[47] For instance, lac-PTS can be selectively inhibited by *p*-chloromercuribenzoate (pCMB), whereas gal-PTS is insensitive to the sulfhydryl reagent.[24]

4. Sucrose and Fructose

Sucrose is transported by a specific high affinity sucrose-PTS (suc-PTS) in *L. lactis* K_1 (K_t = 22 μM) and DR_{1251}.[48,49] Suc-PTS activity is inducible in *L. lactis* K_1 and detectable only after growth on sucrose. Most strains, however, are unable to metabolize sucrose, suggesting plasmid linkage of the suc-PTS.[5] Sucrose-6P is hydrolyzed intracellularly by an inducible sucrose-6P hydrolase to form Glc-6P and fructose. Fructose is phosphorylated by an ATP-dependent (manno)-fructokinase to fructose-6-phosphate (Fru-6P), and subsequently degraded by the glycolytic pathway. When fructose is present extracellularly, this sugar is transported most probably by a separate PTS (fru-PTS).[28]

Sucrose transport has also been studied in various streptococci such as strains of *S. mutans*.[50,51] Again evidence has been obtained for non-PTS mediated transport (K_t = 3 mM) as well as for suc-PTS activity (K_t = 60-250 μM).[50]

5. Maltose, Xylose, and Other Sugars

Maltose-PTS activity was undetectable in cells of *L. lactis* ML_3 and 65.1 grown on maltose.[16] Uptake of the disaccharide is observed upon addition of arginine (as a source of ATP synthesis), suggesting the involvement of a pmf-driven system.

The ability of *L. lactis* DR_{1253} to grow on xylose appears to be plasmid linked but the mechanism of D-xylose transport has not yet been solved.[5] Also transport of rhamnose and arabinose in some *Lactococcus* species appears to be plasmid-linked.[49]

6. Glucose-6-Phosphate

L. lactis subspecies *lactis* ATCC 7962 and *cremoris* E_8 possess an unique transport system that exchanges inorganic phosphate (P_i) for certain sugar-6Ps.[52-54] This transport system has been studied in intact cells, in membrane vesicles, and in proteoliposomes.[52-58] High levels of transport activity were found after cells were grown in the presence of rhamnose or arabinose. Activity is repressed by a number of sugars including glucose.[53] Homologous phosphate exchange exhibits a K_t of about 200 μM between pH 5.5 and 6.5, which increases somewhat (from 200 to 330 μM) when the pH approaches the pK_2 of phosphate (i.e., 7.2), indicating that $H_2PO_4^-$ is the preferred substrate.[53] Heterologous exchange studies have been performed using membrane vesicles loaded with either P_i or 2-deoxyglucose-6-phosphate (2DG6P).[52,57] The results suggest that the exchange stoichiometry depends on the pH of the medium. At pH 7.0 and above, two molecules of P_i are translocated per molecule of 2DG6P, whereas at pH 5.2 this stoichiometry drops to one.[57] Under all conditions tested the exchange reaction is electroneutral. The K_t for 2DG6P is nearly independent of pH, indicating a random choice among mono- and divalent forms of this substrate. The pH dependence of the exchange stoichiometry can therefore entirely be attributed to the pH effect on the valence

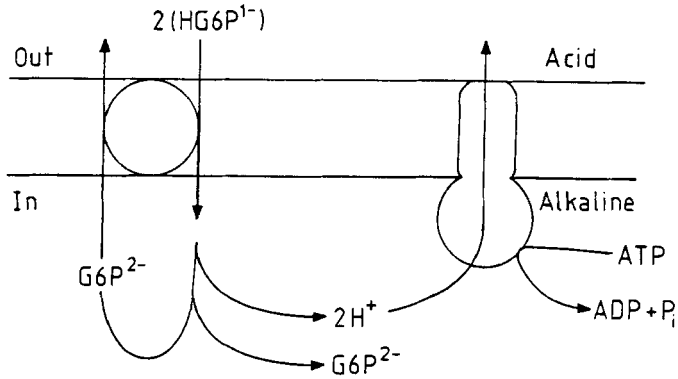


FIGURE 3. A model of anion exchange in which net glucose 6-phosphate accumulation occurs by self-exchange between extracellular monovalent and intracellular divalent glucose 6-phosphate as a consequence of a pH gradient across the membrane.[54]

of the sugar-6-phosphate.[57] Self-exchange of Glc-6P in the presence of a ΔpH (inside alkaline) should in principle result in net accumulation of sugar phosphate via exchange between *one* molecule Glc-6P^{2-} on the inside ($pH_{in} > 7.0$) and *two* molecules of Glc-6P^{1-} on the outside ($pH_{out} < 7.0$) (see Figure 3).[54] The anion antiporter is inhibited by mercurials but not by maleimides.[52]

The functional role of the anion antiporter is not yet clear. This system might be used to scavenge sugar-6Ps from the medium in order to grow on Glc-6P as carbon and energy source,[58] or to excrete these metabolites when intracellular concentrations become bactericidal. Alternatively, the anion antiporter can function in regulating the intracellular P_i concentration which in turn affects glycolysis (see Section II.C).[54]

B. Sugar Metabolism and Product Formation

L. lactis species can be subdivided into two classes:[59] (1) starter strains in milk fermentations which convert lactose rapidly and almost quantitatively (>90%) into L(+)-lactic acid,[60] and (2) strains that ferment lactose slowly and produce a variety of products.[60] During growth of these organisms on lactose usually about 15% of the sugar is recovered as L(+)-lactic acid.[61]

Well-studied representatives of both groups are *L. lactis* ML_3 (and 133) and ATCC 7962, respectively. Differences between these organisms are not only found with respect to lactose metabolism, but also for the transport mechanisms of some essential nutrients, the reaction with group N antiserum and the presence of cyclic adenosine 3′,5′-monophosphate (cAMP) (and associated enzymes).[39,61,62]

With a few exceptions (for instance sugars metabolized by the D-tagatose 6-phosphate pathway) most sugars fermented by lactococci enter glycolysis at the level of Glc-6P or Fru-6P (see Figure 2).[16] When galactose is taken up as free sugar or formed after hydrolysis of free lactose by β-galactosidase, galactose is directed into the Leloir pathway. It has been suggested that the enzymes of the Leloir pathway are repressed during growth of "starter" lactococci on lactose,[63] i.e., when lactose is transported by the lac-PTS. After hydrolysis of Lac-6P by P-β-galactosidase or when extracellular galactose is transported by the gal-PTS, Gal-6P is formed which is converted to D-tagatose 6-phosphate (Tag-6P) by D-galactose 6-phosphate isomerase, then further phosphorylated by D-tagatose 6-phosphate kinase to D-tagatose 1,6-bisphosphate and split to dihydroxyacetone-phosphate (DHAP) and glyceraldehyde 3-phosphate (G3P) by D-tagatose, 1,6-bisphosphate aldolase.[64]

The relative flux of galactose moieties through the tagatose-6P and the Leloir pathway is

determined by the form in which galactose is transported into the cell. As shown in Table 1, the pmf-driven galactose uptake system of *L. lactis* ML_3 has about tenfold higher affinity for galactose than the gal-PTS, suggesting that the Leloir pathway is the predominant initial route of galactose metabolism at low substrate concentrations. In fact, at galactose concentrations in the medium below 1 mM, high intracellular levels of Gal-1P (13.6 mM) in addition to Gal-6P (7.4 mM) are found in *L. lactis* ML_3.[65] On the other hand, *L. lactis* subspecies *cremoris* E_8, which apparently lacks the pmf-driven galactose transport system and accumulates galactose via gal-PTS (K_t = 15 to 27 mM), contains high concentrations of Gal-6P (5.7 mM) and no detectable Gal-1P during growth.[65,66] In the presence of other sugars like glucose or lactose, galactose is not significantly metabolized in contrast to glucose and lactose.[23] The sequential utilization of these sugars seems to result from catabolite inhibition (and not catabolite repression) of enzyme systems(s) specific for galactose metabolism.[23]

In general, the products of sugar metabolism of lactococci depend highly on the growth conditions. Although most organisms carry out obligate homolactic fermentation, product formation is markedly influenced by the imposed growth (glycolytic) rate, the nature of the growth limiting substrate, the specific sugar metabolized, and/or the presence of oxygen in the environment. Homolactic fermentation is generally found under anaerobic conditions and at high growth (glycolytic) rates with glucose and lactose.[61,67,68] When lactose (or glucose) becomes limiting, fermentation switches and acetate, formate, and ethanol can be formed in addition to lactate (mixed acid fermentation) (see Figure 2).[61,67,68] At 5% of the maximum rate of lactose utilization and under aerobic conditions, glycolysis of nongrowing *L. lactis* strains ML_3 and ML_8 can even result in the formation of acetate as major product.[69] This pattern of product formation (under aerobic conditions) is, however, not observed in resting cells metabolizing lactose at maximal rates nor in growing cells. The (quantitative) conversion of carbohydrates to acetate leads to the generation of an additional ATP by substrate level phosphorylation via acetate kinase. Finally, in contrast to the starter strains (*L. lactis* and subsp. *cremoris*) used in the dairy industry, "nonstarter" (or wild type) strains ferment lactose slowly and invariably produce a variety of endproducts.[38]

To maintain the intracellular redox balance under conditions that sugars are metabolized quantitatively to acetate, an exogenous electron acceptor is required. Since lactococci contain flavin-type NADH oxidases and NADH peroxidase, electrons can be donated to oxygen which obviates the need for pyruvate reduction to lactate or reduction of acetyl-CoA to ethanol. The increase in specific activities of NADH-oxidase and NADH-peroxidase (together with superoxide dismutase) in different strains of *L. lactis* and *S. thermophilus* upon aerobic growth enables the cells to reoxidize NADH in the presence of oxygen and to remove toxic oxygen metabolites.[69,70] The oxygen sensitivity observed for some lactococci could be due to an imbalance of oxidase and peroxidase activities which may result in the accumulation of the toxic intermediate H_2O_2.[69] In principle sugar metabolism by lactococci may also yield acetate as a major end product under anaerobic conditions by using fumarate reductase. In this scheme electrons would have to be transferred from a NADH dehydrogenase via (mena)quinones to fumarate reductase. All these components have been found in the cytoplasmic membrane of *L. lactis* subspecies *cremoris*.[71]

C. Regulation of the Glycolytic Pathway

The products of sugar metabolism are largely determined by the fate of pyruvate and NADH for which a number of enzymes compete in order to regenerate NAD^+ (see Figure 2). The control of the flux of pyruvate and NADH is not yet fully understood. However, the fermentation patterns observed are in accordance with a number of enzymatic properties:

1. At limiting rates of glycolysis, i.e., under conditions of mixed acid fermentation, the intracellular concentrations of fructose 1,6-diphosphate (FDP) (activator of lactate

dehydrogenase) and triose phosphates (inhibitors of pyruvate formate-lyase) in *L. lactis* ML_3 are low. Conversely, at high glycolytic rates, i.e., under conditions of homolactic fermentation, the concentrations of these intermediates are high.[67,72]

2. The changes in concentrations of FDP and triose phosphates with varying imposed glycolytic rates are much smaller in *L. lactis* strain ML_8 which does not exhibit the shift in fermentation pattern.[67]
3. In various aerobically grown lactococci the levels of NADH oxidase, NADH peroxidase, and pyruvate dehydrogenase are increased whereas the level of lact 'e dehydrogenase is decreased in comparison to anaerobically grown cells.[69]
4. Upon addition of lactose or glucose the rate of oxygen uptake (i.e., NADH oxidase/peroxidase activity) in washed cell suspensions of *L. lactis* is biphasic which correlates with the build up of factors affecting the *in vivo* activity of lactate dehydrogenase.[69]
5. Metabolism of free galactose by lactococci leads to relatively low levels of FDP and lactate dehydrogenase, resulting in mixed acid fermentation.[65,73] The level of pyruvate-formate lyase in these cells is elevated three- to fourfold compared to growth on glucose.[65]

Although the kinetic properties of several glycolytic (key)-enzymes have been studied in detail, quantitative information on the control of the overall glycolytic activity by individual enzymes is hardly available.[75-77] To quantitate the control (control strength) that enzymes exert on the steady state flux through a metabolic pathway (or on the steady state concentration of an intracellular metabolite) the metabolic control theory has been proposed[78,79] and methods have been developed to determine the quantitative control parameters.[80] Application of the control theory on oxidative phosphorylation in rat-liver mitochondria and on various other metabolic pathways has indicated that control is usually exerted by several steps and not by a single (rate-limiting) enzyme.[81,82] Control analysis of glycolytic enzymes in lactococci is restricted thus far to P-β-galactosidase and glyceraldehyde 3-phosphate dehydrogenase. Upon cloning of the gene for P-β-galactosidase in *L. lactis* ML_3 the expression of this enzyme has been increased by 21 to 54% without affecting the rate of acid production in milk.[83] This indicates that the flux control coefficient (C_{Ei}^{J}) for P-β-galactosidase is zero under the conditions employed. In contrast, the flux control coefficient of glyceraldehyde 3-phosphate dehydrogenase in glycolyzing cells of *L. lactis* is close to 1 indicating that this enzyme can be significantly rate limiting (see Section I.C.3.b.)

The synthesis of many inducible enzyme systems in bacteria is regulated by cAMP.[84] Despite the detection of cAMP and the associated enzymes adenylate cyclase and phosphodiesterase in lactococci, except for *L. lactis* strain ATCC 7962, the involvement of cAMP as control mechanism of metabolic pathways, e.g., glycolysis, has hardly been considered in these organisms.[62] The intracellular concentrations of cAMP in *L. lactis* C_2 cells can be increased about two-fold by prostaglandin E_1 (stimulates adenylate cyclase)-treatment and this high cAMP concentration prevents repression of P-β-galactosidase synthesis by glucose.[62] Elevated levels of cAMP have also been shown to increase lactic acid production by *L. lactis*.[85]

Qualitative information on the control of glycolysis by individual enzymes (in growing, glycolyzing and starving lactococci) has been obtained from analyses of pools of glycolytic intermediates, regulatory properties of enzymes as well as from enzyme levels. The major enzyme systems of the glycolytic pathway that have been studied in this respect will be discussed.

1. Regulation of Phosphoenolpyruvate-Dependent Phosphotransferase Systems

The simplest mechanism of regulation of carbohydrate metabolism by PTSs is sugar exclusion by competition for a common Enzyme II. In case of lactose Enzyme II the apparent

affinity constant for lactose is usually about 100-fold lower than that for galactose.[17] In addition to this type of control which holds (in principle) for any transport system, the hierarchical order of sugar utilization by PTS can be determined by the degree to which sugar specific Enzyme IIIs compete for phospho-HPr {HPr(His~P)}. For instance, the exclusion of TMG has been attributed to the preferential utilization of HPr(His~P) during translocation of glucose by the man-PTS.[16,17]

The accumulation of sugars can also be regulated by expulsion of intracellular sugars.[14,25,30,86] Lactococci (and streptococci) preloaded with non-metabolizable galactoside-phosphate analogues like TMG-P or phosphoisopropyl-1-thio-β-D-galactopyranoside rapidly expel the free sugar analogues upon addition of a metabolizable sugar. Evidence has been presented indicating that the release of free sugar is not due to transphosphorylation nor to some kind of exchange reaction. Rather, expulsion appears to be caused by the intracellular dephosphorylation of the galactoside phosphates, followed by efflux of the free sugar (Figure 4). Since exit of galactoside analogues was prevented by eliminating the gene for lactose Enzyme II or reduced by specific competitive inhibitors of this enzyme, efflux most likely has to be facilitated by the Enzyme II^{lac}.[87] Dephosphorylation of sugar phosphates requires the presence of ATP[25] and an early glycolytic intermediate (like FDP) intracellularly.[15,86,87] Although a sugar-phosphate hydrolase has been purified (and characterized) from *L. lactis* cells it has not been established whether this enzyme is involved in the process of galactoside expulsion.[26]

By analogy to inducer exclusion, which decreases the intracellular inducer concentration by inhibition of uptake, expulsion of galactoside-phosphate analogues from lactococci has been referred to as inducer expulsion mechanism since sugar-phosphates act internally as inducers of the Lac regulon.[17] In addition to the mechanism of inducer expulsion, modulation of the intracellular concentration of sugar phosphates is also possible by release of these metabolites without dephosphorylation of the sugar. As already discussed above, exchange between P_i and various hexose 6-phosphates has been observed in *L. lactis* ATCC 7962 independent of a pmf.[52,53] On the basis of the sugar specificity of the anion antiporter and the man-PTS, the activities of these transport systems have been suggested to be closely related.[52] The affinity constants (K_ts) for sugar-phosphate transport by the antiporter can be ranked in the same order as the K_ts of man-PTS for the corresponding free sugars; i.e., Glc-6P ~ 2-deoxyglucose 6-phosphate (2DG-P) ~ mannose 6-phosphate (Man-6P) < fructose 6-phosphate (Fru-6P) < glucosamine 6-phosphate < some other metabolites for which the affinity constant exceeds 0.5 mM.[52,53] The advantage of releasing sugar phosphates by anion antiport over the coordinate operation of a phosphatase and a sugar exit mechanism is that (in principle) metabolites can be reaccumulated by the antiporter without loss of metabolic energy. The operation of the phosphatase introduces a futile cycle in the energy metabolism of lactococci.[27] A functional role of the devices which affect the intracellular concentrations of sugar phosphates has been sought in preventing accumulation of these metabolites to bactericidal concentrations.[17]

Another level at which sugar metabolism in lactococci can be regulated is by chemical modification of a serine residue on HPr (Figure 4). HPr(Ser) is phosphorylated by an ATP-dependent HPr(Ser) kinase in a reaction which is allosterically inhibited by P_i and stimulated by FDP and various other glycolytic intermediates.[88,89] Phosphorylation of HPr(Ser) may serve to modulate both the rate of sugar transport and the sequence of sugar transport, since: (1) e.g., HPr(Ser-P) cannot serve as substrate for PEP-dependent phosphorylation by Enzyme I and (2) cannot replace HPr(His~P) in the PTS-mediated phosphorylation of sugars. Relief of regulation of PTS activity by HPr(Ser-P) can be achieved by hydrolysis of the phosphoryl bond by a phosphoprotein phosphatase (stimulated by P_i) or by complexation of HPr(Ser-P) with various Enzyme IIIs which allow (to varying degrees) Enzyme I-mediated phosphorylation of HPr(Ser-P). This process yields HPr(Ser-P)-(His~P) which can serve as

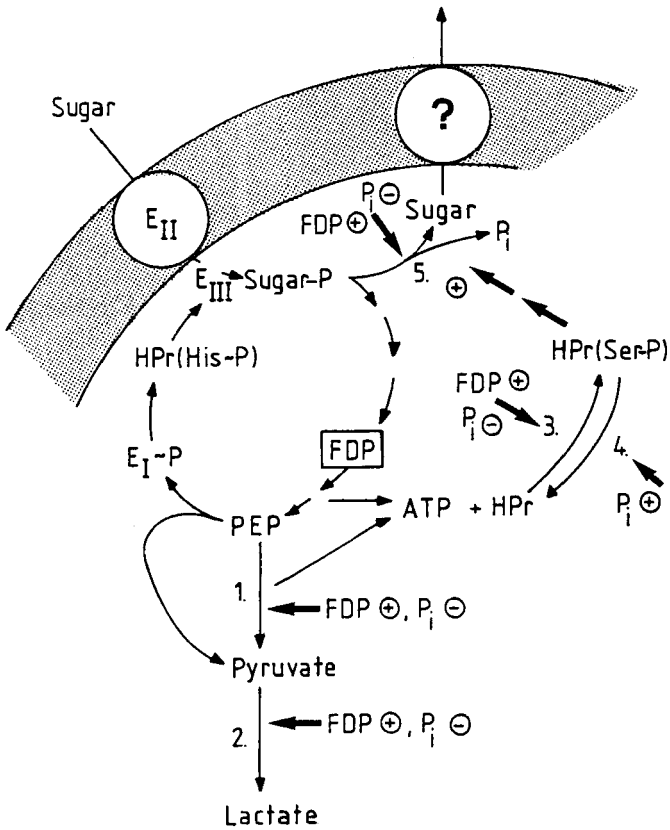


FIGURE 4. Sites of control of carbohydrate fermentation by FDP and P_i in lactococci. The intracellular levels of FDP and P_i modulate the activity of glycolysis by coordinate regulation of pyruvate kinase (1.), lactate dehydrogenase (2.), HPr(Ser) kinase (3.), HPr(Ser-P) phosphatase (4.), and a sugar-phosphate hydrolase (5.) (see Section II.C). High concentrations of FDP result in low levels of "active" HPr, as a result of a high HPr(Ser) kinase activity, and in high pyruvate kinase activity which directs the flow of PEP from sugar uptake by PTS to ATP production. On the other hand, high concentrations of P_i and low concentrations of FDP stimulate HPr(Ser-P) phosphatase and inhibit HPr(Ser) kinase and pyruvate kinase thereby promoting PEP utilization by PTS.[17] Since FDP and P_i can also modulate the activity of lactate dehydrogenase, product formation (and thus net ATP production) by glycolysis is affected by these intermediates. The role of FDP and P_i on the flow of carbohydrates by regulating the sugar explusion process is also depicted in this figure. Dephosphorylation of sugar-phosphates requires ATP and an early glycolytic intermediate, presumably FDP,[25,86] whereas the activity of the putative sugar-phosphate hydrolase is inhibited by P_i (and PEP) and presumably activated by HPr(SerP).[26]

phosphoryl donor for sugar translocation.[89,90] This regulatory mechanism (see Figure 4) appears to be unique for Gram-positive bacteria.[17]

A similar type of ATP-dependent mechanism for phosphorylation of HPr (by a distinct kinase) has recently been described for *S. salivarius*.[91] The phosphorylated amino acid residue in HPr has been identified as an acid-labile N-3-phosphohistidine {HPr(3-P-His)}. The role of HPr(3-P-His) in the regulation of PTS activity and/or carbohydrate metabolism still has to be established.

Various mechanisms of regulation of PTS activity have thus far only been demonstrated in Gram-negative organisms like *Salmonella typhimurium* and *E. coli*. These mechanisms,

which may also occur in lactococci, will be listed in the following order: (1) noncompetitive inhibition of carbohydrate transport by intracellular sugar phosphates,[17,92] (2) regulation of PTS by the pmf,[15] and (3) regulation by acetate kinase as a result of phosphoryl transfer from acetate kinase via PTS proteins to glucose.[93] ATP, GTP, as well as acetyl-phosphate can serve as phosphoryl donor for this reaction *in vitro*.[17]

2. Regulation of Sugar Metabolism Via the Phosphoenolpyruvate Phosphotransferase System

Phosphoenolpyruvate-dependent phosphotransferase systems have been implicated in the regulation of the metabolism of non-PTS sugars. In *E. faecalis* the enzyme dihydroxyacetone (DHA)/glycerol kinase is phosphorylated in a (reversible) PEP-dependent reaction in the presence of Enzyme I and HPr, which results in approximately tenfold higher activity of the enzyme.[94] The rate of phosphorylation of DHA/glycerol kinase by HPr(His~P) is about 200 times slower than that of Enzyme III^{lac}. The rates of phosphorylation are very similar with HPr(Ser-P)-(His~P) as phosphoryl donor, and DHA/glycerol kinase can then compete effectively with Enzyme III.[94] It should be noted that the bulk of HPr in *E. faecalis* cells utilizing PTS-sugars is usually in the form of HPr(Ser-P). Consequently, in the presence of PTS substrates DHA/glycerol kinase will be relatively inactive. DHA/glycerol kinase can be activated as a result of phosphorylation by HPr(His~P).[17]

In Gram-negative bacteria the utilization of non-PTS sugars such as maltose, melibiose, and lactose is inhibited by the presence of PTS sugars. For the lac carrier of *E. coli* this inhibition appears to result from the binding of unphosphorylated Enzyme III to the (lactose) carrier protein.[95] In the absence of PTS sugars, Enzyme III will be largely phosphorylated and inhibition of lactose transport is relieved. It is not known whether pmf-driven sugar transport systems of lactococci, e.g., the galactoside carrier of *L. lactis* ATCC 7962 and the galactose carrier of *L. lactis* ML_3, are also regulated in this manner.[10,24] This type of regulation could, however, explain the slow rates of lactose metabolism in *L. lactis* strains containing the lac-PTS in addition of a pmf-driven lactose transport system and β-galactosidase but lacking P-β-galactosidase.[38] Finally, as indicated in the section on lactose transport, phosphorylation of substrate may not necessarily be obligatory coupled to translocation via the PTS,[28,37] and evidence for transport (exit) without phosphorylation has also been presented.[16,87]

3. Regulation by Glycolytic Enzymes

a. Phosphofructokinase

Although phosphofructokinase is generally believed to play a key role in the regulation of the glycolytic activity, this enzyme has hardly been studied in lactococci. However, in *L. lactis* subspecies *cremoris* the intracellular concentrations of PEP decrease with increasing growth (glycolytic) rate,[96] and PEP is known to be a potent inhibitor of phosphofructokinase in *L. lactis*. It is possible that the rate of glycolytic activity may be controlled by feedback inhibition of this enzyme by PEP.[96,97] In *L. lactis* subspecies *cremoris* phosphofructokinase in conjunction with fructose-1,6-diphosphatase (inhibited by AMP), may establish a futile cycle for the dissipation of metabolic energy.[96]

b. Glyceraldehyde 3-phosphate Dehydrogenase

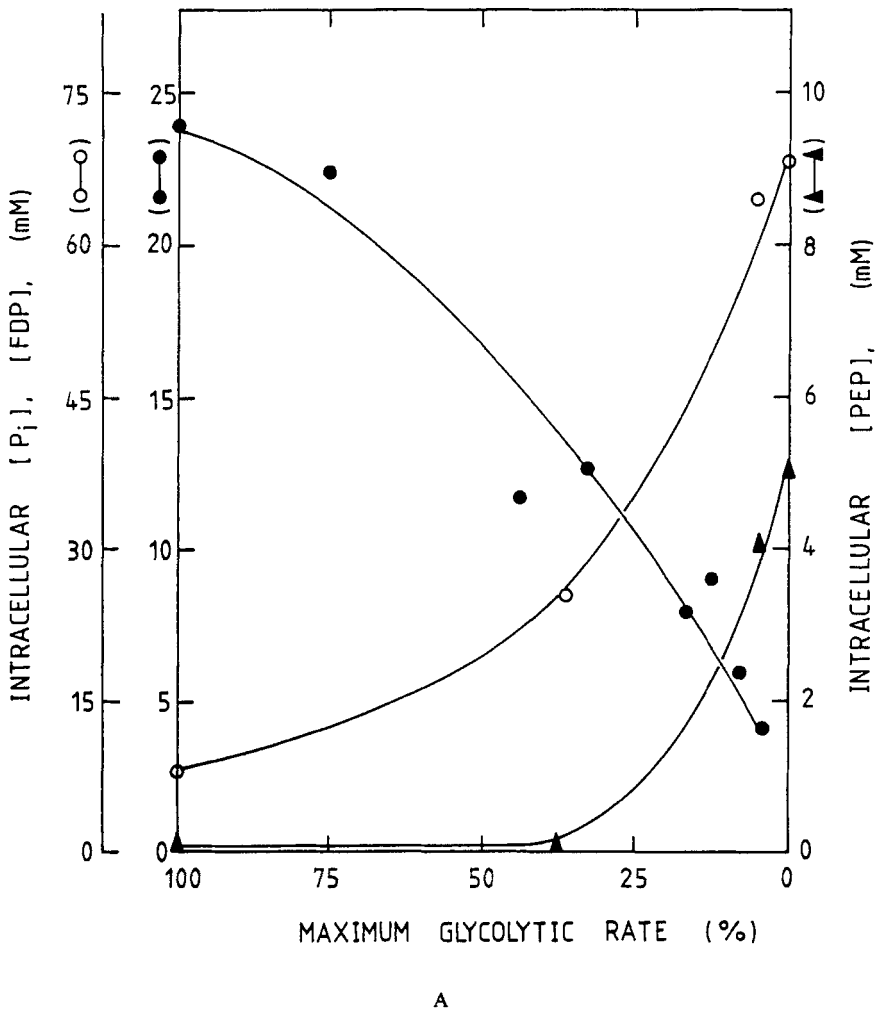
Most lactococci and streptococci possess an NAD^+ – dependent glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) that requires P_i and catalyzes a reversible oxidation of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate. In addition to this enzyme, some oral streptococci also have a $NADP^+$-dependent glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.9) which catalyzes the (P_i independent) irreversible oxidation of glyceraldehyde 3-phosphate (G3P) to 3-phosphoglycerate (3-PG), i.e., without generating ATP.[98] The $NADP^+$-dependent enzyme is present in streptococci which lack the oxidative part of the hexose monophosphate pathway and serves to generate NADPH.

The importance of glyceraldehyde 3-phosphate dehydrogenase in controlling glycolysis in lactococci has recently been investigated.[99] When cells of *L. lactis* are starved for lactose, the capacity of the glycolytic pathway decreases rapidly within a few hours of starvation.[99,100] The reduction in glycolytic activity is accompanied by a decrease in the activities of glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate mutase (E.C. 2.7.5.3), whereas the activities of all other glycolytic enzymes remain essentially unchanged.[99] The gradual change in steady state levels of glycolytic intermediates before and after the glyceraldehyde 3-phosphate dehydrogenase reaction with increasing starvation time indicate that the decrease in glycolysis is primarily due to reduced activity of glyceraldehyde 3-phosphate dehydrogenase. Furthermore, experiments have been performed to quantitate the control that glyceraldehyde 3-phosphate dehydrogenase exerts on the glycolytic flux in nongrowing cells of *L. lactis*. When glycolytic activity was gradually inhibited by addition of iodoacetate (a specific irreversible inhibitor of glyceraldehyde 3-phosphate dehydrogenase) a flux control coefficient of about 0.9 was calculated from the inhibition curves. From the observations, it was concluded that glyceraldehyde 3-phosphate dehydrogenase reaction was the major rate-limiting step for glycolysis in these cells.[99] Interestingly, the increased glycolytic activity of continuously cultured *S. sanguis* (upon shifting from glucose-excess to glucose-limited conditions) has been ascribed to an increase in the synthesis of glc-PTS and glyceraldehyde-3-phosphate dehydrogenase, suggesting that these enzyme systems largely control the pathway flux in this organism.[101]

A decrease in activity of the glycolytic pathway resulting from the inactivation of glyceraldehyde 3-phosphate dehydrogenase has also been observed in various oral streptococci.[102] The natural environment of these organisms, (i.e., the oral cavity) contains H_2O_2 which in itself may not be inhibitory but may cause inhibition when lactoperoxidase and thiocyanate are present. Streptococci can oxidize thiocyanate (SCN^-) to hypothiocyanate ($OSCN^-$) in the presence of H_2O_2 in a reaction catalyzed by lactoperoxidase. The hypothiocyanate ion affects glycolysis (and O_2 uptake) in oral streptococci primarily by inhibition of glyceraldehyde 3-phosphate dehydrogenase.[102] Inhibition by hypothiocyanate could be due to an oxidation of the sulfhydryl group in the active site of glyceraldehyde 3-phosphate dehydrogenase to yield a sulfenic acid or sulfenyl thiocyanate derivative.[103] The high capacity of some oral streptococci to recover from inhibition by hypothiocyanate has been attributed to the presence of a NADH-OSCN oxidoreductase which converts hypothiocyanate into thiocyanate.[102] Raw milk, the natural environment of *L. lactis*, also contains the lactoperoxidase-thiocyanate-H_2O_2 system which has been shown to be inhibitory to the growth of several strains of lactococci.[63] Presumably inactivation of glyceraldehyde 3-phosphate dehydrogenase by hypothiocyanate is responsible for the inhibition of glycolysis of growth in these organisms.

c. Pyruvate Kinase

The role of pyruvate kinase (E.C. 2.7.1.40) in the regulation of glycolysis in growing, glycolyzing, and starving lactococci has been investigated by various laboratories.[21,22,67,104-107] The activity of pyruvate kinase in *L. lactis* modulated by a number of glycolytic intermediates including FDP and P_i (Figure 4).[74,104,108,109] At maximal glycolytic rates, lactococci contain FDP as the major phosphorylated intermediate, whereas the level of PEP is comparatively low (Figure 5). In addition, the intracellular concentration of P_i is also very low under these conditions, at least in washed cell suspensions metabolizing glucose (Figure 5A).[67,104] Conversely, at submaximal glycolytic rates, the cells are depleted of FDP and the concentrations of P_i and PEP increase (Figure 5A). Since the concentrations of FDP (activator) and P_i (inhibitor of pyruvate kinase) fluctuate reciprocally with the glycolytic rate, it has been proposed that sugar fermentation by lactococci is largely controlled by the activity of pyruvate kinase.[16,104,106] Furthermore, PEP is not only a substrate for pyruvate



A

FIGURE 5. (A) Fluctuations in intracellular concentrations of FDP, P_i, and PEP with respect to the rate of carbohydrate fermentation in glycolyzing cells of *L. lactis* strain ML_3. Data in panel A calculated from data in reference 67. (B) Fluctuations in intracellular concentrations of FDP, P_i, and PEP with respect to the rate of carbohydrate fermentation in growing cells of *L. lactis* subspecies *cremoris* Wg_2. *L. lactis* subsp. *cremoris* was grown in chemostat culture with lactose as growth-limiting substrate as described.[100]

kinase but is also the high-energy phosphoryl donor for sugar transport by PTSs, and therefore control of pyruvate kinase activity is required.[16,110]

The maintenance of PEP-pool intermediates [3-PG, 2-phosphoglycerate (2-PG) and PEP] during starvation has been attributed to (1) the inactivation of pyruvate kinase by the high concentrations of P_i and (2) the absence of early glycolytic intermediates, specifically FDP.[21,104,106] The slow utilization of PEP by pyruvate kinase can provide the cells with maintenance energy during prolonged periods of starvation and also permits the rapid accumulation of PTS-sugars when these sugars become again available.[21,111] Maintenance of a large PEP-pool however does not appear to be essential for survival of lactococci during carbohydrate starvation.[100]

Control of pyruvate kinase by FDP and P_i *in vivo* has been investigated further in cells of *L. lactis* subspecies *cremoris* grown in chemostat culture.[100,105] Upon decreasing the imposed growth (glycolytic) rate from 0.38 to 0.04 h^{-1}, the intracellular concentration of

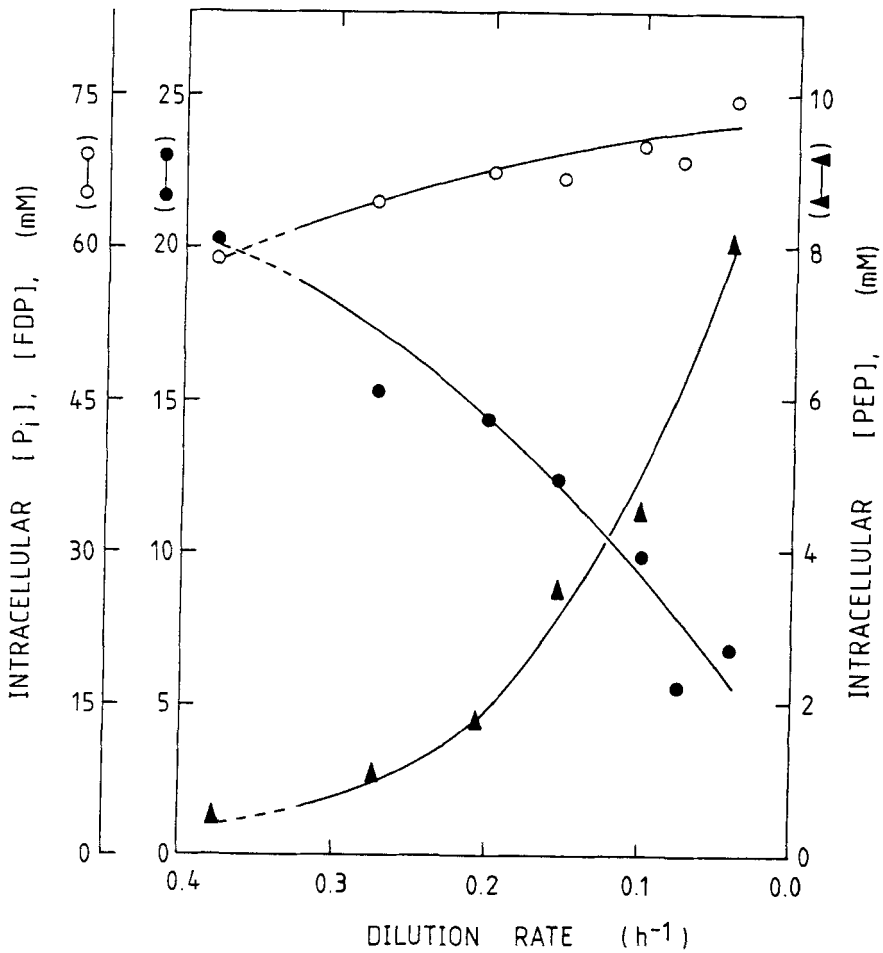


FIGURE 5B.

FDP decreases whereas the concentration of PEP increases (see Figure 5B) similar to what has been observed in glycolyzing cells of *L. lactis* (see Figure 5A). The intracellular concentration of P_i remains at a level of 60 to 70 mM, indicating that pyruvate kinase can be active despite the presence of high concentrations of this potent inhibitor. Upon lactose exhaustion these cells are rather rapidly depleted of PEP-pool intermediates even though the internal P_i concentration may be as high as 70 to 230 mM.[100,105] These experiments indicate that the inhibition of pyruvate kinase at the onset of (lactose) starvation of chemostat-grown *L. lactis* subspecies *cremoris* cells may be attributed to the decrease in FDP and other early glycolytic intermediates rather than to an increase of intracellular P_i.[100] Control experiments have verified that differences between the experiments presented in Figures 5A and 5B are not due to differences in the *Lactococcus* species used. These experiments indicate that general conclusions about regulatory mechanisms of metabolic pathways should be drawn with caution since the regulation pattern may vary with the experimental conditions.

d. Lactate Dehydrogenase

The role of lactate dehydrogenase (E.C. 1.1.1.27) in determining product formation of glycolyzing lactococci has already been indicated above. In *L. lactis* NAD-dependent lactate dehydrogenases have been identified for the L(+) and the D(−) isomers of lactic acid.[76] In contrast to D-lactate dehydrogenase, L-lactate dehydrogenase requires FDP for catalytic

reduction of pyruvate.[75,76] Other factors that have been implicated in the regulation (inhibition) of L-lactate dehydrogenase include pH, PEP, adenine nucleotides, and P_i.[72,76] When assayed in the presence of FDP (10 mM), the K_m for pyruvate of L-lactate dehydrogenase from different lactococci varies between 1.0 to 2.5 mM.[65,75] L-lactate dehydrogenase is affected at the level of enzyme synthesis by the carbohydrate source and the presence of oxygen in the medium.[65,69]

e. Pyruvate-Formate Lyase

Pyruvate-formate lyase activity measurements in cell-free extracts are often hampered by the oxygen sensitivity of this enzyme.[107] To circumvent this problem, pyruvate-formate lyase activity has been measured in intact cells by using [^{14}C]-formate to assay formate-pyruvate exchange.[65] Similar to the *S. mutans* enzyme,[107,112] pyruvate-formate lyase from *L. lactis* is inhibited by G3P and DHAP (triose-phosphates).[65] Various hexose phosphates tested had no effect on pyruvate-formate lyase. The K_m for pyruvate is 6 to 8 mM, which is approximately fivefold lower than the corresponding K_m of lactate dehydrogenase under similar conditions.[65]

D. Exit of Lactic Acid and Other End Products

During carbohydrate fermentation, lactococci produce large amounts of lactic acid. Fermentation of 1 mol of hexose yields 2 mol of lactate. Since rates of lactic acid production of 1 μmol per min × (mg of cell protein) have been reported, it is clear that accumulation of this organic acid would rapidly lead to a dramatic fall in internal pH.[113,114] The efflux of lactic acid in *L. lactis* subspecies *cremoris* has been shown to occur via a H^+/lactate symporter which operates with a pH dependent variable stoichiometry (see also Section V.B).[115] At low external lactate concentrations and at medium pH above 6, this efflux process results in the net transport of protons across the cytoplasmic membrane and to the generation of a pmf. The potential energy of the outwardly-directed lactate gradient is thus converted into an inwardly electrochemical gradient of protons.

The mechanism of acetate excretion has not yet been studied, however, it is assumed that this weak organic acid diffuses rapidly across the membrane. Acetate can be used for the artificial generation of a pH gradient across the membrane, since the membrane is highly permeable to the undissociated acetic acid but essentially impermeable to the anionic species.[116]

III. CITRATE TRANSPORT AND METABOLISM

Citrate metabolism by a number of strains of *L. lactis* yields the volatile compounds diacetyl and acetoin, which are important for flavor development in fermented milk products.[117] Although citrate degradation yields pyruvate, citrate cannot be used as an energy source and carbohydrates are required for growth. The enzymes involved in citrate degradation are citrate lyase, oxaloacetate decarboxylase, acetolactate synthase, diacetyl synthase, acetolactate decarboxylase, diacetyl reductase, and acetoin reductase.[117] The citrate-fermenting ability has been linked to the presence of a 5.5 MDa plasmid (cit).[118,119] Possibly, however, only the citrate transport system is plasmid encoded.[118] Citrate uptake is catalyzed by an inducible (most likely pmf-dependent) transport system with a K_t of 40 to 50 μM.[120]

IV. ARGININE TRANSPORT AND METABOLISM

Various lactococci have the ability to metabolize arginine (Arg) to ornithine (Orn), ammonia, and carbon dioxide (ratio 1:2:1) via the arginine deiminase pathway which provides these organisms with an additional substrate level phosphorylation site (see Figure 6).[121] The

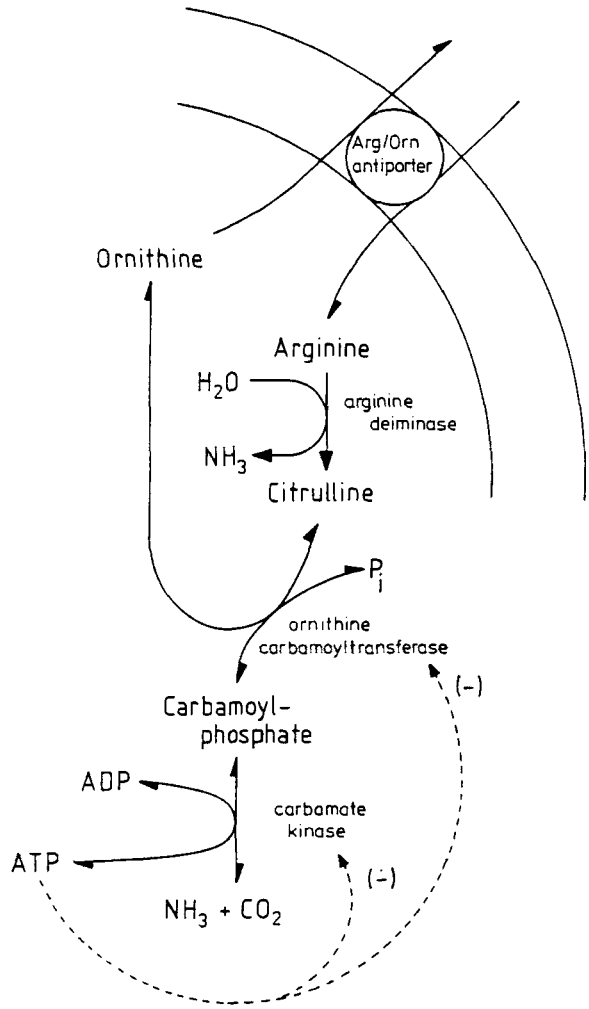


FIGURE 6. Pathways of arginine metabolism in *L. lactis*. A possible regulatory effect of ATP on the pathway activity is marked by the dashed arrows.

arginine deiminase (ADI) pathway comprises three cytoplasmic enzymes: (1) arginine deiminase (L-arginine transferase, E.C. 3.5.3.6), (2) ornithine carbamoyltransferase (carbamoyl phosphate:L-ornithine carbamoyltransferase, E.C.2.1.3.3) (3) carbamate kinase (ATP:carbamate phosphotransferase, E.C. 2.7.2.2), and (4) the arginine/ornithine antiporter which catalyzes the stoichiometric exchange between extracellular arginine and intracellular ornithine across the membrane (see Figure 6).[121,122] Recently, it has been shown that the driving force for arginine uptake and ornithine excretion is supplied by the arginine and ornithine concentration gradients, and no additional metabolic energy is required for the translocation of these solutes.

A. Arginine/Ornithine Antiporter

The arginine/ornithine (Arg/Orn) antiporter has been characterized in membrane vesicles derived from galactose-arginine grown cells of *L. lactis* ML_3[122] and indirectly by studying the mechanism of ornithine transport in sucrose-grown cells of *L. lactis* 133.[123] Arg/Orn antiport as initial step of the ADI pathway has also been demonstrated in *E. faecalis*, *S. sanquis*, and *S. milleri*.[124]

1. Mechanism

Rapid protonophore-insensitive heterologous exchange between external arginine and internal ornithine occurs at a rate which is at least 60-fold higher than that of pmf-driven arginine transport.[122] In proteoliposomes reconstituted with octyl β-D-glucopyranoside-extracted membrane proteins of *L. lactis*, it was found that one arginine exchanges with one ornithine.[122] This 1:1 stoichiometry was also found in whole cells for heterologous exchange between arginine and ornithine[124] and for homologous exchange of ornithine.[123] The Arg/Orn antiporter is in sensu stricto a real antiporter. Release of internal ornithine is extremely slow in the absence of external arginine or ornithine.[123,124] External ornithine or arginine elicits a more than 500-fold acceleration of the rate of ornithine efflux.[125] The V_{max} of arginine uptake increases with increasing internal ornithine concentration, and half-maximum rate of arginine uptake is obtained at an internal ornithine concentration of 42 μM.[125] Heterologous exchange experiments indicate that the K_t for arginine uptake increases with the internal ornithine concentration when varied between 50 and 200 μM. The variation of the V_{max}/K_t ratio with increasing internal ornithine concentration indicates that the Arg/Orn antiporter behaves according to a ping-pong mechanism. Further kinetic studies of homologous exchange of ornithine and arginine in rightside-out and inside-out membrane vesicles using an inhibitor-stop assay, showed that arginine and ornithine share a common binding site with a K_t value of 5.5 and 40 μM, respectively. The kinetics of the Arg/Orn antiporter are identical in both membrane preparations. Rightside-out membrane vesicles of *L. lactis* subsp. *lactis* ML_3 contain approximately 87 pmol of arginine and ornithine binding sites per mg of protein with a dissociation constant (K_D) of 6.6 and 61.8 μM, repectively. With a V_{max} of 30 nmol per min × mg of membrane protein a turnover of about 6 s^{-1} can be calculated.

2. Specificity

Arginine uptake via arginine/ornithine exchange is inhibited by a number of analogues with a specificity which decreases in the following order; L-arginine > L-canavanine > D-arginine > L-homoarginine > L-ornithine ⩾ S-2-aminoethyl-L-cysteine > L-lysine ⩾ D-ornithine.[125] Citrulline is not transported.[122,123] The substrate-specificity on the inner side of the membrane has only been determined for ornithine analogues and was found similar to the specificity reported for the outer side.[122]

3. Molecular Properties

The Arg/Orn antiporter contains reactive sulfhydryl groups and is inactivated by several organomercurials, including pCMB, *p*-chloromercuribenzene sulfonic acid (pCMBS), and mersalyl acid. However, only moderate inhibition is effected by maleïmides, and little or no inhibition is elicited by dithiol-specific agents, such as 2-(3,6-disulfo-2-hydroxy-1-naphtylazo)benzene arsonic acid (thorin) and the lipophilic phenylarsene-oxide.[125] Inhibition by organomercurials is rapidly and completely reversed by addition of dithiothreitol. The reactive sulfhydryl group is exposed to the outer surface of the membrane, and largely inaccessible to inhibitors in inside-out membrane vesicles. Arginine and ornithine are able to protect the carrier against inactivation by organomercurials. Protection is, however, more efficient with arginine. Binding of the amino acid substrates to the carrier is most likely accompanied with a conformational change of the protein such that the reactive sulfhydryl-group(s) become(s) less well exposed. Internal ornithine and arginine are not able to protect against pCMBS inactivation.

The Arg/Orn antiporter is completely repressed when cells of *L. lactis* are grown on a arginine-deficient synthetic medium (see Section IV.B).[125] Membrane vesicles derived from galactose-arginine grown cells display at least a 100-fold higher rate of Arg/Orn exchange than membrane vesicles derived from cells grown on galactose or glucose alone. Ligand

binding experiments with L-[^{3}H−] arginine show negligible binding levels to noninduced membranes. Induced and noninduced membranes have been subjected to analysis of the polypeptide composition using two-dimensional gel electrophoresis.[126] Among several protein bands which display an enhanced expression, one unique prominent protein has been identified in the induced membranes with a molecular weight of around 51,000 Da. This protein is absent in membranes derived from *L. lactis* subsp. *cremoris*, which is unable to metabolize arginine (see also Section IV.B).

B. Regulation of the Arginine Deiminase Pathway

1. Coarse Control

Until recently, studies of the regulation of arginine catabolism in lactococci were principally focussed on the synthesis of the cytoplasmic enzymes (coarse control). In *L. lactis* grown in complex medium, specific activities of arginine deiminase and ornithine carbamoyltransferase are five- to tenfold higher in cells grown in the presence of galactose compared to glucose or lactose.[127] The specific activities of arginine deiminase and ornithine transcarbamoylase can be increased further upon addition of high concentrations of arginine to the growth medium.[127] Carbamate kinase appears to be constitutive and is most likely involved in the synthesis of carbamoylphosphate as a source of pyrimidine biosynthesis.[128]

Complete repression of the ADI pathway and the Arg/Orn antiporter is observed when the cells are grown on a arginine-deficient synthetic medium containing either glucose, galactose or lactose.[125] With galactose as energy and carbon source, it was found that arginine acts as a inducer, whereas ornithine and citrulline are without effect. On the other hand, glucose acts as a repressor, and because this repressive effect can be partially antagonized with cAMP, it is possible that glucose exerts its effect via catabolite repression.[124] Similar results have been obtained for the ADI pathway of *E. faecalis* except that carbamate kinase is also co-induced in these cells.[129] In contrast to *L. lactis*, *E. faecalis* can use arginine as the sole energy source.[129,130]

An important characteristic of *L. lactis* subspecies *cremoris* is its inability to metabolize arginine. Analysis of the activities of ADI pathway enzymes indicates that arginine deiminase is lacking in all *L. lactis* subspecies *cremoris* strains, whereas some strains lack the ornithine carbamoyltransferase.[127] Carbamate kinase is present at levels comparable to those found in *L. lactis*. Arg/Orn exchange activity was not found in *L. lactis* subspecies *cremoris* Wg_2 and E_8.[124,131] The genetic information for the Arg/Orn antiporter as well as the other ADI pathway enzymes is located on the bacterial chromosome since the activities are also detected in the plasmid-cured strain *L. lactis* MG 1363.[131] The co-induction of the Arg/Orn antiporter, arginine deiminase, and ornithine carbamoyltransferase in *L. lactis* suggests that these enzymes might be coded by a single operon, similar to the one encoding the ADI pathway in *Pseudomonas aeruginosa*.[132]

2. Fine Control

Identification of Arg/Orn exchange as the mechanism of arginine transport, and the characterization of this system in fused and reconstituted membranes[122] have made a study on the regulation (fine control) of the ADI pathway possible.[124] Resting cells of *L. lactis*, grown in the presence of galactose and arginine, maintain a high intracellular ornithine pool in the absence of arginine or other exogenous energy sources. Addition of arginine results in a rapid release of ornithine concomitant with uptake of arginine. Subsequent arginine metabolism results intracellularly in high citrulline and low ornithine pools.[123,124] Citrulline is not a substrate for the antiporter and since cells accumulate this amino acid to concentrations exceeding 20 mM, the thermodynamically unfavorable reaction towards ornithine and carbamoylphosphate can be effected by ornithine carbamoyltransferase.[133,134]

The Arg/Orn exchange is not affected by protonophores and ionophores which dissipate the components of pmf, but the overall ADI pathway activity is strongly stimulated by these

compounds.[122,124] These effects can only to a minor extent be explained by effects of the ionophores on the intracellular pH. Several lines of evidence indicate that ADI pathway is regulated indirectly by pmf-induced changes in the internal concentrations of (adenine) nucleotides.[124] Conditions which lower ATP consumption, i.e., inhibition of F_0F_1-ATPase by N,N′-dicyclohexyl-carbodiimide (DCCD) or high (internal) pH, decrease ADI pathway activity, whereas protonophores and ionophores which stimulate ATP consumption increase the activity. The regulation of ADI pathway by ATP {and possible other (adenine) nucleotides} could be exerted on carbamate kinase, for which ADP is a substrate and ATP a product, but possibly also on ornithine carbamoyltransferase (Figure 6). In *P. fluorescens*, ornithine carbamoyltransferase is inhibited by pyrimidine and purine triphosphonucleotides which increase the threshold substrate concentration required for activating the enzyme, whereas nucleotides monophosphates restore the normal kinetic behaviour.[121,135] Whether ornithine carbamoyltransferase in *L. lactis* is regulated allosterically by (adenine) nucleotides is unknown at present.

C. Biosynthesis, Metabolism, and Transport of Basic Amino Acid Derivatives

Although arginine is essential for growth of some *L. lactis* strains, others have the ability to synthesize arginine from citrulline, ornithine, or glutamic acid.[136,137] To evade the essentially irreversible reaction catalyzed by arginine deiminase, citrulline can be converted into arginine via argininosuccinate at the expense of the hydrolyses of ATP to AMP plus PP_i and the conversion of aspartate to fumarate. Little is known about the enzymes catalyzing these reactions, i.e., argininosuccinate synthetase and argininosuccinate lyase, either in lactococci nor in other bacteria.[121]

E. faecalis ATCC 11700, devoid of arginine decarboxylase is able to use agmatine (Agm) as the sole energy source by the sequential action of a three-enzyme system and transport system analogous to the ADI pathway.[138,139] Agmatine deiminase (EC 3.5.3.12), putrescine carbamoyltransferase (EC 2.1.3.6), and carbamate kinase constitute the agmatine deiminase route, which catalyzes the conversion of agmatine into putrescine (Put), ammonia, and carbondioxide with the concomitant formation of 1 mol of ATP per mole of agmatine consumed. Under those conditions putrescine is excreted into the medium. Agmatine uptake and putrescine excretion is catalyzed by a Agm/Put antiporter.[130] The Agm/Put antiporter is induced when the cells are grown on agmatine. Under those conditions, the Arg/Orn antiporter is almost completely absent. As far as the physiological substrates concerned, the substrate specificity of the Agm/Put antiporter is distinctly different from that of the Arg/Orn antiporter. The Agm/Put antiporter mediates homologous exchange of putrescine with a K_t for putrescine of 20 μM.[130] Agmatine is a competitive inhibitor of homologous putrescine exchange with a K_i of 7 μM. The regulatory phenomena observed with the ADI pathway, however, appear to be very similar to those of the agmatine deiminase pathway.[130] ADI pathway mutants of *E. faecalis*, deficient in the Arg/Orn antiporter are still able to utilize agmatine.[125,129,138]

Recently, some new amino acids have been isolated from the intracellular pools of *L. lactis* which have been identified as N^5-(1-carboxyethyl)ornithine {N^5-(1-CE)Orn}, N^5-acetylornithine (N^5-AcOrn) and N^6-(1-carboxyethyl)lysine {N^6-(1-CE)Lys}.[136,137,140,141] Radiotracer experiments have shown that exogenous [^{14}C]-ornithine can serve as precursor for biosynthesis of [^{14}C]-arginine, [^{14}C]-N^5-(1-CE)Orn and [^{14}C]-N^5-AcOrn by cells of *L. lactis* K_i during growth in a defined medium lacking arginine.[137,140] Growing cells of *L. lactis* also have the ability to synthesize N^5-(1-CE)Orn from glutamic acid in the absence of arginine and ornithine.[137,141] The synthesis of N^5-(1-CE)Orn by resting cells requires the presence of ornithine and a metabolizable sugar.[136] The *in vitro* synthesis of this compound by a cell-free extract requires ornithine, pyruvate and NAD(P)H on the basis of which a pathway of (enzymatic) synthesis of N^5-(1-CE)Orn by a reductive condensation mechanism has been

proposed.[136,137,141] The biosynthesis of N^5-AcOrn is most likely catalyzed by ornithine carbamoyltransferase by using acetylphosphate as an analog of carbamoylphosphate.[137] The biosynthesis of N^5-(1-CE)Orn has only been observed in certain *Lactococcus* strains, i.e., *L. lactis* 133, K_1, C_{10}, and DRC_1 (but not in ML_3, C_2, and DL-11), suggesting plasmid linkage of the gene(s) encoding the enzyme(s) for this amino acid.[137] A survey among streptococci showed that N^5-(1-CE)Orn was not detectable in the amino acid pool of *E. faecalis*, *S. sanquis*, *S. mutans*, and *S. salivarius*. Since N^5-(1-CE)Orn is excreted in the medium during growth, preliminary experiments have been carried out to characterize the transport mechanism.[137] Accumulation of N^5-(1-CE)Orn by resting cells of *L. lactis* 133 is energy dependent and optimal at a medium pH of 7.

The physiological role(s) of the newly discovered amino acids is not yet known, despite the fact that these compounds are present in high concentrations (up to 20 mM). It has been proposed that the ornithine derivatives may modulate the activity of the enzymes involved in arginine biosynthesis. Alternatively, these compounds may be detoxification products formed by the organism to maintain the intracellular ornithine concentration at an acceptable level. Synthetic isomers of N^5-(1-CE)Orn are now available by chemical synthesis, which will facilitate further studies on the function of this compound.[137,140]

V. GENERATION OF A PROTONMOTIVE-FORCE

A. Ca^{2+}-Mg^{2+}-Stimulated, Membrane-Bound ATPase Complex

Lactococci are impaired in the synthesis of porphyrins and these organisms are unable to generate a pmf by electron flow in electron transfer systems. When grown in the presence of hematin some lactococci synthesize prophyrin and develop a rudimentary cytochrome-linked electron transport chain.[142,143] In a related organism *E. faecalis* cytochrome-linked electron transfer to fumarate appears to be coupled to proton translocation.[144]

A considerable fraction of the ATP formed by substrate level phosphorylation is consumed by the membrane-bound Ca^{2+}-Mg^{2+}-stimulated F_0F_1-ATPase complex for the generation of the electrochemical gradient for protons ($\Delta\tilde{\mu}_{H^+}$). The energy present in the $\Delta\tilde{\mu}_{H^+}$ exerts a force on the protons [the proton-motive force, pmf, $\Delta\tilde{\mu}_{H^+}/F$ or Δp] which is composed of an electrical potential ($\Delta\psi$) and a chemical gradient of protons across the cytoplasmic membrane ($Z\Delta pH$) which (in mV) equals $2.3(RT/F)(pH_{in} - pH_{out})$, in formula,

$$(\Delta\tilde{\mu}_{H^+}/F) = \Delta p = \Delta\psi - Z\Delta pH \quad (1)$$

Unlike aerobic heterotrophs the lactococcal ATPase functions as a hydrolase rather than a synthase. The driving force for H^+-extrusion by the ATPase is supplied by the free energy for hydrolysis of ATP, i.e., the phosphate potential $\Delta G'_p$. At thermodynamic equilibrium the following relationship exists between Δp and $\Delta G'p$,

$$\Delta p = \Delta G'_p/nF = \Delta G^\circ + 2.3RT/nF \log\{[ATP]/[ADP] \cdot [P_i]\} \quad (2)$$

in which ΔG° is the standard free energy of ATP hydrolysis, and n the number of protons translocated per molecule ATP hydrolyzed. Estimates of the H^+/ATP stoichiometry (n) have for long been and still are a matter of controversy. A pmf of different magnitudes and composition across the cytoplasmic membrane of *L. lactis* ATCC 7962 was artificially (pH jump) generated by rapidly changing the external pH in combination with or without a K^+ diffusion potential in the presence of valinomycin or a diffusion potential of membrane permeable anions.[145,146] The magnitude of the ΔpH was estimated from the distribution of the weak acid salicylic acid, whereas the $\Delta\psi$ was estimated from the K^+ or anion potential measured from the internal and external concentrations of these ions. The net proton entry

that can be attributed to the coupling of H^+ influx and ATP synthesis has been estimated by comparing cells treated and untreated with the ATPase inhibitor, DCCD. Below a pmf of 180 to 190 mV the initial rate of H^+ influx is the same for DCCD-treated and untreated cells, and increases linearly with the magnitude of pmf. Above a pmf of 180 to 190 mV a marked acceleration of proton influx was observed in untreated cells concomitant with synthesis of ATP. However, in DCCD-treated cells H^+ influx still showed a linear dependence on the pmf without eliciting significant ATP synthesis.[145,146] Since the $\Delta G'_p$ of resting cells of *L. lactis* ATCC 7962 is 8.4 kcal/mol, corresponding with about 370 mV when expressed in electrical units, the reversal (threshold) potentials of pmf and $\Delta G'_p$ suggests a H^+/ATP stiochiometry of about 2. Both components of the pmf (i.e., electrical potential and the chemical gradient of protons) have been shown to be equivalent in driving ATP synthesis.[147] In a subsequent study, the H^+/ATP stoichiometry was estimated from the magnitudes of the pmf and $\Delta G'_p$ in glycolyzing cells of *L. lactis* ATCC 7962. These data indicated a $\Delta G'_p/\Delta p$ ratio of about 3 at pH 5, a value which rose to 4.3 as the external pH was raised to 7.[148] Since the pmf was maximal at pH 5 and the $\Delta\psi$ could be converted almost quantitatively into ΔpH, it was assumed that a near equilibrium state existed at pH 5. The conclusion that the actual H^+/ATP stoichiometry is 3 in glycolyzing cells heavily depends on this assumption.

The $\Delta G'_p$ and the pmf have also been measured in resting and growing cells of *L. lactis* subspecies *cremoris* Wg_2.[149] In resting cells ATP synthesis could be driven by a valinomycin-induced K^+ diffusion potential. In the subsequent phase of ATP hydrolysis, the $\Delta G'_p/\Delta p$ ration has been estimated to be 2.5. The H^+/ATP stoichiometry was concluded to be most likely two but this number might be an underestimation. Upon imposition of an artificial membrane potential by means of a (cat)ion-diffusion gradient, protons tend to move down the electrical potential thereby creating a reversed pH gradient. Such a pH gradient has not been taken into account and the magnitude of the total pmf might have been overestimated. The $\Delta G'_p/\Delta p$ ratio of growing cells of *L. lactis* subspecies *cremoris* was found to be close to 5, suggesting a far from equilibrium state under these conditions.[149]

B. The Energy Recycling Model

Besides ATP hydrolysis by F_0F_1-ATPase, two alternate mechanisms for pmf generation and maintenance have been demonstrated in fermentative bacteria: ion transport decarboxylases and carrier-mediated excretion of metabolic endproducts. The ion transport decarboxylases studied thusfar are membrane bound enzymes which can convert the chemical energy of highly exergonic decarboxylation reactions into electrochemical energy of sodium ions (Na^+).[150] Recently, also a proton-translocating decarboxylase has been detected.[151] Transport decarboxylases have not yet been found in lactococci and this mechanism of energy transduction will therefore not be discussed here. The second pmf-generating mechanism, carrier-mediated excretion of metabolic end products has been studied extensively in *L. lactis* subspecies *cremoris* (see Figure 7).[115,152] During glycolysis this organism continuously produces lactate at a high rate (up to 1000 nmol/mg of protein × min). Lactate is weak acid with a pK of 3.86. At an internal pH above 7 almost all lactate is dissociated and in the anionic form. This anionic form is not membrane permeable unlike undissociated lactate and can only pass the membrane by a carrier-mediated process. If this efflux of lactate occurs in symport with protons the energy of the lactate concentration gradient will be converted into electrochemical energy of protons (energy recycling) (see Figure 7). Basically, the mechanism of energy recycling is the reverse of solute uptake in which a pmf is converted into a solute gradient.[153] A prerequisite for pmf-generation by endproduct efflux is that the efflux process is electrogenic and carrier-mediated, and both parameters have been described in *L. lactis* subsp. *cremoris*.[71,115,154] The number of protons translocated with lactate, the H^+/lactate stoichiometry, was found to vary between 1 and 2 with the external

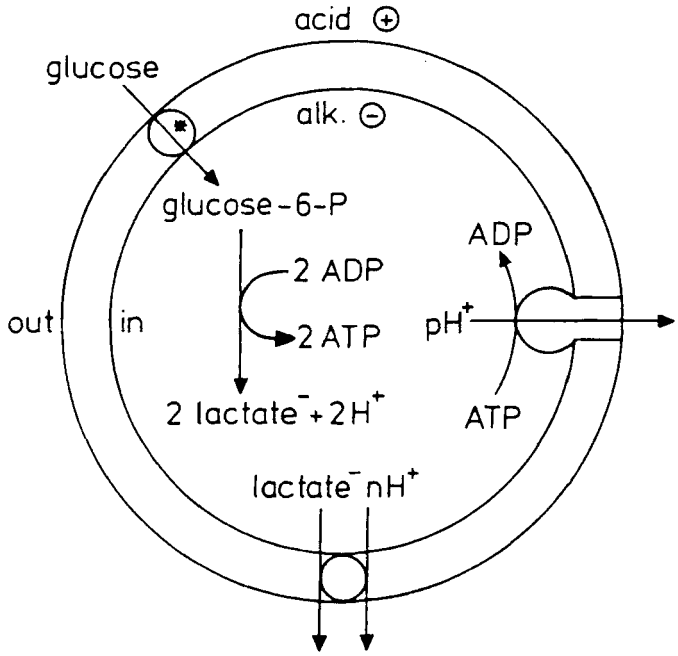


FIGURE 7. Schematic presentation of the energy-recycling model in lactococci. Glucose is taken up by the phosphoenolpyruvate-dependent group translocation system (PTS).

pH and lactate concentration.[113] Furthermore, lactate efflux from lactate-loaded cells is able to drive pmf-dependent amino acid uptake.

The energy-recycling model has been studied in *L. lactis* subspecies *cremoris* cells growing in batch or chemostat culture with lactose as sole energy source.[113,114,155] On the assumption that the lactate concentration gradient is very close to thermodynamic equilibrium with the pmf, H^+/lactate stoichiometries have been determined from these entities. An increase in H^+/lactate stoichiometry has been observed with increasing external pH and/or decreasing external concentration of lactate.[113,114] The energy gained by lactate excretion can be expressed in ATP equivalents provided that the stoichiometries of H^+/lactate efflux and H^+/ATP of the F_0F_1-ATPase are known.[114,115] Per mole of hexose metabolized 2 mol of ATP are synthesized by substrate level phosphorylation and 2 mol of lactic acid are produced. When lactate is excreted in symport with 1 H^+, the cell will have no advantage from the efflux process. However, when the H^+/lactate stoichiometry is 2 and two protons are translocated per ATP hydrolyzed, the theoretical energy gain of lactate excretion can be as high as 50% (see Figure 8).[115]

Variable H^+/lactate stoichiometry has also been observed for lactate influx in whole cells of *E. faecalis*.[156] The initial rate of lactate influx, however, did not saturate at external concentrations exceeding 100 mM. Lactate influx into inside-out membrane vesicles of *E. faecalis* drives ATP/$^{32}P_i$ exchange.[157] In another strain of *E. faecalis* it has been suggested that lactate is excreted as undissociated acid with a K_t of 2 mM.[158]

C. Magnitude and Composition of the Protonmotive Force in Growing Cells

The magnitude and composition of the pmf in growing and glycolyzing lactococci is comparable provided that the media are comparable with respect to the concentrations of K^+, Na^+, and H^+, and weak acids (e.g., lactic acid, acetic acid) (see Section VI).[113,159-163] Due to the relatively low electrical capacitance of the cytoplasmic membrane

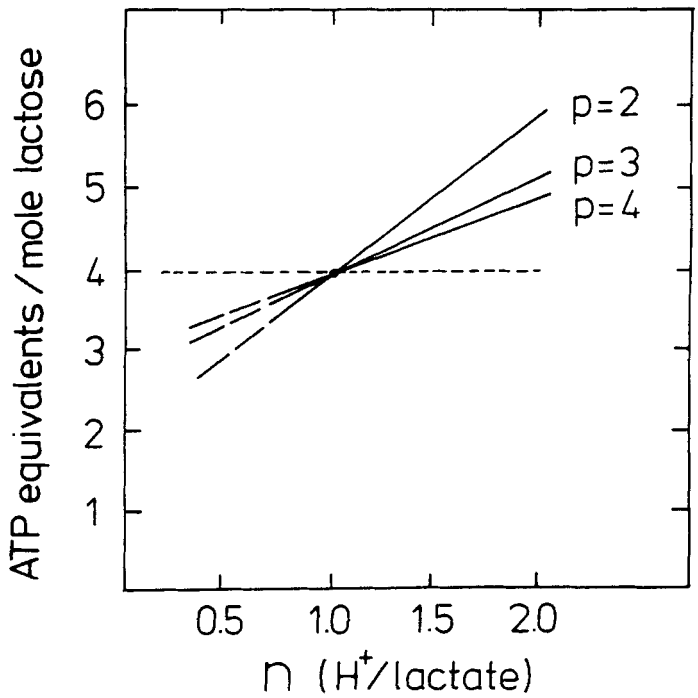


FIGURE 8. Effect of the H^+/lactate stoichiometry (n) on the theoretical number of ATP equivalents produced per mol of lactose consumed in *L. lactis* subsp. *cremoris* Wg_2. p is the number of protons translocated per mol of ATP synthesized. (From ten Brink, B. and Konings, W. N., *J. Bacteriol.*, 152, 682, 1982. With permission.)

with respect to the differential buffering capacitance of the bulk phase (external medium and cytoplasm) proton translocation results in a rapid generation of the $\Delta\psi$ component of the pmf whereas the ΔpH component builds up slowly. Subsequent (net) uptake of cations (other than protons) is required to raise the ΔpH at the expense of $\Delta\psi$. In most, if not in all neutrophilic bacteria the cation which best fulfills this role is K^+. K^+ is taken up electrogenically, thereby depolarizing the $\Delta\psi$. As a result the system will be out of thermodynamic equilibrium and more protons can be extruded to reestablish thermodynamic equilibrium. This will lead to an increase of the internal pH and an increase of the ΔpH.[164] The maximal effect of K^+ ions in raising ΔpH is already achieved at about 2 mM, i.e., at the V_{max} of the corresponding K^+ transport system(s).[161,165,166]

In lactococci high concentrations of Na^+ (>50 mM) tend to lower the pmf.[161,166] This effect of Na^+ could be due to the increased requirement of metabolic energy (possibly ATP) in order to pump Na^+ out and to keep the intracellular Na^+ concentration low.

The effect of the proton concentration (external pH) on the magnitude and composition of the pmf in lactococci is exemplified by the pH dependence of pmf in growing cells of *L. lactis* subspecies *cremoris* Wg_2 (see Figure 9) (see also Section VI). The ΔpH component decreases with increasing external pH such that the intracellular pH remains fairly constant, i.e., slightly alkaline (see Figure 10). The decrease in ΔpH is only partially compensated by an increase in the $\Delta\psi$, resulting in a lower pmf at more alkaline pH. Essentially the same results have been obtained for other lactococcal strains under growing or glycolyzing conditions.[159,160,167,168] Differences in recorded values of pmf may be attributed to the methods employed to assay $\Delta\psi$ and/or the model used to correct for nonspecific binding of the probe used to calculate $\Delta\psi$.[169]

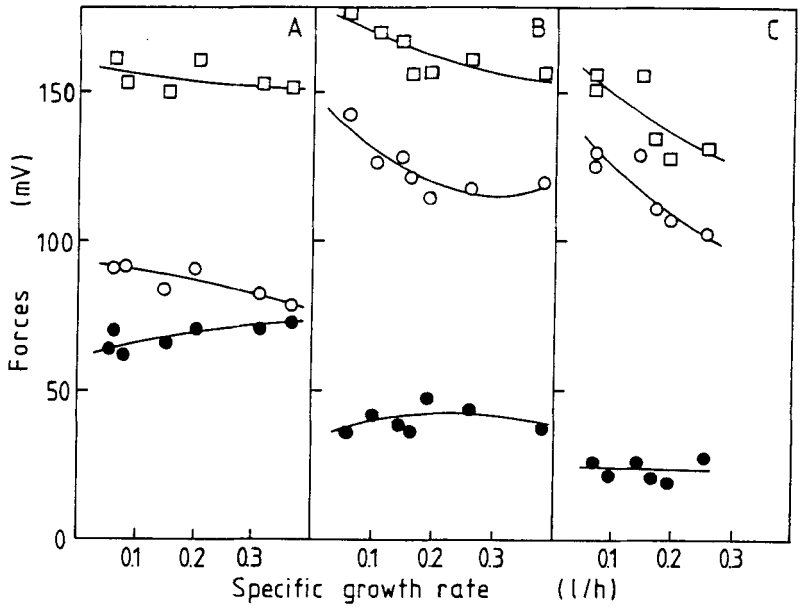


FIGURE 9. Effect growth rate on the composition (○, $\Delta\psi$, and ●, ΔpH) and the magnitude of the pmf (□) in *L. lactis* subspecies *cremoris* Wg_2 growing in lactose-limited chemostat cultures at pH 5.7 (Panel A), pH 6.4 (Panel B), and pH 7.0 (Panel C). The growth rate was varied by changing the dilution rate of the culture. (From Otto, R., ten Brink, B., Veldkamp, H., and Konings, W. N., *FEMS Microbiol. Lett.*, 16, 69, 1983. With permission.)

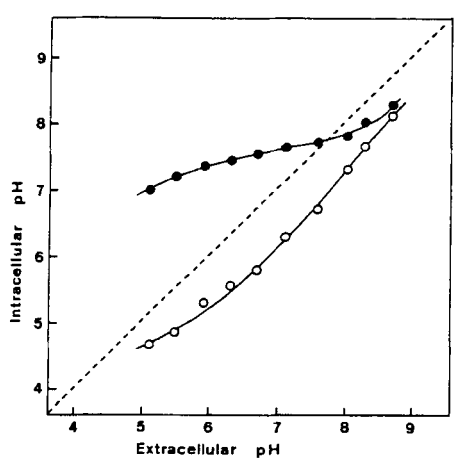


FIGURE 10. Effect of extracellular pH on the intracellular pH of glycolyzing cells of *L. lactis* ML_3 in the absence (●) and the presence (○) of nigericin. (From Poolman, B., Driessen, A. J. M., and Konings, W. N., *Microbiol. Rev.*, 51, 498, 1987. With permission.)

In the presence of high concentrations of weak acids the pmf can be decreased.[170] The cytoplasmic membrane is permeable to the lipophilic, undissociated forms of these acids and essentially impermeable to the hydrophilic charged forms. The relative amount of the dissociated and undissociated forms is determined by the pK_{app} of the acid and the pH of the medium. Consequently, cells which maintain the intracellular pH alkaline relative to the pH of the outside medium, accumulate weak acids thereby decreasing the cytoplasmic pH and thus the magnitude of the ΔpH.

The effect of the growth rate on the pmf in *L. lactis* has been studied in chemostat and batch cultures under conditions of pH control.[160,171] With *L. lactis* subspecies *cremoris* Wg_2 growing under lactose limitation in continuous culture, an increase in growth rate is associated with a decrease in the $\Delta\psi$ component of the pmf (see Figure 9).[171] On the other hand, when the growth rate of batch grown *L. lactis* ATCC 7962 is varied by adding different sugars as carbon sources, the magnitude and composition of pmf is not affected.[160] For *L. lactis* the magnitude and composition of the pmf remains essentially constant during the exponential growth phase as long as the medium pH is kept constant.[113,160] However, when pH control is not employed (e.g., during batch cultivation) the external pH decreases, and this in turn leads to an increase in both ΔpH and pmf.[114]

Upon exhaustion of the carbohydrate source of batch or chemostat growing cells of *L. lactis* subspecies *cremoris*, the pmf drops to zero within 60 to 90 min of starvation.[100,149,171] The decrease in pmf is associated with a depletion of the intracellular pool of sugar phosphates and a decrease in ATP level. With cells starved for carbohydrate in growth medium, the PEP-pool intermediates also fall rapidly to zero.[100,105] On the other hand, when *L. lactis* cells are removed from the growth medium and washed in buffer, the PEP-pool in these starved cells remains high for several hours.[16,21,100,110] This PEP-pool and a low activity of pyruvate kinase provides the cells with low concentrations of ATP (<0.1 mM) for prolonged periods of time. In these resting cells the low ATP levels are sufficient to generate a ΔpH of about −100 mV at pH 6.0.[177] The maintenance of a large PEP-pool by starved cells allows rapid accumulation of sugars by PTSs when these become available again.[110] Starved *L. lactis* subspecies *cremoris* cells, in which the PEP-pool has fallen to undetectable levels, can however synthesize ATP and generate a high pmf within a few minutes upon renewed addition of carbohydrates.[100,105]

VI. pH HOMEOSTASIS

The mechanism of pH homeostasis is lactococci has several features in common with the mechanisms described in the more intensively studied *E. faecalis*.[164,167,172-174] For example,

1. The extrusion of protons by the F_0F_1-ATPase and the electrogenic uptake of K^+ raises the cytoplasmic pH. The requirement for K^+ to raise the cytoplasmic pH is generally observed for pH homeostasis in (neutrophilic) bacteria and is attributed to the depolarization of the membrane potential which facilitates protons to be pumped out (see also Section V.C).[164] In principle any cation can replace K^+ in raising the cytoplasmic pH, provided uptake is rapid and electrogenic. These conditions have been demonstrated in *E. faecalis* by using lipophilic (organic) cations or a (mutant) strain which accumulates Na^+ via a leak pathway in response to the membrane potential and shows defective Na^+ extrusion.[167]
2. The activity of the F_0F_1-ATPase decreases with increasing (internal) pH. Experiments performed with inside-out membrane vesicles indicated that the ATPase has an optimum pH at 6.5, whereas the activity is very low at pH values above 7.7.[167,172]
3. The synthesis of the F_0F_1-ATPase is regulated by the internal pH. Upon lowering the cytoplasmic pH of growing cells from 7.5 (or above) to 6.5 the level of F_0F_1-ATPase is increased approximately fivefold.[172]

4. A mechanism to acidify the cytoplasm at alkaline pH values, besides the proton release during glycolysis, should be present since *E. faecalis* is able to maintain the cytoplasmic pH at 7.8 to 8.2 when the medium pH ranges from 8.4 to 9.5.[175] Although Na^+/H^+ antiport activity has been observed in *E. faecalis*, this system does not likely function at alkaline pH due to a reversed ΔpH (pmf) of +77 mV at pH 9.5.[175,176]
5. The net formation of either acidic or basic end products by cells metabolizing lactose and arginine, respectively, does not seem to be essential for pH homeostasis at alkaline pH values, since the intracellular pH becomes even more acid relative to the outside medium with arginine than with a glycolytic substrate.[161,162,167]

The absolute requirement for K^+ to alkalinize the cytoplasm[161,166] and the decrease in F_0F_1-ATPase activity with increasing pH[177] suggest that the mechanism of pH regulation in *L. lactis* could be similar to that of *E. faecalis*.[164,167] Differences in pH homeostasis of *L. lactis* and *E. faecalis* are found with respect to the absolute value at which the internal pH is regulated and the mechanism of regulation of the internal pH at acidic pH values.[161,163,167] Interestingly, *L. lactis* cells metabolizing arginine maintain the cytoplasmic pH relatively constant between pH 5 to 7, but the internal pH values are 0.3 to 0.4 pH units lower than in glycolyzing cells.[162] Both with arginine and a glycolytic substrate the intracellular pH of *L. lactis* (and *E. faecalis*) becomes acid relative to the outside medium above pH 7.5 (see Figure 10).[162,167]

Regulation of the cytoplasmic pH in lactococci is necessary since the medium pH decreases continuously during growth as a result of massive acid production. Inhibition of metabolism and loss of viability at low pH values most likely results from a decrease of the internal pH below a threshold rather than from the decrease in external pH.[163,170] Inhibition of cellular functions at acidic pH values has also been attributed to deleterious effects exerted directly by fermentation end products such as organic acids (increase of proton permeability, inactivation of enzymes by high concentrations of acids intracellularly) and alcohols (chaotropic effects).[170] These and the observations that the intracellular pH plays a fundamental role in the regulation of solute transport (see Section VIII) suggest that under certain conditions in fermentative bacteria the function of proton pumps in maintaining a neutral to slightly alkaline cytoplasm can be as important as the generation of a pmf. This is strongly illustrated by the ability of some anaerobes to grow under certain conditions in the absence of a pmf.[170,178] For instance, *E. faecalis* is able to grow in the presence of protonophores and/or ionophores which short circuit the currents for H^+, K^+, and Na^+, provided the medium pH exceeds 7, and high concentrations of essential nutrients and K^+ and low concentrations of Na^+ are present.[178] A high pmf is essential for these organisms at low concentrations of essential nutrients as indicated by the severe inhibition of growth upon reducing the concentration of amino acids from 1 to 2 mM to 0.1 mM in the presence of the ionophores.[178]

VII. MODEL SYSTEMS FOR SOLUTE TRANSPORT

Although whole cells provide a model system for *in vivo* studies of solute transport in lactococci,[179] for detailed studied of these mechanisms at the molecular level it is desirable to make use of isolated membrane vesicles. The advantages of this system are twofold: (1) vesicles are devoid of interfering cytoplasmic enzymes and (2) the structural and functional properties of the cytoplasmic membrane are retained. Kaback devised a procedure for the isolation of closed cytoplasmic membrane vesicles by osmotic lysis of sphaeroplasts of *E. coli*.[180] These membrane vesicles have the same polarity of the cytoplasmic membrane as in intact cells. Several procedures for the preparation of membrane vesicles of enterococcal species have appeared in the literature,[181,182] but none of these procedures yield membrane vesicles with a homogeneous orientation of the cytoplasmic membrane. For this purpose the

procedure of Kaback has been considerably modified. Lysis of lysozyme-treated cells occurs only in high ionic strength buffer, yielding functional membrane vesicles of lactococci which are homogenous in orientation.[71]

The pmf supplies the energy for many energy-transducing functions of membranes, such as solute transport across the membranes.[183] Except for menaquiones and the NADH dehydrogenase, lactococcal membrane vesicles contain no electron carriers such as cytochromes or dehydrogenases.[71] It is therefore not possible to generate a pmf by electron flow. The major pmf-generating system present in these membranes is the F_0F_1-ATPase complex. The catalytic site of the ATPase is located on the inner surface of the membrane of these vesicles which have the same orientation as the cytoplasmic membrane of intact cells. Since the membrane is impermeable to ATP addition of exogenous nucleotide to these membrane preparations does not result in the generation of a pmf. One must therefore resort to artificial methods for the generation of pmf, such as valinomycin-mediated potassium efflux or diffusion gradients of weak acids.[116,184] By these procedures it is possible to generate a pmf across the lactococcal membrane, and the uptake of a number of amino acids has been demonstrated in response to the potential applied.[71] Although these studies have shown that membrane vesicles of lactococci retain the property of a diffusion barrier, the endogenous ion leak of the membranes is in general too high to maintain an imposed pmf for a long period of time.

In vesicles of many aerobic bacteria a pmf can be generated by supplying membrane localized pmf-generating systems (e.g., electron transfer chains) with a continuous source of energy. A similar experimental system has been developed for lactococci[185-188] and other fermentative bacteria[188,189] by combining the technology of reconstitution procedures for pmf-generating systems and methods to induce fusion between biological membranes and liposomes. In these fused membranes a constant pmf can be generated for a much longer period of time compared to a imposed pmf. A detailed description of the membrane fusion inducing techniques and properties of the pmf-generating systems is beyond the scope of this review. For this purpose the reader is referred to a recent review on this topic.[190]

The properties of solute transport systems of *L. lactis* species has been studied in membrane vesicles fused with proteoliposomes containing one of the following pmf-generating systems:

1. Bacteriorhodopsin, a light-driven protonpump isolated from the cytoplasmic membrane of halophilic bacteria. Most reconstitution procedures for bacteriorhodopsin result in the formation of proteoliposomes in which an everted polarity of the pmf, i.e., inside positive and acid is obtained upon illumination. Bacteriorhodopsin is therefore best suited for studies on the properties of solute extrusion systems (see Section IX.B).[187,191]
2. Cytochrome c oxidase, a redox-linked protonpump isolated from the mitochondrial inner-membrane. This protonpump interacts asymmetrically with the electron donor cytochrome c. Upon reconstitution into proteoliposomes, only cytochrome c oxidase molecules with their cytochrome c binding site located on the outer surface are able to accept electrons from reduced cytochrome c (see Figure 11). By these constraints the generation of a pmf, inside negative and alkaline is assured[185,186], and by its ease of use over a wide pH range the cytochrome c oxidase system is often the system of choice.
3. Reaction centers extracted from chromatophores of phototrophic bacteria. As in the case of cytochrome c oxidase, the light-dependent pmf-generating reaction centers exhibit an asymmetric interaction with cytochrome c, which in combination with membrane permeable quinones assures the generation of a pmf with the *in vivo* polarity.[192,193] Moreover, this pmf-generating system can be used in an anaerobic environment, and the magnitude of the pmf can be adjusted by varying the light intensity.[188]

Reconstituted proteoliposomes containing one of these pmf-generating systems are fused

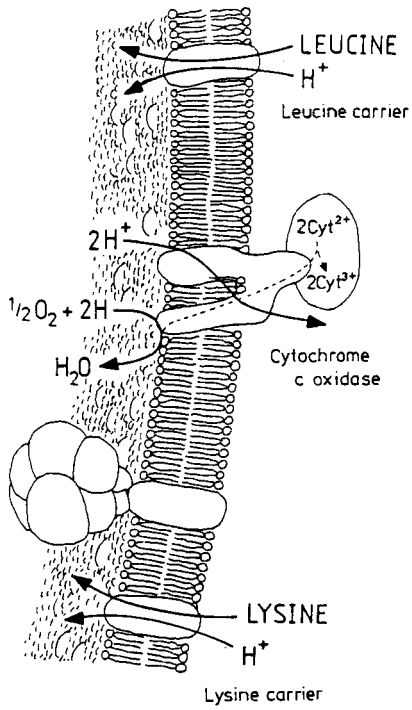


FIGURE 11. Scheme of the insertion of cytochrome c oxidase in the cytoplasmic membrane of *L. lactis*.

with membrane vesicles derived from *L. lactis* by one of the following procedures. In low pH-induced fusion the pH of a suspension of proton pump containing proteoliposomes and bacterial membrane vesicles is lowered to a pH of 6.0 or lower, and the suspension is allowed to remain at that pH for several minutes.[187,194] Under those conditions the membranes tend to fuse giving rise to hybrid membranes containing both the bacterial membrane proteins and the protonpump initially incorporated into the proteoliposomes. In freeze/thaw-sonication-induced fusion a mixture of proteoliposomes containing a pmf generator and bacterial membrane vesicles is rapidly frozen in liquid nitrogen and subsequently is allowed to thaw slowly at room temperature. Under those conditions membrane fusion occurs. The turbid membrane suspension which contains mainly multilamellar membrane vesicles is dispersed by brief ultrasonic irradiation, finally resulting in a homogenous mixture of unilamellar fused membranes.[185,186]

The development of a procedure for the incorporation of a functional pmf-generating system into bacterial (biological) membranes has had a great impact on bioenergetic studies in lactococci. In general a pmf of more than 100 mV can be sustained for at least 30 min, allowing detailed studies on the mechanism of energy coupling to solute transport. The potential applications of these fused membranes will be illustrated in the following section on amino acid and peptide transport in lactococci.

VIII. TRANSPORT OF AMINO ACIDS AND PEPTIDES

The major amino acid source for lactococci growing in milk is the milk protein casein. Caseins are present in milk as micelles with an open, large random structure which makes them readily susceptible to proteolysis by proteinases produced by lactococci. Considerable attention has been paid to the study of the biochemical and genetic properties of proteinases

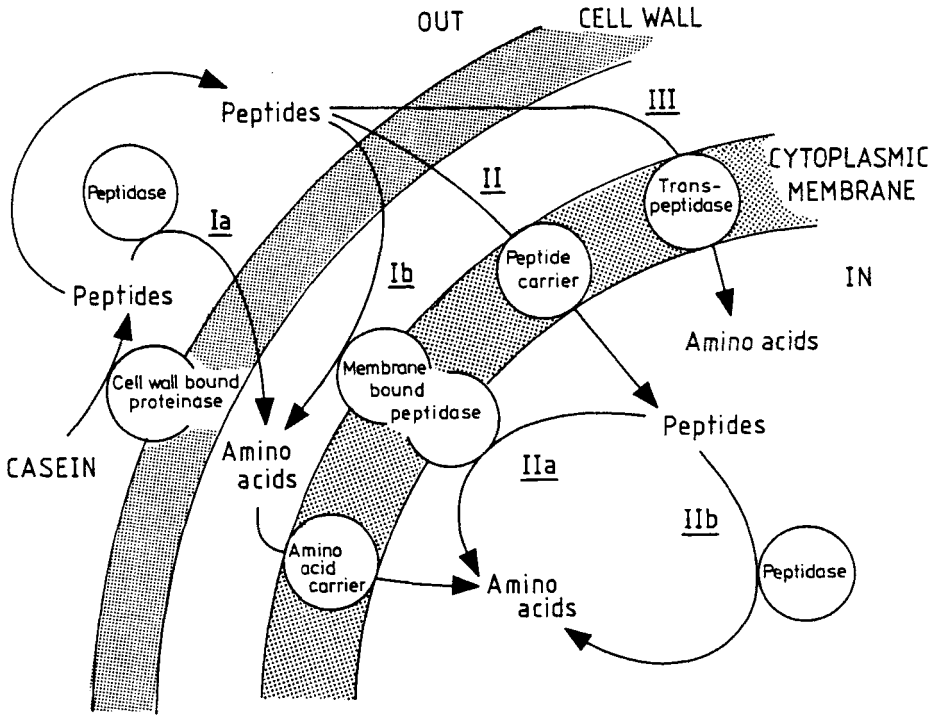


FIGURE 12. Schematic presentation of the process involved in the case in hydrolysis and the possible mechanisms for uptake and hydrolysis of peptides. In Model I, peptide hydrolysis occurs extracellularly by soluble peptidases (Ia) or peptidases associated with the external surface of the membrane (Ib). Uptake of the liberated amino acids into the cells is catalyzed by amino acid carriers. In Model II, peptide uptake occurs via specific peptide carriers and subsequent intracellular hydrolysis by membrane associated (IIa) or soluble peptidases (IIb) Model III, peptides are simultaneously translocated and hydrolyzed by a membrane-bound trans-peptidase complex. Further detailed discussion is given in Section VIII.A.

and peptidases.[195,196] Proteinases are mainly bound to the cell wall and are primarily responsible for the degradation of extracellular proteins (Figure 12). A complexity of proteinase activities by lactococci has been detected and these activities result in the liberation of a variety of small peptides with different numbers of amino acid residues. These peptides are subsequently degraded by peptidases of which also a wide array have been recognized. These enzymes have been divided into different classes, depending on their substrate specificity and mode of action, e.g., amino-, di-, tripeptidases, and arylamidases. Although many of these enzymes are located intracellularly, both cytoplasmic membrane and cell wall-bound forms have also been described.

Numerous amino acids are either stimulatory or essential for growth of lactococci. These amino acids have to be translocated across the membrane. Also, peptides can enhance the growth rates and yields of lactococci, although some strains of *L. lactis* subspecies *cremoris* grow poorly on peptide-containing media.[197,198] Since several peptidases are located intracellularly, peptides have to be translocated across the membrane in order to be hydrolyzed. Extensive information on the biochemical properties of the proteolytic enzymes has been accumulated over the past years, but the information on the mechanism of amino acid and peptide transport into lactococci is limited.

Three types of amino acid transport systems can be recognized in lactococci (see Figure 1): (1) pmf-driven transport, in which amino acid uptake is primarily coupled to the pmf; (2) exchange or antiporter transport, in which the driving force for transport is supplied by

Table 2
AFFINITY CONSTANTS AND SPECIFICITY OF AMINO ACID TRANSPORT SYSTEMS IN *LACTOCOCCUS LACTIS* STRAIN ML_3 AND SUBSPECIES *CREMORIS* Wg_2

Transport system	Affinity (μM)	Energy coupling mechanism
L-leucine	6.5	pmf-driven[188,199-201]
L-isoleucine	8	
L-valine	12	
L-alanine	52	pmf-driven[199,200,207]
glycine	330	
L-serine	42	pmf-driven[199,200,207]
L-threonine	285	
L-lysine	16	pmf-driven[219]
L-arginine	5.5	antiport[122,125]
L-ornithine	40	
L-glutamic acid	1.8	~P-linked[161,222]
L-glutamine	2.5	
L-asparagine	3.0	~P-linked[223]
L-aspartate	250	unknown[223]

the chemical gradient of one or both amino acids; and (3) phosphate-bond linked transport, in which the driving force for uptake is supplied by ATP or another high-energy phosphate-bond intermediate.

These types of amino acid transport systems will be discussed in separate sections, except for exchange transport which is exemplified by the Arg/Orn antiporter and discussed in the section on arginine metabolism and transport.

A. Protonmotive Force Driven Amino Acid Transport

1. Leucine, Isoleucine, and Valine

An in-depth analysis of the properties of the branched chain amino acid carrier (referred to as the leucine carrier, see the following) of lactococci has been carried out in membrane vesicles of *L. lactis* subspecies *cremoris* fused with proteoliposomes containing cytochrome c oxidase or reaction centers by a procedure outlined above.

a. Specificity

The leucine carrier of *L. lactis* subspecies *cremoris* Wg_2 catalyzes the translocation of L-leucine, L-isoleucine, and L-valine in symport with one H^+ (see Table 2).[188,199-201] Equilibrium ligand binding studies indicate that cytoplasmic membranes of *L. lactis* subspecies *cremoris* contain approximately 42 pmol leucine binding sites per mg of protein with a K_D of 1.5 μM at pH 6.0[201] The leucine carrier has a single binding site for H^+ with a pK_{app} of about 7.0. The affinity of the carrier for leucine decreases with decreasing external H^+ concentration.

The structural requirements for substrate binding to the leucine carrier have been deduced from the inhibitory effects of branched chain amino acid analogues on the initial rate of pmf-driven leucine uptake. The carrier has a 15-fold higher affinity for L isomers than for D isomers with an aliphatic side chain of at least three methyl groups. The amino group, hydrogen atom, and carbonyl-group bond to C-α are essential and the carboxylic acid group can be modified within certain limits. Methionine which resembles norleucine, i.e., a non-branched aliphatic side chain, is also transported by the leucine carrier with a low affinity of about 100 μM.[201]

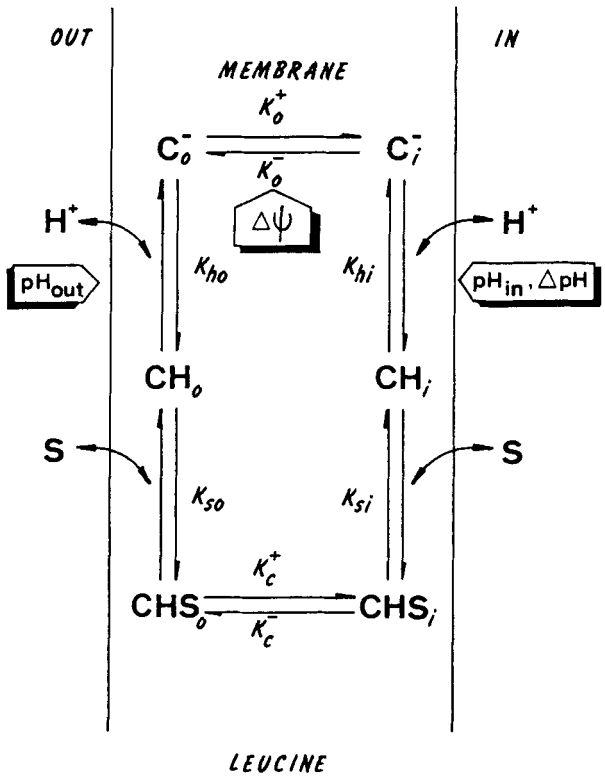


FIGURE 13. A model for the translocation cycle of H^+/leucine co-transport. In the presence of a pmf, the carrier (C) becomes protonated on the outer surface (H), followed by binding of leucine (S). Reorientation of the ternary [carrier-H^+-leucine] complex takes place, and on the inner surface release of leucine occurs. After deprotonation, the unloaded carrier is reoriented from the inner to the outer surface in order to repeat this cycle. This step is accelerated by the $\Delta\psi$. Deprotonation and protonation of the carrier are affected by the internal and external pH, respectively. (From Driessen, A. J. M., de Jong, S., and Konings, W. N., *J. Bacteriol.*, 169, 5193, 1987. With permission.)

b. Mechanism

Analysis of the pH and pmf dependency of various modes of facilitated diffusion suggest that H^+ and leucine binding to and from the carrier occurs by a sequential ordered mechanism.[201] The essential features of this four-step model for leucine transport are (Figure 13) H^+ binding at the outer surface of the membrane occurs prior to leucine binding, the carrier-H^+-leucine complex reorients, at the inner surface of the membrane release of leucine is succeeded by deprotonation of the carrier and finally, the unloaded carrier subsequently reorients and the cycle can be repeated. In the absence of a pmf the carrier functions symmetrically. In the presence of a pmf the rate of leucine transport is increased. The role of both components of the pmf, $\Delta\psi$ and ΔpH on the kinetic mechanism of leucine transport has been analyzed in detail. $\Delta\psi$ increases the rate of leucine transport by accelerating the reorientation of the unloaded carrier. The $\Delta\psi$ dependency of the rate of leucine transport has been further analyzed with membrane vesicles fused with reaction center proteoliposomes.[188] In these fused membranes the $\Delta\psi$ can simply be varied with the light intensity under conditions that the ΔpH is collapsed by the ionophore nigericin, which catalyzes an electroneutral exchange between K^+ and protons. The initial rate of leucine transport increases exponentially with increasing $\Delta\psi$. Although in these experiments the kinetic param-

eters of leucine transport were not determined, it was previously demonstrated that the K_t is independent from $\Delta\psi$, thus the V_{max} of leucine transport is expected to vary in a similar way.[201] ΔpH has an effect on the step which involves the deprotonation of the carrier on the inner surface of the membrane. ΔpH causes a shift of the equilibrium of carrier intermediates to the deprotonated species. The rate of leucine transport increases with increasing intracellular pH with a pK_{app} of H^+ release of approximately 7. Since this pK_{app} is within the physiological intracellular pH range, the carrier evinces the internal pH *in vivo*.[199,201] The intracellular pH thus determines the extent of deprotonation of the leucine-H^+-carrier complex, and has to be designated as a catalytic pH effect.[164,201] It should be emphasized that none of these steps, e.g., the deprotonation on the inner surface of the membrane and the reorientation of the unloaded carrier are uniquely rate limiting.

The stoichiometry of H^+ and leucine cotransport has been found to be one under many conditions, while under other conditions values far below one are estimated.[188,199,200] These deviations from one-to-one stoichiometry may be attributed to passive diffusion of the solute across the membrane.[200] Membranes exhibit a significant passive permeability (leak) for various amino acids. The diffusion constant is a function of the hydrophobicity of the side chain of the amino acid and is relatively high for the hydrophobic branched chain amino acids. As a result of this leak process a steady state for accumulation of these amino acids is rapidly reached before thermodynamic equilibrium is achieved. When the activity of the leucine carrier is reduced even lower steady state accumulation levels are achieved. In contrast, hydrophilic amino acids, like alanine and serine, reach a steady state of uptake which is close to thermodynamic equilibrium (see Section VIII.A.2.). The stoichiometry of H^+ and leucine cotransport is, however, independent from the magnitude of $\Delta\psi$.[188]

c. Role of Lipid Environment

The major lipid species in *L. lactis* subspecies *cremoris* membranes are acidic phospholipids (phosphatidylglycerol (PtdGro) and cardiolipin (Ptd_2Gro)), glycolipids and glycerophosphoglycolipids.[202,203] Phosphatidylethanolamine (PtdEtn) is completely absent. The role of the phospholipid composition on both pmf-driven and counterflow activity of the leucine carrier has been investigated.[202] Alterations in phospholipid composition can be achieved by using the membrane fusion technique (see Section VII). The leucine carrier is only active in lipid bilayers containing aminophospholipids, such as PtdEtn and phosphatidylserine (PtdSer), or glycolipids, such as mono- and digalactosyldiglycerides or native glycolipids. Phosphatidylcholine (PtdCho) or mixtures of PtdCho with PtdGro, Ptd_2Gro, or phosphatidic acid do not permit leucine carrier activity. In mixtures of PtdCho and methylated derivatives of PtdEtn, carrier activity decreases with increasing degree of methylation of PtdEtn. The lipid composition of the membrane only affects the V_{max} of leucine transport, whereas the K_t remains largely unchanged. Since aminophospholipids are completely absent in cytoplasmic membranes of lactococci, glycolipids will be most likely the physiological relevant lipid species. The role of membrane fluidity in modulating the activity of the leucine carrier has been studied in membrane vesicles of *L. lactis* subspecies *cremoris* fused with PtdCho/PtdEtn liposomes which contain increasing levels of cholesterol.[204] Cholesterol reduces the rate of leucine transport, an effect which is paralleled by a relative increase in membrane viscosity.

d. Molecular Properties

The V_{max} of leucine transport by *L. lactis* ML_3 was at least tenfold higher when the organism was grown on a chemically defined medium compared to growth in a complex medium. Possibly peptides or other compounds present in the complex medium act as a repressor(s) for the leucine transport system.[205] Membrane vesicles derived from cells with an elevated leucine carrier activity have been used for the isolation and partial purification

of the leucine carrier.[206] Partial purification of the carrier has been accomplished by simple procedures employing partial solubilization and ion-exchange chromatography. Some characteristics of the partially purified leucine carrier have been determined. No specific inhibitors of the leucine carrier have been found thus far. The system appears to be insensitive towards thiol-specific reagents, but strongly inhibited by the histidyl specific reagent diethylpyrocarbonate.[207] The carrier can only be detected and quantitated by activity measurements after reconstitution into liposomes. Reconstitution of the partially purified leucine carrier into proteoliposomes has been accomplished by the octylglucoside dialysis method. These proteoliposomes exhibit both pmf-driven and counterflow activity of leucine. Although the leucine carrier has not yet been purified to homogeneity, analysis of this preparation by sodium dodecyl sulfate polyacrylamide gel electrophoresis demonstrates a high degree of purity.

e. Branched Chain Amino Acid Transport by Streptococci and Enterococci

Transport studies of branched chain amino acids have also been carried out in other strains of *L. lactis*[208] and several streptococci, i.e., *S. agalactiae*,[209] *S. thermophilus*,[210] and *S. pneumoniae*.[211] It has been suggested that more than one transport system is present for these amino acids. Whole cells of *S. thermophilus* and *S. agalactiae* display biphasic kinetics of branched-chain amino acid uptake with apparent K_t values for leucine uptake of 6 to 10 μM and 250 to 310 μM, respectively. However in view of the above described high permeability of the cytoplasmic membrane for leucine it is not unlikely that the apparent low affinity is due to passive diffusion of leucine, as shown for in *L. lactis* subspecies *cremoris*.[200,201] Some evidence for pmf-dependent transport of branched-chain amino acids in these streptococci has been presented. Firm conclusions with respect to the mechanism of energy coupling to branched-chain amino acid transport in these organisms are not yet possible since only indirect evidence based on the effects of inhibitors of cellular metabolic processes has been presented.[209,210]

2. Alanine, Glycine, Serine, and Threonine

The mechanism and specificity of neutral amino acid transport has been studied in membrane vesicles of *L. lactis* subspecies *cremoris* Wg_2 fused with cytochrome c oxidase proteoliposomes[199,200,207] and in whole cells of *L. lactis*.[212]

a. Specificity

Careful kinetic analysis of neutral amino acid transport by membrane vesicles of *L. lactis* subspecies *cremoris* suggest that serine and threonine share a common transport system which is distinct from the common transport system for alanine and glycine (see Table 2).[207] Serine, alanine, and glycine markedly reduce the uptake of the nonmetabolizable alanine analogue α-aminoisobutyric acid (AIB) by whole cells of *L. lactis* ML_3.[212] Serine is, however, less efficient than alanaine and glycine in inhibiting AIB uptake, and the poor K_t for AIB uptake, i.e., 1 mM, makes uptake of this substrate more susceptible to inhibition by other low affinity substrates, such as serine. No K_i values have been provided and although some cross-specificity appears to exist, it seems more likely that in *L. lactis* separate systems are operational for serine and alanine.

b. Role of the Internal pH

Experiments performed with imposed Δ pH and $\Delta\psi$ in membrane vesicles of *L. lactis* subspecies *cremoris* suggest that uptake of alanine and serine is strictly coupled to the pmf.[199] The apparent H^+/amino acid stoichiometry of the transport process was estimated from the steady-state level of amino acid accumulation and pmf and drops from 0.9 to 1.0 to values as low as 0.2 when the external pH is raised from 5.5 to 8.0.[199,200] This drop in the apparent

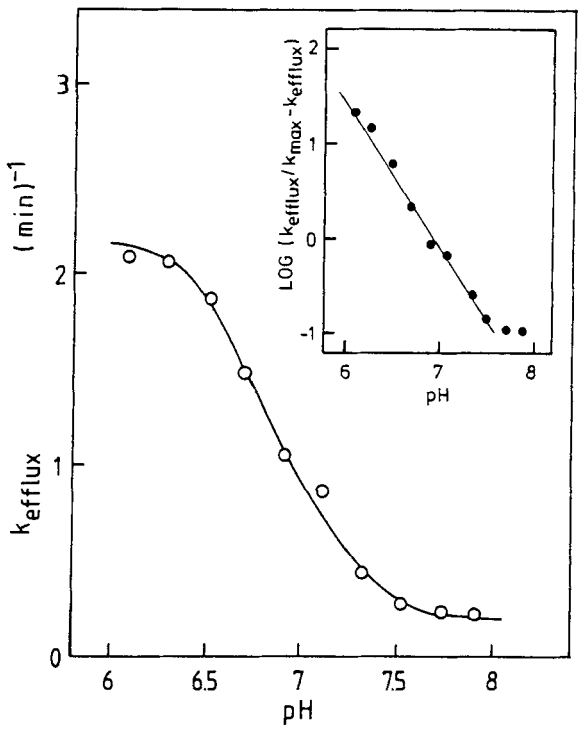


FIGURE 14. pH dependence of alanine efflux by membrane vesicles of *L. lactis* subsp. *cremoris* in the absence of a pmf. The first order rate constant for alanine efflux (K_{efflux}) was calculated from the time required for half-maximal release of alanine. The inset shows a Hill plot of the activation effect of protons on the activity of the alanine transport system, indicating a Hill coefficient of 1.6.[125]

stoichiometry was found to be caused by the low activity of the alanine and serine transport systems at high pH and the extent of passive diffusion of these amino acids across the membrane. As a result the overall rate of transport is too low at high pH to reach a thermodynamic steady state. A similar pH dependency has been observed for several modes of facilitated diffusion of alanine and serine, displaying an apparent pK of 7.0 (see Figure 14). Unlike the V_{max} of serine and alanine transport which decreases with increasing pH, the K_t of transport is not pH dependent. A Hill-plot from the activating effect of the H^+ concentration on alanine efflux suggests that the carrier is modulated by protonation of at least two highly cooperative proton-binding groups (see Figure 14).

The modulating effect of pH on the activity is caused by a prominent internal pH dependency of these carriers. Although uptake of alanine and serine is strictly coupled to the pmf, a stimulation of the initial rate of uptake of both serine and alanine, as well as the final level of uptake has been observed upon collapsing of the ΔpH by nigericin. This increase was not due to a change in apparent H^+/amino acid stoichiometries which has a value of around unity. In the absence of nigericin, the internal pH is equal to the sum of the external pH and ΔpH, while in the presence of nigericin the internal pH equals the external pH. It should be emphasized that nigericin does not affect the pmf since a collapse of the ΔpH is accompanied by a compensatory increase in Δψ.[199,200,213] Experiments performed at various external pH values in the presence and absence of nigericin, indicated that the initial rate of uptake of alanine and serine decreases with the internal pH with an aparent pK of 7.0.[199] The internal pH effects on alanine and serine transport are the same

for entry and exit as well as for exchange. It is therefore unlikely that pH predominately acts on a rate determining (de)protonation step in the catalytic mechanism. The pH therefore appears to exert allosteric effects on the transport protein.[163,199]

This strong internal pH dependency leads to some peculiar relationships between pmf, uptake, efflux and the steady-state concentration of AIB in whole cells of *L. lactis*.[212] Low concentrations of K^+ were found to inhibit AIB uptake, whereas efflux of AIB from the cells was stimulated. These apparently conflicting observations can now be explained by the finding that K^+ causes a depolarization of the $\Delta\psi$, which is compensated by an increase of the pH gradient (see also Section VI).[164] This latter effect results in a decreased activity of the alanine transport system, and because the outwardly directed passive flux of AIB remains unchanged, the internal concentration of AIB decreases.[200]

c. Neutral Amino Acid Transport by Streptococci and Enterococci

Uptake of alanine and/or AIB has also been studied in *E. faecalis*,[214-216] *S. pyogenes*,[217] and *S. bovis*.[218] The substrate specificity of the alanine transport system of *E. faecalis* and *S. pyogenes* are distinctly different for the system in *L. lactis* subspecies *cremoris*.[199,207] Alanine, glycine, threonine, and serine are transported by *E. faecalis* ATCC 9790 with K_t values between 30 and 100 μM.[214] Accumulated alanine is released by the addition of other neutral amino acids, and uptake of all four amino acids is reduced in a cycloserine-resistant mutant, suggesting the presence of a common transport system for these amino acids in *E. faecalis*. The involvement of a pmf in the accumulation of neutral amino acids is indicated by the effects of ionophores on amino acid transport, and the accumulation of glycine and threonine in starved cells upon imposition of a ΔpH and/or $\Delta\psi$.[214] AIB uptake in *S. pyogenes* type 12 follows biphasic kinetics with apparent K_t values of 48 and 480 μM.[217] The low affinity form is only detected in starved cells, and the existence of multiple alanine transport systems has been suggested. Uptake via the high-affinity system is pmf dependent, in contrast to uptake via the low affinity system. Competitive inhibition studies on AIB uptake via the high affinity system indicate that glycine, alanine, and serine are substrates for this system, with K_i values between 70 and 500 μM. Threonine is inhibitory with a very low K_i, i.e., 3.2 mM. A conclusive statement about the substrate specificity is only possible when mutual inhibition and correlating K_i and K_t values have been demonstrated. No strict requirement for K^+ and Na^+ has been observed, suggesting a H^+/amino acid symport mechanism.

In *S. bovis*, a ruminal bacterium, uptake of the neutral amino acids threonine, serine, and alanine by both whole cells and membrane vesicles is strictly Na^+ dependent, and occurs most likely by Na^+/amino acid symport mechanism.[218] High levels of amino acid transport are observed in the presence of a sodium-motive force, and further stimulation of the uptake rate can be achieved by the simultaneous imposition of a $\Delta\psi$. A Hill-plot of the Na^+-activating effect on serine transport indicates the presence of two Na^+-binding sites. The affinity for Na^+ must be very low, since more than 40 mM Na^+ is required for half-maximum activity. The K_t values for threonine, serine and alanine are found to be 6.3, 8.6, and 8.8 μM, respectively. As in *L. lactis* subspecies *cremoris*, serine and threonine are accumulated by a common transport system, whereas alanine is transported by another system.

3. Lysine

The properties of the Arg/Orn antiporter have been described in the section on arginine metabolism and transport. The Arg/Orn antiporter catalyzes strictly a one-to-one exchange, and in order to compensate for the fraction of arginine which is used for biosynthesis, cells require an alternative transport system for ornithine or arginine. This requirement might be fulfilled by the high affinity lysine carrier which also has specificity for ornithine. The characteristics of the lysine carrier have been studied in membrane vesicles of *L. lactis*

subspecies *cremoris* Wg_2 fused with cytochrome c oxidase proteoliposomes. Membranes of this organism were chosen for this study in order to avoid interference of the Arg/Orn antiporter. This antiporter which displays some affinity for lysine, is absent in *L. lactis* subspecies *cremoris*, and is thus better suited for studies on lysine transport.

Lysine transport is catalyzed by a pmf-dependent system, with a K_t of approximately 16 μM (see Table 2).[219] The lysine carrier exhibits a sharp substrate specificity which is distinct from that observed for the Arg/Orn antiporter. L-lysine transport is inhibited by a number of analogues which decrease the uptake rate in the following order; L-lysine > L-S-2-aminoethylcysteine > L-homoarginine > D-lysine > DL-δ-hydroxylysine ≫ L-arginine, L-canavanine, L-citrulline, and L-ornithine. Ornithine is a poor substrate for the lysine carrier with a K_i value in the submillimolar range.

Lysine uptake is primarily coupled to the $\Delta\psi$ component of the pmf. Experiments with imposed pH gradients demonstrate that lysine uptake occurs most likely by a H^+/lysine symport mechanism. In contrast to a number of other amino acid transport systems in lactococci[163] there are no indications that the lysine system is subjected to regulation by the internal or external pH. The V_{max} of lysine transport is fairly slow compared to uptake of other amino acids, and in conjunction with the low passive permeability of the membrane for lysine it takes long before a steady state is reached, i.e., at least 30 min. Another peculiar feature of this transport system is that the rate of lysine efflux is similar to the rate of lysine/lysine exchange. This enables the cell to maintain a sufficiently high intracellular lysine pool under conditions that the extracellular arginine concentration is high.

Lysine transport has also been studied in *E. faecalis* ATCC 8043.[220] Lysine uptake occurs via two systems, one energy-requiring system specific for lysine with a K_t of about 0.3 μM, and an energy-independent system specific for lysine and arginine with a lower affinity, i.e., 5 to 10 μM.[220] The latter system is most likely the Arg/Orn antiporter.[124] Hydroxylysine resistant mutants lack the high-affinity lysine transport system.

4. Other Amino Acids

Aside from the previously discussed amino acid transport systems, membrane vesicles of *L. lactis* subspecies *cremoris* Wg_2 fused with cytochrome c oxidase proteoliposomes also accumulate L-histidine, L-proline, L-methionine, L-cysteine, L-tyrosine, and L-phenylalanine, indicating that uptake of these amino acids is coupled to the pmf.[71,125,207] Up to now, uptake of these amino acids by lactococci has been poorly characterized and limited information about these uptake systems is available. Histidine uptake by membrane vesicles of *L. lactis* subspecies *cremoris* follows the time course of an imposed $\Delta\psi$.[71] Phenylalanine uptake by intact cells of *L. lactis* is inhibited by tyrosine and tryptophan.[205] Methionine is translocated by the leucine carrier.[201] At present, no information is available on a possible high-affinity methionine transport system.

Two transport systems for disulfide and sulfhydryl amino acids have been distinguished in *S. mutans*.[221] Glutathione disulfide (GSSG), L-cystine, and reduced glutathione, i.e., γ-glutamyl-cysteinyl-glycine (GSH) compete for uptake with apparent K_t values of 20 to 100 μM. After uptake, GSSH and L-cystine are reduced, resulting in the accumulation of GSH and L-cysteine within the cells. L-cysteine is taken up by a separate system with a K_t of 300 to 400 μM. This transport system also accepts a number of competing substrates with decreasing affinities: L-cysteine < L-penicillamine < L-alanine < D-cysteine. K_t values for these competing substrates range from 1 to 4 mM. Uptake of GSH and L-cysteine is dependent on the presence of glucose, and blocked by inhibitors of the glycolysis.

B. Phosphate Bond-Driven Amino Acid Transport

In addition to the pmf-driven and exchange transport systems, a third group of transport systems have been recognized in lactococci which are driven by phosphate-bond energy

(see Figure 1 and Table 2). These transport systems catalyze essentially irreversible vectorial reactions which differ in several respects from the reversible pmf-driven transport systems. Phosphate-bond-driven transport systems have been observed for the translocation of inorganic phosphate (see Section IX.A)[162] and the amino acids glutamate[161,222] glutamine, asparagine,[223] and possibly aspartate[223] in *L. lactis* and subspecies *cremoris*. Although not all these transport systems have been studied to the same extent, several common properties have been recognized:

1. Driving force. Transport of these solutes proceeds in the absence of a pmf.[161,162,222,223] The concentration gradients in cells can be up to 10^5 which exceeds the thermodynamic limits set by the pmf (assuming that one H^+ accompanies the translocation of one solute molecule). Although the exact nature of the energy source has not yet been established, uptake of these metabolities requires the production of metabolic energy by glycolysis or by the ADI pathway, possibly ATP or another metabolite derived thereof. An imposed pH gradient or electrical potential does not support uptake of these solutes. Furthermore, the activities of these transport systems decrease upon reduction of the intracellular levels of ATP by arsenate. None of these amino acids are accumulated by lactococcal membrane vesicles in the absence nor in the presence of a high pmf.
2. Regulation by the internal pH. Despite the dependence on phosphate-bond energy, variations in the magnitude of the ΔpH have been shown to be reflected directly in the activity of these transport systems.[161-163,223] The apparent relationships between the rates of transport and the ΔpH can, however, entirely be explained by internal (and external) pH effects. The activity of the phosphate-bond-driven transport systems increase sharply with increasing internal pH with apparent pK values of 7.0 to 7.3.
3. Unidirectionality. The rates of exit (efflux and exchange) are at least two orders of magnitude lower than the rates of uptake independent of the presence of metabolic energy.[162,222]
4. Transinhibition. The activity of the transport system for inorganic phosphate of *L. lactis* decreases with increasing concentration of inorganic phosphate intracellularly.[162] A similar type of regulation may affect the activity of other phosphate-bond-dependent transport systems when the intracellular pools of the corresponding metabolities are filled, but this phenomenon has not yet been demonstrated. Transinhibition might act as a regulatory device in these transport systems to prevent accumulation to unacceptably high internal levels.

1. Glutamate and Glutamine

In lactococci glutamate and glutamine are taken up by a single kinetically distinguishable transport system (see Table 2).[222] The pH dependence of the K_t for glutamate transport indicates that glutamic acid instead of glutamate anion is the transported species. The observed K_ts for glutamate uptake were 3.5, 11.2, 77, and 1200 μM at pH 4.0, 5.1, 6.0, and 7.0, respectively. However, recalculation of the K_t based on the concentration of the undissociated species glutamic acid yielded K_t values of 1.8 ± 0.5 μM independent of the external pH. The affinity of the transport system for glutamine is 2.5 μM and also pH independent. The kinetic characteristics of glutamate (and glutamine) transport systems in other bacteria have not yet been described. The absolute preference of the transport system for glutamic acid imposes severe limitations on the growth of *L. lactis* and subspecies *cremoris* at alkaline pH values.[205] At pH 7 or above the maximum specific growth rates of these organisms can be predicted (calculated) from the kinetic parameters of glutamate transport, the requirement for glutamate and the concentrations of amino acids (which competitively inhibit glutamate uptake) in the medium (see Figure 15). Growth limitation

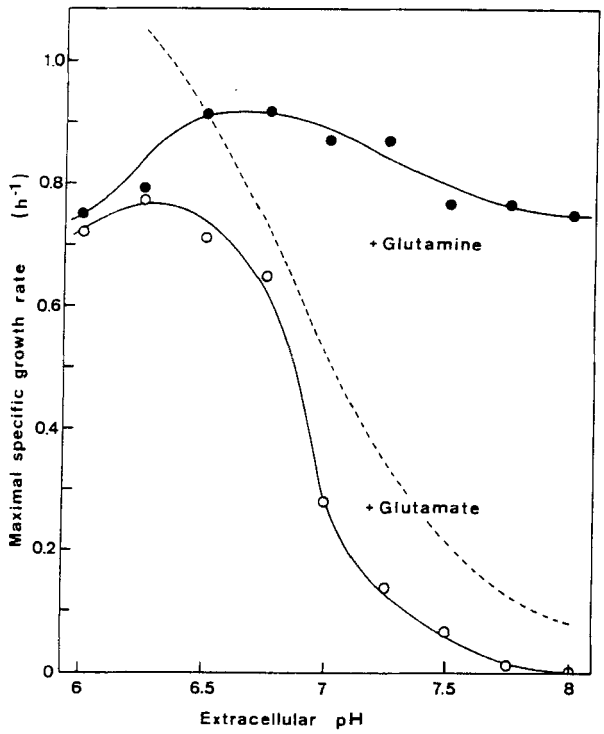


FIGURE 15. pH dependence of growth of *L. lactis* ML_3. Cells were grown in a chemically defined medium at 30°C with L-glutamate (○) or L-glutamine (●) as sources of L-glutamate for biosynthesis. The dashed line indicates the pH dependence of the predicted growth rate with L-glutamate assuming that uptake of undissociated species of glutamate, e.g., glutamic acid is growth-rate limiting. (From Poolman, B. and Konings, W. N., *J. Bacteriol.*, 170, 700, 1988. With permission.)

at alkaline pH values can be overcome by replacing glutamate by glutamine as source of glutamate in the growth medium. Since the K_t for glutamine uptake is independent of the external pH the cells are able to scavenge this amino acid from the growth medium effectively also at high external pH.

2. Aspartate

A kinetic analysis of aspartate uptake indicates that aspartate just like glutamate is taken up as the acidic species, e.g. aspartic acid. Aspartatic acid is accumulated with a very low affinity compared to the other solutes (K_t = 250 μM, Table 2).[163,223] The relatively weak competitive inhibition of aspartate transport by asparagine and glutamine suggest the presence of a separate low affinity transport system for aspartate. The mechanism of energy coupling to aspartate transport is still uncertain. Deënergized cells of *L. lactis* are able to accumulate aspartate whereas under the same conditions virtually no uptake of glutamine, glutamate and asparagine is observed. Aspartate uptake by deënergized cells could possibly occur by exchange with the high endogenous intracellular Asp pool.[100,136,137] Membrane vesicles of *L. lactis* fused with liposomes display a low but significant rate of homologous exchange of aspartate, and heterologous exchange between aspartate and glutamate (or glutamic acid).[223] Whether this aspartate/aspartate exchange activity is mediated by the aspartate uptake system has yet to be established.

3. Asparagine

In *L. lactis* asparagine appears to be accumulated by a high affinity transport system (K_t = 3.0 μM) which is distinct from the transport systems for glutamate/glutamine and aspartate (see Table 2).[223]

4. Transport of Acidic Amino Acid by Streptococci and Enterococci

Transport of acidic amino acids has also been studied in *E. faecalis*. In this organism glutamate uptake is mediated by a high-affinity system (K_t of 30 μM) and a low-affinity system (K_t of 12 mM).[224-226] Although glutamate transport by the low-affinity system is inhibited by glutamine, this transport system does not resemble the glutamate/glutamine transport system of *L. lactis*, since the initial rate of glutamate uptake was independent of the presence of an energy source, and thus most likely represents exchange of labeled extracellular amino acid with a preexisting intracellular glutamate pool.[224] The high-affinity system is competitively inhibited by aspartate (K_i of 7 μM)[226] which is a general property of dicarboxylic acid transport systems in bacteria. The glutamate/aspartate transport system of *E. faecalis* is rather insensitive towards variations in the internal pH unlike the glutamate/glutamine transport system of *L. lactis*.[226] Aspartate and glutamate uptake in *E. faecalis* is also essentially unidirectional and requires most likely ATP and not the pmf.[226] Phosphate-bond-dependent transport of glutamate as the anion has also been observed in *S. mutans*.[227] This system is specific both for glutamate and aspartate, and the rate of glutamate transport increases with increasing intracellular pH.

C. Peptide Transport

The limited availability of [^{14}C-]labeled peptides and the large number of possible combinations of amino acids in di- and oligopeptides has forced many workers to study peptide transport by indirect means. Uptake of [^{14}C-] labeled peptides in most cases is followed by a rapid efflux of the amino acids which are liberated after peptide-hydrolysis by intracellular peptidases. This severely limits the applicability of [^{14}C-]labeled peptides in studies on peptide transport in whole cells.[228,229] The removal of peptides from the medium is therefore often followed by labeling the remaining peptides with dansylchloride or fluorescamine, which are subsequently assayed by thin-layer chromatography or HPLC techniques.[230,231]

Three different mechanisms might be involved in the uptake of peptides (Figure 12): (1) peptides are externally cleaved by extracellular peptidases and the liberated amino acids are transported into the cell via specific amino acid transport systems, (2) peptides enter the cell via specific peptide transport system, and are subsequently intracellularly hydrolyzed by peptidases, and (3) peptides are hydrolyzed during the translocation step and the amino acids are released at the inner surface of the membrane.

In general, peptide uptake by lactococci is an energy-requiring process and abolished by protonophores and inhibitors of the membrane-bound ATPase.[232-234] Lactococci have been shown to possess separate transport systems for amino acids, di-, and oligopeptides, which rules out Model (1) as a major mechanism (Figure 12).[233,235] The rates of peptide utilization and transport can vary drastically between different strains of lactococci.[234-236] Some strains of *L. lactis* subspecies *cremoris* actually lack the ability to utilize dipeptides as a source of essential amino acids possibly because of the absence of dipeptide transport systems.[233] Growth studies on peptide-containing media and uptake of [^{14}C-]labeled peptides have indicated that the size exclusion limit for peptide transport across the membrane is four to five amino acid residues.[233,235]

In recent years more information has been gathered on the molecular mechanism of dipeptide transport. The characteristics and energy requirements for uptake and hydrolysis of leucyl-leucine have been studied in whole cells of *L. lactis* and subspecies *cremoris*.[232,234,236,237] L-[^{14}C-] leucyl-L-leucine uptake is mediated by a distinct transport system,

with a high affinity for di- and tripeptides which contain an N-terminal amino acid with a β-methyl group in the side chain.[234] The K_t for leucyl-leucine uptake by *L. lactis* subspecies *cremoris* E_8 increases more than tenfold when the external pH is raised from 5.2 to 7.4, whereas the V_{max} remains largely unchanged. Leucyl-leucine hydrolysis and uptake are strongly inhibited by protonophores, by DCCD and by arsenate, an inhibitor of ATP synthesis by substrate level phosphorylation.[232,234] At low external pH, nigericin completely abolishes leucyl-leucine uptake, whereas valinomycin is without any effect. At high external pH, however, nigericin appears to be less effective, and it was suggested that the intracellular pH is an important controlling factor for the activity of this transport system. Uptake of leucyl-leucine decreases with decreasing internal ATP-concentration which might imply that leucyl-leucine transport is driven by a phosphate-bond high energy intermediate or ATP, similar to the above discussed glutamine/glutamate transport system. Recent data, however, showed that at high pH leucyl-leucine uptake is inhibited by valinomycin and protonophores comparable to the uptake levels observed with deenergized cells.[238] This could be due to dipeptide uptake as a result of the inwardly directed dipeptide concentration-gradient and the intracellular hydrolysis of the dipeptide. Deenergized cells in which glutamic acid uptake is completely abolished (see also Section VIII.B), display a slow but progressive influx of leucyl-leucine and alanyl-glutamic acid.[238] The results appear to be more in line with an energy-coupling to pmf. Direct evidence for the role of the pmf as a driving force for dipeptide transport has recently been obtained in studies with membrane vesicles of *L. lactis* fused with cytochrome c oxidase proteoliposomes. Uptake of alanyl-glutamic acid by the fused membranes is inhibited by ionophores which dissipate pmf, and these ionophores cause efflux of accumulated dipeptide (see Figure 12, Model II). Quantitative evaluation of the accumulation of this anionic peptide in response to pmf indicates that transport is electrogenic with a H^+/alanyl-glutamic acid stoichiometry of one. This is the first observation that dipeptide transport can occur in bacterial membrane vesicles. Peptidase activity is virtually absent in these fused membranes. Accumulation of alanyl-glutamic acid occurs therefore in an intact form and the accumulated dipeptide is freely exchangeable with a variety of externally added dipeptides, including leucyl-leucine. The system exhibits a broad substrate specificity, and it seems that in *L. lactis* and subspecies *cremoris* (at least) one transport system is operational with a broad substrate specificity including zwitterionic and acidic di- and tripeptides. In agreement with this conclusion is the observation that a spontaneous L-alanyl-L-β-chloroalanine resistant mutant of *L. lactis* ML_3 displays greatly reduced uptake rates for a variety of di-and tripeptide.[238]

Separate transport systems for di-, oligo-, and anionic peptides have been identified in *E. faecalis*.[229,239] The dipeptide transport system requires both a free N-terminal α-amino group and C-terminal γ-carboxylgroup. On the other hand, the oligopeptide transport system, which shows a much lower activity, requires only a free N-terminal α-amino group. Toxic peptides, such as alanylphosphin, are primarily accumulated via this system, and mutants have been selected with these peptides which lack the oligopeptide transport system which translocates tri-, tetra-, and pentapeptides.[229] Both systems display little specificity for the amino acid composition or sequence. The anionic peptide transport system displays a specificity which is restricted to peptides with N-terminal glutamyl or aspartyl residues. Uptake of glutamate as glutamyl-peptides is more than 20-fold faster than uptake of the single amino acid.[239] Dipeptides are translocated in intact form by the dipeptide transport system as demonstrated with the peptidase-resistant substrate glycylsarcosine.[229] The peptide carriers are expressed to different levels in various strains of *E. faecalis*.[229] The exact mechanism of energy coupling is far from clear although a role of the pmf in dipeptide accumulation via the dipeptide carrier seems to be established.[231]

Transport and metabolism of arginine-containing peptides is of particular importance for oral streptococci. Salvia contains arginine-containing peptides, such as the tetrapeptide Sialin,

which act as pH rising factors.[239] In addition, arginine-containing peptides are liberated by a trypsin-like activity exhibited by a number of dental plaque bacteria.[241] These compounds stimulate glycolysis and delay the decline in plaque pH following exposure to a fermentable carbohydrate in a similar manner as arginine.[242] Arginine-containing peptides are externally cleaved by *S. mitis*[243,244] and *S. sanquis*,[245] thereby avoiding the need for a specific transport system for these positively charged peptides. A cell wall bound aminopeptidase which specifically hydrolyzes N-terminal arginine residues from low molecular weight peptides have been identified in *S. mitis* ATCC 9811.[234,244] Arginine is immediately converted into ornithine, and it appears that the aminopeptidase is subjected to a similar regulatory control as the ADI pathway enzymes (see Section IV.B).[246] It has been suggested that in addition to the aminopeptidase, arginine deiminase, and most of the ornithine carbamoyltransferase are cell wall bound.[247] The cell fraction experiments presented only allow a firm conclusion with respect to the localization of arginine deiminase. Furthermore, it seems unlikely that energy-rich compounds such as carbamoylphosphate are released externally and have to be recaptured by the cells. The Arg/Orn transport system was found in *S. sanquis* 12, but not in *S. mitis* strain BMS.[125] The presence of this system may be strain dependent or a different transport mechanism could be operative in *S. mitis*, possibly a citrulline/ornithine antiport system. Resting cells of *L. lactis* ML_3 are unable to use arginine containing peptides as a substitute for arginine in the ADI pathway.[238] Although these peptides are hydrolyzed by cell-free extracts, the inability of the cells to metabolize these peptides is due to the absence of a transport system for peptides with positively charged residues.

The apparent lack of side-chain specificity of some peptide transport systems certainly requires more attention. Characterization of peptide uptake using isolated membrane vesicles of *L. lactis* fused with cytochrome c oxidase proteoliposomes will facilitate a rapid elucidation of the actual number and specificity of peptide transport systems.

IX. TRANSPORT OF INORGANIC CATIONS AND ANIONS

A. Phosphate

The regulation by inorganic phosphate (P_i) of key glycolytic enzymes is well documented (see Section II.C).[107] A regulatory role by P_i has also been invoked in the reverse reaction catalyzed by an ATP-dependent HPr kinase and HPr(Ser-P) phosphatase (see Figure 4).[90,248] In lactococci no uniform mechanism for P_i transport seems to be operational. Transport of P_i in *L. lactis* ATCC 7692 can occur by P_i/P_i and P_i/sugar 6-phosphate exchange via the anion antiport system discussed in Section II.A. P_i uptake in this strain can also occur via another system which has not been further characterized.[53] P_i/sugar 6-phosphate exchange activity has not yet been detected in other lactococci except for *L. lactis* subspecies *cremoris* E_8.[53,61,162] P_i uptake by *L. lactis* strain ML_3 and subspecies *cremoris* Wg_2 appears to be catalyzed by a phosphate-bond linked transport system (see also Section VIII.B).[162] A general characteristic of these transport systems is that transport proceeds in the absence of a pmf as long as ATP is synthesized by either the ADI pathway or glycolysis. With a fermentable sugar as energy source, an excessive conversion of the accumulated P_i into organic phosphate compounds is observed. In the presence of arginine, most of the accumulated P_i is recovered in the free intracellular P_i pool. The transport system is essentially unidirectional, and does not catalyze P_i/P_i exchange. P_i concentration gradients of up to 10^5 can be established. The extracellular pH has no significant effect on the V_{max} and the K_t (6.2 μM) under conditions that the intracellular pH is kept constant, indicating that both HPO_4^{2-} and $H_2PO_4^-$ are substrates for this transport system. The exact mechanism of energy coupling is, however, still unknown. P_i transport is not affected by the $\Delta\psi$ and proceeds therefore most likely electroneutral, i.e, in symport with cations. The activity of the P_i transport system is controlled by the intracellular P_i concentration, most probably by a feedback inhibition mech-

anism allowing regulation of the free intracellular P_i pool. Protonophores and ionophores which dissipate the pH gradient across the membrane inhibited P_i transport only at acidic pH, suggesting that the activity of the transporter is also controlled by the intracellular pH. The pK_{app} of this regulatory phenomenon ranges from 7.0 to 7.3, depending on the energy source.

In *E. faecalis* P_i translocation is an electroneutral process which is not directly coupled to the pmf, but rather involves ATP or another energy phosphorylated intermediate.[226,249,250] This transport system thus appears to be very similar to the one described system above. In this organism the rate of arsenate (phosphate-analog) transport increases with intracellular pH at internal pH values above 7.0 and is maximal at pH 7.5.[226]

In *S. pyogenes*, a transport system for P_i is operational with properties which differ from those of the P_i transport systems discussed above.[251] This system catalyzed P_i/P_i exchange at a rate which is much faster than unidirectional uptake or efflux, but unlike the anion antiporter of *L. lactis* ATCC 7962 since it and does not catalyze heterologous exchange between P_i and organic phosphate esters. Uptake of $^{32}P_i$ and exchange with externally added P_i occurs rapidly in starved cells with a low affinity of 1.1 mM. The V_{max} of P_i uptake is markedly reduced upon the addition of fermentable sugars or arginine, most likely by reducing the free intracellular P_i pool thereby preventing P_i/P_i exchange. This reduction is not reversed by ionophores or DCCD. On the other hand, the inhibitory effect of glucose can be completely reversed by inhibitors of the glycolysis. The K_t for P_i uptake shifts to larger values in the presence of arginine, suggesting the involvement of another negative effector which nature is yet unclear. The physiological role of this P_i/P_i exchange system is rather puzzling since this mechanism cannot by itself give rise to alterations in the free intracellular P_i pool.

B. Calcium

In general, bacteria maintain a low intracellular calcium concentration by the use of a specific extrusion system for Ca^{2+}. Initial studies performed by Kobayashi et al.[181] indicated that in *E. faecalis* an ATP-driven calcium pump is operational. Inside-out membrane vesicles of *E. faecalis* accumulate Ca^{2+} with ATP as energy source. Uptake of Ca^{2+} is essentially insensitive to DCCD, protonophores, and ionophores. The transport system exhibited a pH dependent K_t for Ca^{2+} uptake which varies between 0.15 to 1 mM. It was concluded that in *E. faecalis* Ca^{2+} efflux is catalyzed by an ATP-linked primary pump.[181] In a latter stage, similar Ca^{2+} transport systems have been described in *L. lactis* ATCC 9762 and *S. sanquis* Challis V288.[252-254] The affinities of these systems, and that of *E. faecalis* ATCC 9790 for Ca^{2+} are several orders of magnitude higher, i.e., about 0.5 μM.[253] The apparent K_m for ATP of the Ca^{2+}-ATPase of *S. sanquis* is approximately 0.1 mM. Membrane vesicles of *L. lactis* and the other lactic acid bacteria have been extracted with octyl-β-D-glucoside in the presence of *E. coli* phospholipid and glycerol as a stablizing compound.[252] Reconstitution of the extracted proteins into proteoliposomes by detergent dilution yields a preparation which catalyzes ATP-dependent Ca^{2+} uptake. Accumulated $^{45}Ca^{2+}$ can be rapidly chased by an excess of externally added cold Ca^{2+}. Ca^{2+} transport by any of these preparations is not affected by protonophores or the ionophores, valinomycin, and nigericin. In inside-out membrane vesicles[253] and reconstituted proteoliposomes[252] of *E. faecalis* DCCD partially inhibited Ca^{2}- transport, but this effect has been attributed to a nonspecific effect of this compound. Transport of Ca^{2+} by any of these three preparations is completely blocked by micromolar levels of orthovanadate, a characteristic of many eukaryotic E_1E_2 ion-motive ATPases.[255]

Membrane vesicles of *L. lactis* subspecies *cremoris* fused with proteoliposomes containing the light-driven protonpump bacteriorhodopsin accumulate Ca^{2+} in response to the pmf generated by bacteriorhodopsin upon illumination.[187,191] In these fused membranes, bacteriorhodopsin generates a pmf, inside acid and positive, upon illumination. Ca^{2+} transport by

these fused membranes does not require ATP and is solely dependent on the ΔpH component of the pmf. This was demonstrated by the use of ionophores and artificially imposed pH gradients. These results are indicative for an electroneutral Ca^{2+}/H^+ antiporter mechanism. The presence of this extrusion system for Ca^{2+} in the lactococcal membrane might be a strain-dependent feature.

C. Potassium

Potassium is an important constituent of the cytoplasm. K^+ ions activate a number of enzymes and are required for ribosomal protein synthesis. Moreover, the circulation of K^+ (and Na^+) ions plays an important role in several homeostatic mechanisms, such as the regulation of the intracellular pH and osmotic strength. Virtually no information is available on the mechanism(s) of K^+ transport in lactococci, except for a study on the uptake of a K^+ analog, i.e., thallous ion (Tl^+), by *L. lactis*.[256] The effect of K^+ on the magnitude and composition of the pmf has been discussed in a previous section, and it seems reasonable to assume that an electrogenic K^+ transport system is operational in lactococci. K^+ transport has been fairly well studied in *E. faecalis*, and will be reviewed in this section.

1. Ktr I

Transport studies with whole cells of wild type and mutant strains of *E. faecalis* have revealed two distinct K^+ transport systems in this microorganism. The major K^+ transport system, designated KtrI, is constitutive and resembles the Trk system of *E. coli* (see Figure 16).[165] Cells of *E. faecalis* accumulate K^+ or Rb^+ ions at rates as high as 70 nmol/mg cell dry weight × min, with a K_t of 0.2 mM for K^+.[165] Optimal activity is at pH 7.0[165,257] and K^+ can be accumulated up to 5 × 10^4-fold. K^+ accumulation via the KtrI system is electrogenic and cells have to generate both ATP and a pmf.[165] The KrtI is assumed to be a K^+/H^+ symporter that functions only when it is phosphorylated, although the experimental support for this idea is circumstantial.[165] A K^+-ATPase of *E. faecalis*, with properties similar to the KtrI system has been purified to homogeneity.[258] The purified enzyme exhibits the following characteristics: (1) it consists of a single polypeptide component exhibiting a molecular weight of 78,000 Da on sodium dodecyl sulfate polyacrylamide gels.[258,259] The gene encoding for this K^+-ATPase has recently been cloned, and DNA sequencing revealed that this gene encodes for a protein of 583 amino acids with a calculated molecular weight of 63,070 Da,[259] (2) the enzyme is inhibited by micromolar concentrations of vanadate,[258] (3) an acylphosphate intermediate is formed with the γ-phosphate of ATP as part of the reaction cycle. This chemical form of the phosphoenzyme has so far only been found as a reaction cycle intermediate of ion-motive ATPases (E_1E_2-ATPases).[254] The amino acid residue subjected to phosphorylation has been identified as an aspartic acid, giving rise to the formation of β-aspartyl phosphate,[260] and (4) the ATPase catalyzes electrogenic K^+ transport in proteoliposomes, with a K^+/ATP stoichiometry of one, a K_t for K^+ of 1.4 mM, and a V_{max} of 0.1 μmol/mg of the purified protein × min.[258,261]

The identity of the purified K^+-ATPase being the KtrI system has been questioned.[262] However, many characteristics of the K^+-ATPase coincide with those reported for the KtrI system.[165,261] With the reconstituted K^+-ATPase no evidence has been obtained for H^+-translocation[261] by this transport system suggesting that the system functions as a K^+-ATPase rather than a H^+/K^+-ATPase.[83]

The primary structure of this protein has regions of homology with the KdpB subunit of the K^+-ATPase of *E. coli*.[259] Also the hydropathy profiles and the secondary structure predictions demonstrate many structural similarities between both proteins. This K^+-ATPase most likely functions as a pmf-regulated, ATP-driven pump that catalyzes the electrogenic translocation of K^+ in *E. faecalis*.

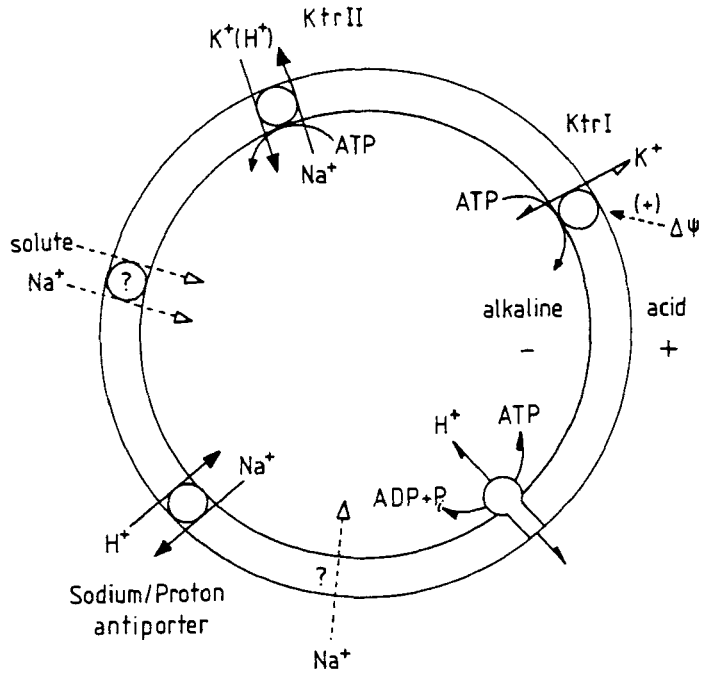


FIGURE 16. A model for potassium and sodium circulation in Enterococcus faecalis. Ktr I is assumed to be an ATP-driven K^+ transport system regulated by the pmf. This system might also be involved in K^+ efflux, although there is no direct evidence which supports this notion. The Na^+-stimulated ATPase that exchanges intracellular Na^+ for extracellular K^+ is denoted by Ktr II. It is not clear whether the Ktr II system is specific for K^+ or whether this system can also accept H^+ or other ions. An alternative route for Na^+ efflux is indicated by the Na^+/H^+ antiporter which is driven by the pmf. The route by which Na^+ enters the cells is not known (?), although Na^+ might enter the cell as a coupling ion in transport of certain solutes.

2. Ktr II

The second K^+ transport system, KtrII, is a Na^+-stimulated ATPase that exchanges cytoplasmic Na^+ for external K^+ (see Figure 16).[257,262] The system was first reported in a mutant of *E. faecalis* deficient in the generation of a pmf.[257] The characteristics of the KtrII system can be summarized as follows: (1) K^+ is preferred above Rb^+, although the K_t for K^+ is relatively low, i.e., about 0.5 mM, (2) the activity is very low at pH values below 7, and maximal at pH 8.5, (3) Na^+ ions stimulate K^+ transport and should be present on the inside of the cells. The system catalyzes a one-to-one exchange between Na^+ and K^+, (4) K^+ uptake requires the generation of ATP by the cells, whereas a pmf is not necessary for activity, and (5) the system is inducible and expressed under conditions that the external Na^+ concentration is high, or conditions which cause a low pmf.

It has been suggested[262] that the KtrII system is a manifestation of the Na^+-stimulated ATPase which was supposed to mediate exchange between Na^+ and H^+.[182,263-265] Mutants lacking the Na^+-ATPase also lack the KtrII system, and both systems are simultaneously recovered in revertants.[262] Both systems are also simultaneously induced when the cells are grown on media rich in Na^+, or when grown in the presence of protonophores.[262,266] A quantitative correlation exists between the expression of the activity of the Na^+-stimulated ATPase and the initial K^+ uptake rate.[262] A KtrII-like type of activity has not yet been reported in lactococci, although K^+ transport in *L. lactis* is stimulated by Na^+.[256]

D. Sodium

Bacterial cells extrude Na^+ ions mostly by a Na^+/H^+ antiport mechanism, such that the sodium concentration in the cytoplasm is kept low. The inwardly directed Na^+ gradient might serve as a driving force for transport systems which catalyze Na^+/solute symport, such as the transport systems for alanine and serine of *S. bovis* discussed in Section VIII.A.2.c. Moreover, the Na^+/H^+ antiporter is considered to be involved in the regulation of the intracellular pH. At present, there are no reports on Na^+ transport in lactococci. Initially, Na^+ extrusion by *E. faecalis* was thought be catalyzed by means of a Na^+-stimulated ATP-driven Na^+/H^+ antiporter.[182,263-265] Na^+/H^+ antiport activity, independent from ATP, has been observed both in whole cells as well as in everted membrane vesicles.[182,265] However, this activity was attributed to an artefact caused by proteolytic damage to the ATPase.[263] The Na^+-stimulated ATPase was thought to be composed of two subunits, i.e., a Na^+/H^+ antiporter and an associated catalytic subunit. Proteolytic treatment would detach both subunits, resulting in the appearance of Na^+/H^+ antiport activity. More recent studies indicate that this activity is the KtrII transport system, which mediates ATP-dependent exchange between Na^+ and K^+.[262] The KtrII system thus interlinks K^+ and Na^+ transport (see Section IX.C). This KtrII system is inducible but cells which lack this activity are still able to extrude Na^+.[176] Na^+ extrusion in cells lacking the KtrII activity is most likely catalyzed by a Na^+/H^+ antiporter, since extrusion depends on an energy source which is blocked by DCCD and protonophores. Moreover, Na^+ extrusion can be elicited by the imposition of a pH gradient.[176] Na^+ efflux is partially inhibited by valinomycin, and an electrogenic Na^+/H^+ exchange mechanism has been proposed. In analogy with K^+ transport a dual mechanism for Na^+ extrusion seems to be operational.

X. CONCLUDING REMARKS

Lactococci are fermentative bacteria which generate metabolic energy by substrate level phosphorylation during sugar fermentation and/or arginine metabolism and by pmf generation by ATP-hydrolysis and lactate efflux. During these metabolic processes the lactococci are capable of maintaining a high phosphate potential ($\Delta G'_p$ of up to ~460 mV; intracellular ATP concentration up to 2.5 mM), a high pmf of up to −150 mV and an internal pH of around pH 7. When the energy supply stops, the phosphate potential and the pmf rapidly collapses and the internal pH falls to values which can be acidic or alkaline relative to the external pH, depending on its actual value. In the absence of fermentable substrate, lactococci lose gradually their viability mainly due to a loss of the glycolytic activity. In this starvation period also the internal pools of many metabolities, including most of the amino acids, are released in the external medium. Upon addition of fermentable substrate to viable starved lactococci the phosphate potential, the pmf, the internal pH, and the metabolite pools are rapidly restored to the levels found in growing cells.

A limited number of sugars are taken up by transport systems which require PEP as energy donor and are independent of the pmf. During starvation, sufficient PEP or its precursors are maintained in the cell in order to make initiation of glycolysis possible. Also arginine can be taken up by a pmf independent exchange system. Since lactococci have limited biosynthetic capabilities many solutes besides the energy sources have to be taken up. Many of these solutes, including essential and nonessential amino acids are accumulated by pmf-driven transport systems. Lactococci may also acquire amino acids by the uptake and subsequent hydrolysis of peptides. Some dipeptides can also be taken up by pmf-driven transport systems. Some amino acids as well as various inorganic ions are taken up by unidirectional transport systems which rely on ATP or a releated energy source. The interplay of the membrane-bound ATPase and ion-transport systems ensures a tight control of the intracellular pH (to values around 7) in order to fine tune and balance metabolic energy production with metabolic energy consumption in the growing cells.

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