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pH Homeostasis in Lactic Acid Bacteria

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ABSTRACT

The ability of lactic acid bacteria to regulate their cytoplasmic or intracellular pH is one of the most important physiological requirements of the cells. Cells unable to maintain a near neutral intracellular pH during growth or storage at low extracellular pH may lose viability and cellular activity. Despite the importance of pH homeostasis in the lactic acid bacteria, however, an understanding of cytoplasmic pH regulation has only recently begun to emerge. This review describes the specific effects of low pH on lactic acid bacteria, reports recent research on the physiological role of intracellular pH as a regulator of various metabolic activities in lactic acid bacteria, and presents the means by which lactic acid bacteria defend against low intracellular pH. Particular attention is devoted to the proton-translocating ATPase, an enzyme that is largely responsible for pH homeostasis in fermentative lactic acid bacteria.
(**Key words**: pH, pH homeostasis, lactic acid bacteria)

Abbreviation key: $\mathbf{pH_{out}}$ = extracellular pH, $\mathbf{pH_{in}}$ = intracellular pH, **ΔpH** = pH gradient, **H^+-ATPase** = proton-translocating ATPase.

INTRODUCTION

The means by which microorganisms tolerate variations of environmental pH has been an active area of investigation [for reviews, see (16, 41, 46, 51, 76)]. pH homeostasis is an especially important concern for lactic acid bacteria used as dairy starter cultures because these obligate acid-producers must cope with low pH, high acid environments during ordinary growth in milk. This paper reviews the effects of low pH, and in particular, low intracellular pH ($\mathbf{pH_{in}}$), on growth and metabolism of lactic acid bacteria and discusses how cytoplasmic pH regulates cell metabolism and how cytoplasmic pH is regulated in the lactic acid bacteria.

GROWTH OF LACTIC ACID BACTERIA IN MILK

In general, growth of lactic acid bacteria continues as long as 1) carbohydrates, amino acids, and other nutrients are available; 2) toxic or inhibiting compounds, such as hydrogen peroxide, are removed, degraded, or diluted; or 3) the hydrogen ion concentration is maintained above the level that a specific strain can tolerate. For example, growth of *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, and *Streptococcus thermophilus* in milk occurs until the pH reaches approximately 4.5, despite nonlimiting concentrations of nutrients and the relative absence of inhibitory compounds (21, 61, 98). Low pH (or high lactic acid) is frequently growth-limiting for lactic acid bacteria that are grown in milk or in weakly buffered bacteriological media (12, 61).

Although lactic acid bacteria may frequently be isolated from acid habitats (69), such as sour milk, and are commonly thought to favor low pH environments, except for certain *Lactobacillus* species, lactic acid bacteria are probably best characterized as neutrophiles. For example, optimal growth rates for the mesophilic dairy starter cultures, *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* have been reported to occur within an external or extracellular pH ($\mathbf{pH_{out}}$) range of 6.3 to 6.9 (12, 13, 37). The optimal pH_{out} for the thermophilic

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lactic acid bacterium *S. thermophilus* was between 6.5 and 7.5 (9, 72). Among the lactic acid bacteria used as dairy starters, only the lactobacilli (*Lactobacillus helveticus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) appear to grow optimally at acid pH; maximal growth occurs at pH 5.5 to 5.8 (9, 95).

Not only do most lactic acid bacteria grow more slowly at low pH, but acid damage and loss of cell viability may also occur in cells held at low pH. In fermented dairy products, such as yogurt or cultured buttermilk, whether the lactic acid bacteria are viable or injured by the lactic acid and low pH environment, once the desired pH is reached frequently is technologically irrelevant. Moreover, inhibition of the starter culture by lactic acid and low pH acts to prevent, in part, overacidification of the finished product. In the production of yogurt, for example, fermentation lowers the pH of the milk from 6.5 to between 4.0 and 4.5, and the milk coagulates. Unrestricted growth and fermentation in yogurt (or postacidification) by the lactic culture may result in excessive acid and flavor defects. In other cases, however, maintenance of culture viability under acid conditions is desired and necessary. Cultures and culture adjuncts (e.g., *Lactobacillus acidophilus*) that are added to yogurt for therapeutic value must remain viable during storage at low pH and, ultimately, must survive the acid stomach environment. Mixed genus, thermophilic cultures used for Italian cheese manufacture (i.e., *S. thermophilus* and *Lb. helveticus*) are usually propagated or cultivated together; that the former is more acid-sensitive than the latter may result in variable strain ratios. In the industrial production of starter cultures, sensitivity to acid is undesirable because high cell densities may not be achieved, and cell viability may be impaired (77).

ACID DAMAGE DURING GROWTH AT LOW pH

The basic knowledge that starter cultures grow best at neutral or near neutral pH, that acid environments are damaging to cells, that starters tend to lose activity during prolonged storage at low pH, and that overripened or overacidified cultures perform slowly or result in inferior productions has long been recognized (54, 55, 77, 84). Harvey (37) first studied the damage to *L. lactis* ssp. *lactis* ML3 resulting from growth at low pH. Maximum specific growth rates occurred at pH_{out} 6.3. Cell damage, determined as the ratio of the transient growth rate to the normal growth rate, began to occur at medium pH or pH_{out} 5.0, and the specific growth rate approached zero at pH_{out} 4.0. A reduction in the specific activity of the enzymes hexokinase and acetate kinase, as well as the overall glycolytic activity, also occurred at pH <5.0. Although pH_{in} was not measured, Harvey (37) suggested that the cells were able to maintain constant pH_{in} as long as pH_{out} was >5.0. Many other workers (18, 55, 72, 98) have reported that growth of lactic streptococci and lactococci decreases sharply when the medium pH reaches 5.0. Similarly, pH-induced damage also occurred in the fecal coccus, *Enterococcus faecalis* (formerly *Streptococcus faecalis*). Below pH 5.0, derangement of membrane structures and solute leakage occurred (60). Although the changes were reversible in both *L. lactis* ssp. *lactis* and *E. faecalis*, a lag in fresh media occurred following exposure to these low pH (37, 60).

Bender et al. (11) studied the effect of gross membrane damage caused by acidification in various oral streptococci. Damage was assessed by measurement of the release of magnesium ions from cells that were incubated at specific pH at room temperature. Magnesium was not lost from *Streptococcus mutans, Streptococcus sanguis,* and *Streptococcus salivarius* at pH near neutrality, but magnesium efflux occurred after the pH was lowered to <4.0. At pH 2 or 3, release of magnesium was rapid and extensive. In contrast, *Lactobacillus casei* leaked magnesium only at pH <3. Those workers (11) suggested that differences in the susceptibility to gross membrane damage caused by acidification appeared to vary among organisms and was correlated with the degree of acid tolerance.

Conventionally prepared, milk-based (without added buffers) starter cultures are especially prone to acid damage because the final medium pH usually is <5.0. Such cultures, if they are not used soon, behave poorly in fermented milks, resulting in slow or sluggish culture performance (39, 84). To minimize these acid-damaging effects in dairy starter cultures, the starter culture industry has devoted considerable effort to development of

TABLE 1. Optimal pH for intracellular enzymes from lactic acid bacteria.

Enzyme	Organism	pH Optimum	Reference
β-D-Galactosidase	*Streptococcus thermophilus*	7.1	(32)
β-D-Galactosidase	*S. thermophilus*	8.0	(92)
Phosphofructokinase	*Lactobacillus bulgaricus*	8.2	(56)
β-D-Phosphogalactoside galactohydrolase	*Lactococcus lactis* ssp. *cremoris*	7.0	(68)
Acylglycerol acylhydrolase (lipase)	*Lactococcus lactis* ssp. *lactis* *L. lactis* ssp. *cremoris*	7.0–8.5	(40)
Pyruvate kinase	*L. lactis* ssp. *lactis*	6.9–7.5	(22)
D-Tagatose 1,6-diphosphate aldolase	*L. lactis* ssp. *lactis* *L. lactis* ssp. *cremoris*	7.0–7.3	(23)
Glucose-6-phosphate dehydrogenase	*Lactobacillus casei*	6.8	(64)
6-Phosphogluconate dehydrogenase	*Lb. casei*	6.8	(64)
Aminopeptidase	*L. lactis* ssp. *cremoris*	7.0	(94)
Intracellular proteinase	*L. lactis* ssp. *lactis*	7.5	(71)
Aminopeptidase	*Lb. casei*	6.5	(27)
Dipeptidase	*Lb. casei*	7.6	(27)
Carboxypeptidase	*Lb. casei*	7.2	(27)
X-Prolyl dipeptidyl peptidase	*L. lactis* ssp. *lactis*	8.5	(106)
X-Prolyl dipeptidyl aminopeptidase	*L. lactis* ssp. *cremoris*	7.0	(44)
X-Prolyl dipeptidyl aminopeptidase	*Lb. bulgaricus*	6.5	(15, 67)
X-Prolyl dipeptidyl aminopeptidase	*Lactobacillus acidophilus*	6.5	(15)
H^+-ATPase[1]	*S. thermophilus*	7.0–7.5	(73)

[1]Proton-translocating ATPase.

pH-controlled culture propagation systems that prevent low pH in the medium. Several different means of controlling medium pH have been employed [reviewed by Sandine (105)]. One option is to utilize highly buffered media, usually containing phosphate or citrate salts (98). One such commercial product, PHASE 4™, releases the buffer components over time (88). Thunell et al. (99) reported that PHASE 4™ medium preserved activity and viability of unfrozen cultures for 1 mo at refrigerated storage. Other systems, called "external pH-controlled systems", rely on the addition of base (usually ammonia or ammonium hydroxide) to the starter culture tank (31, 82). The optimal pH set for the control of pH_{out} depends on the strain of bacteria.

pH HOMEOSTASIS

Growth of Bacteria in Acid Environments

In general, microorganisms are able to grow over a wide pH range from 1.0 to 11.0 (76). Despite this remarkable tolerance, most bacteria maintain a neutral cytoplasm. Even acidophiles, for which the optimal pH for growth may be as low as 2.0, have pH_{in} near 7.0 (62). Streptococci, lactococci, and other lactic acid bacteria generally grow and remain viable within a medium pH range of 4.5 to 7.0 (41). Not surprisingly, therefore, many of the enzymes involved in carbohydrate and amino acid metabolism have pH optima in a neutral range, as indicated in Table 1.

During growth and fermentation, the pH of the medium decreases because of the accumulation of organic acids, primarily lactic acid. However, the pH within the cytoplasm of fermenting lactic acid bacteria remains more alkaline than the medium surrounding the cells (41), largely because the cells rapidly excrete protonated lactic acid, via a carrier-mediated process, into the extracellular medium (30, 51). In addition, the membrane is relatively impermeable to extracellular protons (and lactate molecules) that are produced during fermentation. Accordingly, a pH difference between the cytoplasm and the medium, a pH gradient (**ΔpH**) is formed. The formation and main-

tenance of ΔpH is important not only for pH homeostasis but also as a component of the proton motive force (66).

The generation and ultimate collapse of ΔpH in *S. thermophilus* is illustrated in Figure 1. A similar pattern also occurs with lactococcal cells [Figure 1; (72)]. As pH_{out} decreases from 6.8 to between 5.0 and 5.2, near neutral pH_{in} is maintained. However, as pH continues to decrease, the cells are apparently unable to maintain ΔpH; i.e., apparently, at a certain point (pH_{out} 5.0 to 5.2), a large ΔpH cannot be maintained, ΔpH begins to collapse, and cell viability is impaired. In contrast, pH_{in} also decreases in lactobacilli but at a rate that apparently permits the cells to generate and to maintain a large ΔpH (63, 72).

The pH_{in} affected growth and numerous metabolic activities in lactic acid and related bacteria. In 1978, Harold and van Brunt (36) demonstrated that the maintenance of a neutral or slightly alkaline pH_{in} was required by *E. faecalis* for rapid growth. Similarly, Kobayashi et al. (49) reported that optimal growth of *E. faecalis* occurred when the pH of the medium was 6.5 to 7.8, corresponding to pH_{in} 7.5 to 7.7. When pH_{in} was reduced from 7.5 to 6.6, the growth rate of *E. faecalis* was reduced by 50% (50). Booth (16) suggested that this bacterium could tolerate reductions in pH_{in} of only 1 pH unit less than its optimum of 7.5.

Optimal growth of lactic acid bacteria also occurs at near neutral pH_{in}. Hugenholtz et al. (38) determined that the growth rate of *L. lactis* ssp. *cremoris* E8 was maximum at pH_{out} 6.2 (corresponding to an approximate pH_{in} 7.0). Otto et al. (75) further reported that growth of *L. lactis* ssp. *cremoris* Wg2 at pH_{in} 6.7 to 7.0 was independent of pH_{out} (from pH_{out} 5.7 to 7.0). Ten Brink and Konings (96) showed that pH_{in} of chemostat-grown *L. lactis* ssp. *cremoris* Wg2 remained between 7.5 and 6.9 when pH_{out} was varied from 7.5 to 5.5, respectively. Similarly, pH_{out} of batch-grown cells of *L. lactis* ssp. *cremoris* Wg2 decreased from 6.8 to 5.3, and a maximal ΔpH of .65 pH units occurred at pH_{out} 5.7 (96). The ΔpH fell to zero, however, soon after growth had stopped.

The use of ionophores, such as gramicidin D, have been useful in understanding the role of pH_{in} on growth and metabolism. These substances act by allowing specific ions to cross the cytoplasmic membrane. For example, gramicidin D allows exchange of monovalent cations (e.g., H^+, Na^+, and K^+), thereby causing the ion gradient to collapse. When *E. faecalis* was grown in the presence of gramicidin (such that pH_{in} = pH_{out}), growth occurred only when the pH was maintained within a narrow range of 7.0 to 7.8 (36). In the absence of gramicidin D, *E. faecalis* was able to grow within a range of pH_{out} 4.5 to 9.5 (45). Those authors (45) concluded that maintenance of neutral pH_{in} was critical for growth of *E. faecalis*. Similarly, *S. thermophilus* lowered the pH of Elliker medium to only 6.5 in the presence of gramicidin, whereas control cells (without gramicidin) grew to pH 4.5 (Nannen and Hutkins, 1991, unpublished data).

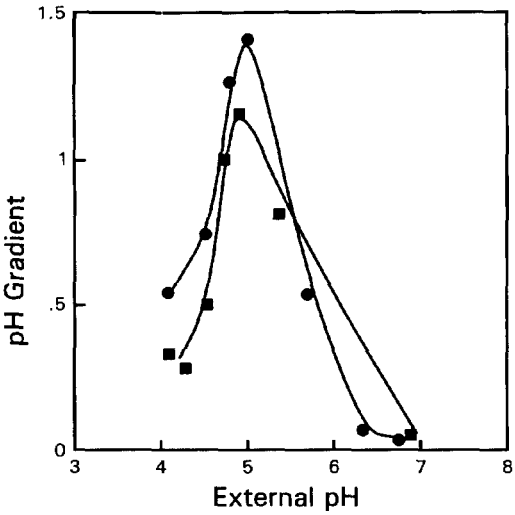

Figure 1. Formation of a pH gradient during growth of *Streptococcus thermophilus* 573 (■) and *Lactococcus lactis* ssp. *lactis* C2 (●) in simulated milk medium. Procedures were those discussed by Nannen and Hutkins (72).

Critical pH for Growth of Lactic Acid Bacteria

Recently, several studies have focused on the actual critical or minimum pH_{in} compatible for growth of acid-producing organisms. Available literature (8) indicates that such critical pH exist and vary among species. Kashket (41) stated that this variation between species is probably due to a slightly different comple-

ment of enzymes and transport carriers (41). The relatively small amount of data relating pH_{in} to cell viability, however, suggests a need for greater understanding of acid tolerance in the lactic acid bacteria (16).

Some lactic acid bacteria, such as the lactococci and the dairy streptococci, maintain neutral or near neutral pH_{in} as pH_{out} decreases as a result of fermentation (78). In contrast, lactobacilli and ruminant streptococci (i.e., *Streptococcus bovis*) are thought to maintain high ΔpH but to allow a reduction of pH_{in} (85, 86). For example, Poolman et al. (80) demonstrated that *L. lactis* ssp. *lactis* ML3 maintains pH_{in} around 7.0 even at pH_{out} 5.0. Maloney (58) reported that, at pH_{out} 6.0, *L. lactis* ssp. *lactis* maintained ΔpH of approximately 1.2 pH units (pH_{in} = 7.2), and, at pH_{out} 5.0, ΔpH and pH_{in} had increased to approximately 1.9 and 6.9, respectively. Kashket et al. (43) also reported ΔpH >1 pH unit (1.1 to 1.4) in growing and fermenting cells of *L. lactis* ssp. *lactis* at pH_{out} 5.0 to 5.1. Kashket (41) further reported that pH_{in} of *Lb. acidophilus, Lb. delbrueckii* ssp. *bulgaricus*, and *Lb. casei* decreased as pH_{out} decreased. However, cells maintained a large ΔpH and grew until pH_{in} around 4.4 was reached.

McDonald et al. (63) reported similar results for batch-grown cells of *Lactobacillus plantarum*, which grew until final pH_{in} 4.6 to 4.8 was reached. In contrast, the less aciduric *Leuconostoc mesenteroides* responded similarly to the lactococci and grew only to pH_{in} 5.4 to 5.7. Recently, Nannen and Hutkins (72) reported that the critical pH_{in} (defined as the pH_{in} at which growth stops and the ΔpH begins to collapse) was 5.0 to 5.5 for *L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, and *S. thermophilus*. These organisms generally maintained near neutral pH_{in} as pH_{out} decreased to <6.0, whereas pH_{in} in *Lb. casei* decreased, even though the latter maintained a large ΔpH (>1.0), even at low pH_{out} (<4.0). These data suggest that cessation of growth coincides with both a low pH_{in} and the collapse of a large ΔpH.

PHYSIOLOGICAL ROLES OF pH_{in}

Somero (91) suggested that significant changes in the rates of a wide variety of metabolic activities were correlated with small changes, within tenths of a pH unit, of pH_{in}. In the lactic acid bacteria and related species, nutrient transport and metabolism, protein synthesis, glycolysis, and nucleic acid synthesis appear to be regulated by pH_{in}.

Effect on Ion Transport

Kashket and Barker (42) showed that, as pH_{in} of *L. lactis* ssp. *lactis* decreased, the exchange rate of potassium ions for protons increased; i.e., cells exchanged intracellular protons for extracellular K^+ at low pH_{in}, thereby increasing pH_{in}. At pH_{out} 5.0, an increase in the ΔpH (and pH_{in}) by the K^+ transport system compensated for decreased electrochemical potential. However, at pH_{out} 7.0, the addition of K^+ had no affect on the magnitude of the ΔpH.

Phosphate ion transport activity in *L. lactis* ssp. *lactis* was shown by Poolman et al. (80) to be regulated by pH_{in}. Maximal uptake of phosphate ion in cells grown in lactose occurred at pH_{out} between 5.5 and 8.0 (pH_{in} approximately 7.2 to 7.5). However, as pH_{in} was reduced to ≤6.0, the initial rate of phosphate uptake approached zero. That inhibition of phosphate transport by agents that dissipate the ΔpH (e.g., nigericin or carbonyl cyanide *m*-chlorophenylhydrazone) occurred at acidic pH, but not at alkaline pH, suggested that phosphate transport activity was regulated by pH_{in} (80).

Effect on Amino Acids and Peptide Transport

As reviewed recently by Poolman et al. (78), numerous amino acid and peptide transport systems apparently are regulated by pH_{in}. van Boven and Konings (100, 101, 102) determined that peptide hydrolysis and peptide uptake were dependent on activities associated with the cytoplasmic membrane of *L. lactis* ssp. *cremoris* Wg2. Although the ΔpH component of the proton motive force appeared to regulate peptide hydrolysis, uptake of leucyl-[^{14}C]leucine was controlled by pH_{in} (and, in part, by the ATP pool). When pH_{in} was decreased from 7.3 to 5.4 by addition of nigericin at pH_{out} 6.4, uptake decreased from 16.5 to .9 nmol/min per mg of protein. In contrast, nigericin had no effect when added to cells at pH_{out} 7.8 (pH_{in} 7.2 to 7.5), showing

that pH_{in}, and not pH_{out} or the ΔpH per se, determined the rate of uptake of this peptide (101).

The uptake of amino acids and peptides by *L. lactis* ssp. *lactis* was also studied by Rice et al. (81). The rate of leucine uptake reached a maximum between pH_{out} 7.0 and 7.5, and the rate of glycine uptake was maximum at pH 6.5. The maximum rate of uptake of dipeptides occurred at pH 6.0; however, maximum uptake of tetrapeptides occurred within a larger range between pH 6.0 and 8.0. At pH_{out} <5.0, the uptake of glycyl-leucine by *L. lactis* ssp. *lactis* decreased, possibly because of the concomitant decrease in pH_{in}.

Dependency on pH_{in} has also been found for the glutamate-glutamine uptake system in *L. lactis* ssp. *lactis* (79). Those authors (79) reported that the initial rate of glutamate uptake increased more than 30-fold when pH_{in} was raised from 6.0 to 7.4. Driessen et al. (26) examined the effect of pH_{in} on L-leucine uptake in *L. lactis* ssp. *cremoris* membrane vesicles and intact cells. In those experiments, the magnitude of the proton motive force was held constant, and pH_{in} was varied by the use of an ionophore, nigericin. A 50% decrease in the maximum velocity of L-leucine uptake, from .8 to .4 nmol/min per mg of protein, occurred when ΔpH was reduced from .6 to 0 at pH_{out} 6.0, even though the affinity constant (K_t) was not affected (26).

Uptake of several amino acids by *Lb. casei* 393 was affected by pH_{in} (93). As pH_{in} was decreased by the addition of uncouplers or ionophores to cells at pH_{out} 6.0, transport of glutamine, glutamate, and arginine decreased. No effect was observed by the presence of uncouplers at pH_{out} 7.5, indicating that pH_{in}, not pH_{out} or ΔpH, regulated transport rates.

The results just discussed demonstrate that primary regulation of amino acid and peptide transport occurs by pH_{in}, not by ΔpH or pH_{out} (78, 79, 102). Although the activity of some amino acid uptake systems (e.g., those for alanine and serine) increases with decreasing pH, Poolman et al. (78, 79, 80) have made the important suggestion that the low uptake rates of essential amino acids at low pH_{in} (<7.0) may account for the growth inhibition of lactic acid bacteria that is observed at low pH.

Uptake of DNA

Recently, Clavé and Trombe (20) suggested that DNA uptake in competent *Streptococcus pneumoniae* cells was strongly dependent on pH_{in}. That bacterium is able to become 100% competent, and, once activated, the uptake of DNA was not driven by a membrane potential. However, Clavé and Trombe (20) suggested that DNA transport was dependent on the intracellular ATP concentration and was regulated by pH_{in}. The optimal pH_{in} for the uptake of DNA was 8.3.

Effect on Proteolysis

De Giori et al. (25) reported that pH had a strong influence on the proteolytic activity of lactic acid bacteria in milk systems. The optimal pH for proteolysis in lactococci was at an external range of pH 5.2 to 5.6, whereas *Lb. casei* showed high proteolytic activity at pH 4.8 to 5.2. However, pH_{in} were not investigated.

REGULATION OF pH_{In}

The actual means by which cells are able to maintain constant pH_{in} despite major fluctuations in the medium pH is an area of intense research (16, 46). The relative impermeability of the membrane to extracellular protons provides cells with some protection and stabilizes pH_{in}. Despite this physical barrier, however, most organisms have evolved additional mechanisms of controlling and regulating pH_{in}. Several possible methods have been proposed (16) to control or to maintain pH_{in} optimally, including 1) synthesis or cytoplasmic buffer, 2) proton symport systems, 3) production of acids and bases, and 4) proton pumps.

Existing Cytoplasmic Buffers

The cytoplasm of most microorganisms has a relatively high buffering capacity. Measurement of 50 to 100 nmol of H^+ pH unit per mg of cell protein have been reported (16) for the buffering capacity of cells at pH_{in} 7.0. Krulwich et al. (53) studied the buffering capacity of bacilli grown at different pH ranges. The data suggest that the buffering capacity of the cytoplasm played little or no role, however, in ultimately determining the pH range for growth of these bacteria. The intracellular buffering capacity may be considered, there-

fore, to be a limited response used by cells to counter variations in pH_{in} (16, 34), which suggests that other mechanisms are utilized by bacteria.

Proton Symport Systems

In 1979, Michels et al. (65) proposed a model of energy recycling to explain how fermenting lactococci could generate a proton motive force. In that system, an organism excretes protonated fermentation end products, such as lactic acid, with a proton:acid stoichiometry ratio >1; thus, a ΔpH and proton motive force are generated (51). The continuous production of relatively large quantities of metabolic end products, coupled to protons, by fermentative bacteria could contribute significantly to a proton motive force and to overall production (or conservation) of energy (65, 97) because protons pumps would be spared ATP. In lactococci, the extrusion of protons is thought to be achieved by an electrogenic H^+-lactate symporter (41).

Production of Acids and Bases

The production of acidic or alkaline metabolic products by organisms growing in media at varying pH may be another important means of pH regulation (16). For example, synthesis of decarboxylases and deaminases may be involved in pH regulation. Many lactic acid bacteria produce these enzymes (e.g., histidine decarboxylase produced by *Lactobacillus buchnerii* and arginine deiminase produced by *L. lactis* ssp. *lactis*). Casiano-Colón and Marquis (19) reported that ammonia released from arginine via the arginine deiminase system protected lactic acid bacteria against acid damage. Marquis et al. (59) suggested that arginolysis at low pH occurs as a general adaptive response by these organisms to acid environments. A similar system involving the malolactic system was reported by Daeschel (24). Decarboxylation of the dicarboxylic malic acid and subsequent production of monocarboxylic lactic acid by *Lb. plantarum* (and other malolactic bacteria) consume an intracellular proton and elevate pH. *Lactobacillus plantarum* and *Leuc. mesenteroides* cells grown in MRS-glucose medium supplemented with malic acid, therefore, achieved higher growth rates than when they were grown in conventionally buffered media.

Proton-Translocating ATPase

Although those mechanisms may represent important means to control or to regulate pH_{in}, perhaps the most important homeostatic system in fermentative bacteria is the membrane-bound, proton-translocating ATPase (**H^+-ATPase**), which extrudes protons out of the cell via ATP hydrolysis. This reaction requires energy (in the form of ATP) because expulsion of protons from a relatively alkaline environment (i.e., the cytoplasm) into an acidic environment (i.e., the medium) requires movement of protons against a concentration gradient. Although the resulting proton or electrochemical gradient (also referred to as the proton motive force) can be used to drive uptake of solutes, the main function of H^+-ATPase in glycolytic, nonrespiring bacteria (i.e., lactic acid bacteria) is the maintenance of ΔpH (34). In most aerobic, respiring microorganisms, this enzyme operates in the opposite direction to generate ATP. Although ATP synthase activity may also occur in lactic acid bacteria (57), under physiological conditions, H^+-ATPase functions primarily as a proton pump (52).

The H^+-ATPase from bacteria, as well as eukaryotic organisms and organelles, have very similar function and structure. In addition, available gene (*unc* or *atp*) sequence data have revealed significant homology. Several reviews on the structure of bacterial H^+-ATPase have recently been published (2, 28, 89, 90, 103), and procedures for purification of the enzyme have been presented (3, 89). Detailed genetic studies have also been reviewed (28, 29, 104).

Structure of ATPase. The H^+-ATPase complex consists of two main portions a hydrophilic, peripheral membrane protein, called F_1, and the hydrophobic F_0, which is integrated within the membrane (29). In *Escherichia coli*, the complex has a molecular weight of approximately 530,000 Da (90). The F_1 is the catalytic portion and is an extrinsic membrane protein consisting of five different subunits. The catalytic site of the ATPase is located at the inner surface of the membrane. The F_0, a transmembrane complex consisting of three subunits, mediates the translocation

between two compartments (i.e., the cytoplasm and the medium). The passage of the proton through F_0 is, in turn, regulated by the F_1 portion. Abrams (1) showed that the enzyme may be detached from the membrane by repeated washings, first with solutions containing high salts and then followed by a solution of low ionic strength and containing no Mg^{2+}. Evidence was also presented demonstrating that binding of the enzyme to the membrane was dependent on multivalent cations, such as Mg^{2+}, Mn^{2+}, and Ca^{2+}. Abrams and Baron (4) further determined that the addition of Mg^{2+} ions increased the strength of attachment of the enzyme to the membrane but did not increase the total number of binding or catalytic sites.

H^+-ATPase in Streptococci, Lactococci, and Lactobacilli. Harold et al. (35) demonstrated that H^+-ATPase extrudes protons, resulting in the alkalinization of the cytoplasm in *E. faecalis*. Abrams and Smith (6) further showed that the activity of H^+-ATPase in *E. faecalis* increased when cells were grown in K^+-limiting medium; .5 m*M* or greater of K^+ ion was required by *E. faecalis* to alkalinize the cytoplasm (47). Some workers (7, 17, 33, 36, 42) have suggested that the mechanism responsible for raising pH_{in} in enterococci and lactococci was the extrusion of protons via the membrane-bound ATPase combined with the electrogenic uptake of K^+. Kobayashi et al. (49) confirmed this suggestion by demonstrating that, as pH_{in} was shifted to <7.5, H^+-ATPase activity in *E. faecalis* was elevated from 1.8 units/mg of protein at pH_{in} 7.6 (pH_{out} 7.3) to 3.0 units/mg of protein at pH_{in} 7.3 (pH_{out} 6.0). The H^+-ATPase activity decreased when the medium returned to alkaline pH. When H^+-ATPase activity was inhibited with N′,N′-dicyclohexylcarbodiimide, cells were unable to keep constant pH_{in}. Kobayashi et al. (49) suggested that the pH_{in} is regulated by changes in H^+-ATPase activity, which is, in turn, dependent on pH_{in}.

Mutants that were defective in the regulation of pH_{in} were studied by Kobayashi and Unemoto (50). These acid-sensitive mutants, isolated from *E. faecalis* 9790 (the parental strain), grew at pH 7.5, but not at pH <6.0. At pH_{out} 6.4, the parent strain maintained a ΔpH of 1.0 unit, whereas the mutant generated a ΔpH of only .5 unit. The acid-sensitive mutant was unable to generate a ΔpH large enough to maintain pH_{in} 7.5. Kobayashi (45) suggested that the defect in the acid-sensitive mutant involved the metabolic extrusion of protons through the proton-translocating ATPase and that the mutants were unable to alkalinize the cytoplasm (45). The cytoplasm of the parental strain was always higher than that of the mutant, at pH <8.0 (45). Cytoplasmic alkalinization by both strains possibly was diminished at pH_{in} >7.7 because of reduced proton extrusion activity by H^+-ATPase.

In contrast to effects that occur at high pH_{in}, amplification of *E. faecalis* 9790 H^+-ATPase activity occurred as pH_{in} decreased (48). The addition of gramicidin D—a protonophore that allows free diffusion of protons across the membrane—to cells grown at pH 7.2 increased H^+-ATPase activity fivefold from 1.90 to 8.51 units/mg of protein. Although the cells were not able to regulate pH_{in} in the presence of protonophores, at pH_{out} <7.6, H^+-ATPase activity increased.

Abrams and Jensen (5) also examined the altered expression of H^+-ATPase in *E. faecalis* in the presence and absence of K^+. Results showed that H^+-ATPase activity in cells grown in K^+-restricted medium (containing .2 m*M* K^+) was more than twofold greater than cells grown in 20.0 m*M* K^+ medium (1.37 vs .59 units/mg of protein). The pH of the medium also affected expression of the enzyme, which increased from .12 units/mg of protein at pH 9.0 to .75 units/mg of protein at pH 6.0.

Research on H^+-ATPase activity in *E. faecalis* strongly suggests that this enzyme regulates pH_{in} (45, 46, 47, 48, 49, 50). The H^+-ATPase activity increases with decreasing pH, and, in addition, greater amounts of enzyme are produced as pH decreases. Although differences exist in the absolute or critical value at which *E. faecalis* and lactic acid bacteria regulate their pH_{in}, a similar mechanism of pH regulation may exist (16, 41, 46).

Recently, H^+-ATPase from several lactic acid bacteria have been characterized, and their role in pH homeostasis has been evaluated. Niskasaari et al. (74) and Rimpiläinen et al. (83) isolated and partially characterized H^+-ATPase from the plasma membrane of *L. lactis* ssp. *cremoris*. Cells were grown to late log phase, which decreased the pH of the medium from 6.7 to 5.2. At pH_{out} 5.2, the specific activity of the H^+-ATPase was 4.03 μmol of

phosphate released/mg of protein per min (83). Given that lactococci grow optimally at near neutral pH_{in}, high enzyme activity at low pH seems to be consistent with research findings on *E. faecalis*. Similar results were reported by Nannen and Hutkins (73); maximum activities occurred at pH_{out} 4.9 to 5.2. However, in the latter study, as pH_{out} decreased below this range, H^+-ATPase activity declined by 25 to 50%.

Several investigators (10, 14, 70, 73) have studied H^+-ATPase from lactobacilli. Although questions related to subunit stoichiometry and structural arrangements of *Lactobacillus* H^+-ATPase remain unresolved, the activity of the enzyme is typical to that of other H^+-ATPase. However, some reports (10, 73) suggest that the pH optimum is lower (approximately 5.0) than for other lactic acid bacteria, which would not be unexpected, because *Lactobacillus* species are more tolerant of low pH than lactococci and streptococci; growth of these bacteria in nutrient-rich media may reduce the pH to as low as 3.0. If acid tolerance is related to protein-expelling activity, lactobacilli H^+-ATPase would be expected to have a more acid pH optima than enzymes from other, more acid-sensitive bacteria.

Bender and Marquis (10) studied the membrane ATPase and acid tolerance of *Lb. casei*. Only after 4 h at pH 3.0 did damage (determined by leakage of Mg^{2+}) begin to occur to *Lb. casei*. Cells that were grown to late exponential phase had ATPase activity of 3.29 units/mg of membrane protein (10, 11). By comparison, *Actinomyces viscosus* was also studied by Bender and Marquis (10). That less acid-tolerant strain had H^+-ATPase activity of only .6 units/mg of membrane protein. The differences in acid tolerance between these bacteria may depend on the relative amounts of ATPase in the cell membranes.

The H^+-ATPase of *Lb. casei* was also studied by Nannen and Hutkins (73). Although activity did not increase with decreasing pH, relatively high basal H^+-ATPase activities and a large ΔpH were maintained, even when pH_{out} decreased to <4.0. They (73) suggested that the maintenance of a large ΔpH at low pH was due to the greater basal production of H^+-ATPase by *Lb. casei*.

CONCLUSIONS

The importance of pH homeostasis and the effects of pH_{in} on metabolic activities in microorganisms have become increasingly recognized. The pH_{in} affected the uptake of nutrients, such as K^+, phosphate, and amino acids. Also, pH_{in} is an important component of the proton motive force and, therefore, has a profound effect on the bioenergetic state of the cell. How pH_{in} is involved or integrated in other metabolic processes and activities, such as DNA uptake, remains an active and interesting area for further research.

The disruption of cytoplasmic membrane activities in fermentative organisms is associated with production of acidic fermentation products. The relative tolerance of these organisms to acidic end products is dependent on the strain of bacteria (41). Mechanisms controlling pH_{in} have been the subject of much research, and evidence now indicates that H^+-ATPase plays a key role in the regulation of pH_{in}. This enzyme directly controlled pH_{in} in *E. faecalis* (45, 46, 47, 48, 49). Continued research focusing on H^+-ATPase in relation to pH necessary for growth of lactic acid bacteria may improve understanding of the physiology of this important group of microorganisms. Future studies on the genetic basis of pH homeostasis may lead to opportunities for development of acid-sensitive and acid-resistant lactic starter cultures for specialized applications.

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