IOWA STATE UNIVERSITY
Digital Repository

1996

Response of Propionibacterium to acid and low pH: tolerance and inhibition

Jill Louise Rehberger

Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

🎨 Part of the Agriculture Commons, Food Microbiology Commons, and the Microbiology Commons

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

Response of *Propionibacterium* to acid and low pH:

Tolerance and inhibition

by

Jill Louise Rehberger

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department: Food Science and Human Nutrition

Major: Food Science and Technology

Major Professor: Bonita A. Glatz

Iowa State University

Ames, Iowa

1996

UMI Number: 9635345

UMI
300 North Zeeb Road
Ann Arbor, MI 48103

ii

Graduate College

Iowa State University

This is to certify that the Doctoral dissertation of

Jill Louise Rehberger

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

TABLE OF CONTENTS

ABSTRACT

Six strains of *Propionibacterium* (three strains of *P. acidipropionici*, one strain of *P. jensenii*, and two strains of *P. thoenii*) were identified as potential acid- and low-pH-tolerant strains from a screen of the culture collection at Iowa State University for acid production and final pH achieved on various carbon sources. The lowest pH at which the strains could initiate growth and remain viable were determined with lactic, propionic, and hydrochloric acid as acidulants. The ability to produce large amounts of acid or achieve low final pH values at the end of culture growth was not directly related to a strain's ability to initiate growth or survive in low-pH conditions.

When grown in batch culture at pH 7, the proton motive force (Δp) of *P. thoenii* P127 was comprised of only the electrical potential ($\Delta \Psi$); the internal pH remained constant at pH 7. During growth at pH 5, the Δp consisted of both $\Delta \Psi$ and pH gradient (ΔpH) and the internal pH decreased slowly throughout the fermentation. Growth at low pH was characterized by low specific growth rate, low internal ATP concentrations, and low propionic and acetic acid yields. The overall Δp values were about the same for cultures grown at low or neutral pH, but the individual components of Δp ($\Delta \Psi$ and ΔpH) were affected by external pH.

Logarithmically growing cells of P127 exposed to mild acid conditions (pH 5.5) for one doubling were more resistant to lethal pH conditions (pH 3.5) than were cells shifted directly from neutral pH to pH 3.5. This acid tolerance response required protein synthesis and allowed the cells to maintain a proton motive force during exposure to pH 3.5. In contrast, the Δp of the unadapted cells collapsed.

Exposure of P127 to acidic pH values caused the cells to manipulate the components of the Δp to maintain a large transmembrane potential. Strain P127 could not initiate growth at pH values below 5.0; in addition, the ΔpH dramatically decreased when cells were exposed to pH values <5.0. This may suggest that the cells' inability to maintain a large enough ΔpH is responsible for growth inhibition.

GENERAL INTRODUCTION

Propionibacteria are fermentative microorganisms that produce propionic

and acetic acids from the metabolism of lactate and/or carbohydrates (21, 75, 90).

Strains of the genus *Propionibacterium* are used industrially as starter cultures for

Swiss-type cheeses (21), as silage inocula (26, 76), as probiotics (59), and for the

industrial production of vitamin B_{12} and propionic acid (75).

Propionic acid is an effective preservative for foods and agricultural

products (25, 38, 43, 75). It extends the shelf-life of the product by inhibiting

molds, bacteria, and yeasts. Currently commercial production of propionic acid is

mainly by petrochemical routes. Fermentation is not used industrially because

propionibacteria grow slowly and produce relatively low concentrations of acid. In

addition, the costs of separation and concentration of the acids make recovery

difficult and expensive. If higher yields could be obtained, the economics of

fermentation could be improved. Fermentation would allow the use of industrial

waste products (whey, corn steep liquor, and beet molasses) as fermentation

substrates. Other antimicrobials such as bacteriocins produced by the organisms

could increase the spectrum of antimicrobial activity of the culture product (34,

56). The ability to label the fermentation product as "natural" could increase its

appeal to some consumers.

A strain of *Propionibacterium* that could tolerate high concentrations of

organic acids and/or low pH might produce more propionic acid and be favored

2

as silage inoculant or probiotic feed additive. In addition, growth at low pH may facilitate downstream processes to remove product acid from fermentation broth.

An examination of the effect of low pH and organic acids on the cell could lead to greater understanding of the mechanism of action of antimicrobials and the process of end-product inhibition by acids during fermentation. The action of organic acids has been attributed both to a generalized inhibition due to low pH and to a more specific inhibition due to action of the anion (37). Some researchers believe that organic acids act as "uncouplers" because the entrance of undissociated acid into the cell followed by its dissociation there allows protons to enter the cell from the external medium (4, 7, 46, 60). The membrane-bound ATPase must function to counteract the futile cycle of protons through the membrane; in this way, the cell becomes deenergized. In contrast, other researchers believe that organic acid toxicity is more closely related to intracellular pH regulation and anion accumulation than to "uncoupling" per se (15, 79, 80, 81, 82).

The specific objectives of this study were to identify strains of Propionibacterium that are potential acid- and low-pH-tolerant variants; to determine the pH values that inhibit growth and survival of these strains; to develop the methods to measure the proton motive force of propionibacteria; to measure the proton motive force of P. thoenii P127 during growth at neutral and acidic pH values; and to measure the proton motive force of cells of P. thoenii P127 during exposure to low pH.

Dissertation Organization

This dissertation follows the alternative format and is divided into three papers. Each paper contains Introduction, Materials and Methods, Results and Discussion, and References sections. Each section is written to conform to the specifications of the journal to which it will be submitted (Journal of Industrial Microbiology and Applied and Environmental Microbiology). Literature review and general conclusion sections are also included. References cited in those sections follow the general conclusion. All experiments, data collection, and data analysis were performed by the candidate.

LITERATURE REVIEW

Characteristics and Metabolism

The genus *Propionibacterium* is divided into two principal groups of organisms, the "classical" propionibacteria and the acnes group. This review will concentrate on the classical propionibacteria, in particular their metabolism, industrial importance, and inhibition by the production of propionic and acetic acid and low pH. This group has been referred to as the dairy propionibacteria and consists of the species *P. freudenreichii, P. thoenii, P. jensenii,* and *P. acidipropionici* (10, 21). These strains have been isolated from soil, silage, raw milk, Swiss cheese, and other dairy products. Some strains are used extensively as dairy starter cultures (39, 49, 75, 90).

The propionibacteria are gram-positive, nonmotile, catalase-positive, nonsporeforming, pleomorphic rods that are anaerobic to aerotolerant (21), and produce propionic acid, acetic acid, carbon dioxide, and vitamin B_{12} from the metabolism of sugar and/or lactate (16, 50, 90).

Propionibacteria are metabolically complex organisms that utilize several different substrates: glucose, fructose, sucrose, maltose, erythritol, glycerol, and others (21, 49, 90). They prefer lactate to glucose and L-(+)-lactate to D-(-)-lactate (16, 39, 74, 90). These organisms produce propionate by a cyclic series of reactions through the intermediate succinate. The theoretical yield from 1.5 moles

of glucose or 3 moles of lactate is 2 moles of propionate, 1 mole of acetate and 1 mole of carbon dioxide (16, 32, 90).

The major pathway of glucose metabolism is via the Embden-Meyerhof pathway (90). Lactate enters the pathway in an oxidation reaction that yields pyruvate, which is then converted to oxaloacetate by a transcarboxylation reaction with (S)-methlymalonly-CoA as CO_2-donor and biotin as CO_2-carrier (Figure 1). In addition, pyruvate can also be converted to phosphoenolpyruvate by pyruvate phosphate dikinase. Through the action of phosphoenolpyruvate carboxytransphosphorylase, carbon dioxide is added to phosphoenolpyruvate in a fixation reaction to yield oxalacetate. In the last two reactions, the function of ATP is performed by inorganic pyrophosphate which is used to phosphorylate fructose-6-phosphate to fructose-1,6 phosphate and serine to phosphoserine (32, 90).

Succinate is formed from oxalacetate through the action of malate dehydrogenase, fumarase, and fumarate reductase. Succinyl-CoA is formed in a CoA transferase reaction, rearranged to (R)-methylmalonyl-CoA, and converted to the (S)-enatiomer by a racemase. Transcarboxylation of (S)-methylmalonyl-CoA yields propionyl CoA; propionate is formed by transfer of CoA to succinate (32, 90).

Under aerobic conditions, intermediary metabolism occurs via the citrate cycle; citric acid cycle intermediates have been isolated in propionibacteria (18, 90). Wood (90) stated that the reactions of the citrate pathway may account for variations in the ratios of propionate to acetate that are often found in cultures.
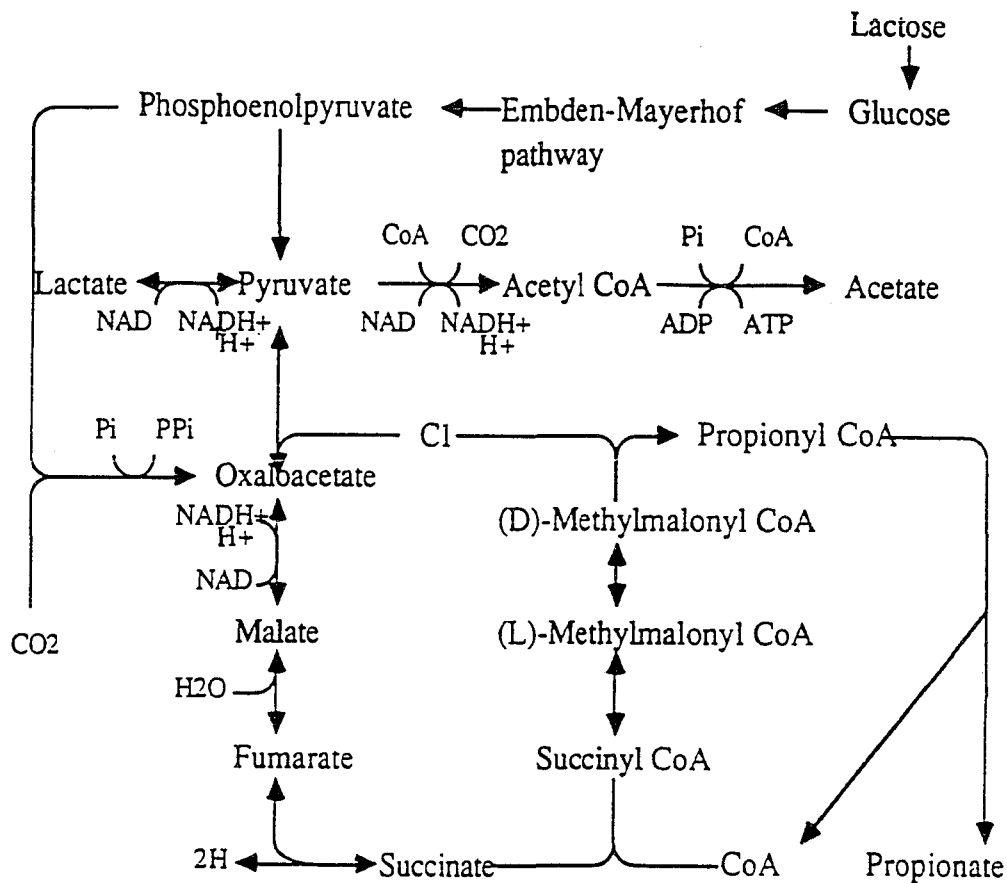
Figure 1: Major metabolic pathway of propionibacteria (adapted from Gottschalk [32]).

In an attempt to determine the effect of pH on these pathways, *P. acidipropionici* was studied in batch culture at pH values controlled at 4.5 to 7.0 with lactose as the substrate (41). The optimum pH for cell growth was 6.0-7.1 (specific growth rate of 0.23 h^{-1}). The growth rate decreased at acidic pH values to a low of 0.08 h^{-1} at pH 4.5. Significant amounts of pyruvate and succinate were produced at neutral pH values. However, the pyruvate was reconsumed and disappeared toward the end of the fermentation. Succinate formation

decreased with pH: 17% (w/w) at pH 7.0 to 8% at pH 5.0. Acetic acid yield was not significantly affected by pH. Propionic acid yield (grams of propionic acid produced per gram substrate used) increased from 33% at pH 6.0-7.1 to 63% at pH 4.5-5.0. The authors noted that at acidic pH values, less energy was diverted to succinate and pyruvate formation and thus the propionate yields were higher.

Propionibacteria are capable of utilizing several amino acids, especially aspartate, alanine, serine, and glycine (51). However, there are large differences in such abilities among species and strains. Propionibacteria metabolize aspartate and propionate via the citric acid cycle to yield succinate, ammonia, acetate, and carbon dioxide (16, 18, 77):

$$3 \text{ aspartate} + \text{propionate} \rightarrow 3 \text{ succinate} + \text{acetate} + CO_2 + NH_3$$

Reactions catalyzed by aspartase and carboxytransphosphorylase are also involved. The utilization of aspartate shifts the balance from propionate production to acetate and carbon dioxide formation from lactate, to maintain the redox balance of the cell (16, 18, 74, 77).

Alanine is metabolized after depletion of lactate (16, 19). Degradation of alanine may be delayed by the high concentration of pyruvate in cells from lactate fermentation, which inhibits alanine dehydrogenase. After depletion of pyruvate, alanine is degraded:

$$3 \text{ alanine} \rightarrow 2 \text{ propionate} + \text{acetate} + CO_2 + NH_3$$

Propionibacteria have at least two proteinases, one produced during exponential growth and the other released at stationary phase (24, 51). These enzymes were found in a study of the caseinolytic activity of several strains of propionibacteria. One enzyme, associated with the cell wall, preferentially hydrolyzed B-casein to α_{s1}-casein. The other had similar activity on both B- and α_{s1}-casein and was located in the cell membrane. Peptidase activity of several species of propionibacteria has been documented as well (24, 51, 68). The activity varies greatly among species and strains and includes aminopeptidase, proline iminopeptidase, proline imidopeptidase, leucine aminopeptidase, carboxypeptidase and endopeptidases. These enzymes supply the necessary amino-nitrogen for cell growth. In addition, by-products of this metabolism are thought to contribute to flavor in Swiss-type cheeses.

Dupuis *et al.* (23) found *P. freudenreichii* subsp. *freudenreichii* to possess both intracellular and extracellular lipase activity. Lipase activity was detected at the beginning of growth (3 h) and reached a maximum at 7.5 h. The activity quickly decreased after exponential growth. Although other species were found to contain intracellular lipases, no others displayed extracellular lipase activity. The researchers also detected esterase activity in several strains of *P. freudenreichii* subsp. *freudenreichii*. These lipases and esterases are also believed to be involved in flavor development during the ripening of Swiss-type cheese by releasing free fatty acids and other precursors to aromatic compounds.

Crow (20) showed that the propionibacteria produce some polysaccharides during glucose and lactose fermentation. The polysaccharides

contain mostly methylpentoses with some glucose and galactose. He observed that the formation of polysaccharides alters the organic acid ratio by increasing the formation of acetate and carbon dioxide without any associated propionate production.

Industrial Importance

Many propionibacteria strains are used as starter cultures in the production of Swiss-type cheeses. They are responsible for eye formation and the characteristic sweet and nutty flavors of the cheese (39, 41, 51). In Swiss cheese production, strains of *Lactobacillus* and *Streptococcus* first convert the lactose in milk to lactate, thus providing a substrate for the propionibacteria. In addition, the proteolytic enzymes of these cultures, especially *Lactobacillus*, hydrolyze milk proteins, producing peptides and amino acids that help stimulate the growth of propionibacteria (3, 51, 74). During the curing process, metabolism of lactate yields carbon dioxide, which contributes to eye formation, and propionate and acetate, which contribute to flavor and also extend the shelf life of the product by inhibiting molds, yeasts, and bacteria. The characteristic flavor of Swiss cheese is also due to amino acids, metabolic pathway intermediates, and short-chain fatty acids produced by the propionibacteria (24, 50, 51).

In addition to their use as dairy starter cultures, the propionibacteria can be used to produce valuable products. Propionic acid is an important preservative for foods and grains (25, 26, 38, 43, 75). It exerts a biostatic action in dilute

aqueous systems and is biocidal at higher concentrations. Propionate inhibits growth of molds, bacteria, and yeasts. The undissociated form of the acid is believed to be responsible for its antimicrobial properties. Thus, it is most effective at a pH lower than the pK_a, 4.87 (25, 38, 43). However, since sodium and calcium salts, which are fully dissociated in solution, also exert an antimicrobial effect, the dissociated form is also believed to be an active species (11, 13).

Buazzi and Marth (11) found that exposure of *Listeria monocytogenes* to a solution of 8% sodium propionate for 60 minutes injured 87% of the population. By studying the recovery of the injured cells in the presence of metabolic inhibitors, they were able to characterize the cellular targets of sodium propionate. Injury did not involve cellular functions related to electron transport, cell wall, cell membrane, mRNA, or protein synthesis. However, there was a reduction of the specific activity of lactate dehydrogenase in the propionate-injured cells. This may be due to propionate inhibiting the synthesis of coenzyme A, which is required for lactate dehydrogenase activity. In addition, the propionate ion itself was the active fraction; 8% NaCl was not inhibitory.

Propionic acid has been found to be more effective than acetate or lactate in inhibiting the acid-tolerant yeasts that cause food spoilage (63). Moon found that some strains of yeast were able to grow in the presence of lactate or acetate but were inhibited by propionate. Even low concentrations of propionate inhibited the transport of amino acids across the cell membrane.

Cherrington *et al.* (14) investigated the use of lactic acid, acetic acid, and BioAdd, a commercial preparation of formic and propionic acids, as potential

decontaminants for scald tank water used in the processing of chicken carcasses. BioAdd was found to have the greatest bactericidal activity against salmonellae. The bactericidal activity increased with temperature and incubation time.

The traditional method of preserving grain is by drying it to 14% moisture (26, 43). However, this process is costly and inefficient because the dried grain must be rewetted before feeding to cattle and sheep. Moist grain has been preserved by spraying with propionic acid, but acid-induced corrosion of equipment is a major disadvantage of this practice.

Raeker et al. (76) compared pure propionic acid, sodium propionate, and spent fermentation broth from propionibacteria for their ability to preserve high-moisture corn. Spent fermentation broth was as effective a fungicide as propionate and prevented the growth of Aspergillus flavus.

An alternative method for grain preservation is applying propionibacteria as a co-inoculant with lactic acid bacteria (26, 38, 43). Use of propionibacteria and Lactobacillus as inoculants for high-moisture corn retarded mold growth and reduced the yeast population over a 60-day period (26). An important advantage of propionic acid treatment over other moist grain storage methods is that its effectiveness is maintained after the grain is removed from storage. In addition, it inhibits the major mycotoxin-producing fungi (28, 38, 76).

There is evidence that propionibacteria have probiotic effects when used alone or together with lactic acid and/or bifibidobacteria (2, 12, 59, 88). Probiotics are viable organisms that are nutritionally or therapeutically beneficial to the consumer when ingested in food or feed supplements. The probiotic effect of propionibacteria is based on the production of propionic and acetic acids, carbon

dioxide, bacteriocins, vitamins, as well as their ability to stay alive during digestion.

Propionibacteria have been used in combination with *Lactobacillus acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* and bifidobacteria for successfully curing disorders of the digestive tract of calves, piglets, and hens (2, 88). Cerna *et al.* (12) invented Proma, a blend of *Lactobacillus plantarum*, *Enterococcus faecium*, *Lactococcus lactis* and *P. freudenreichii* cultures. When Proma was fed daily to calves at 2×10^8 cfu/g, the calves' daily intake was lower while their daily weight gain was higher than for controls. Even after withdrawal, Proma remained effective; the calves continued to gain weight 11 to 22% faster than the control animals.

Mantere-Alhonen (58) found *P. fruedenreichii* subsp. *shermanii* to be an effective probiotic when used alone. Piglets were fed fodder containing *P. fruedenreichii* subsp. *shermanii* that had been cultivated in trypsin-treated whey and silage juice. The animals received 2×10^9 cfu/g daily for 7 weeks. During that time, the animals gained 9.2 to 14.5% more weight, while their fodder demand was 7.2 to 46.1% lower than for the control animals. In addition, the test piglets experienced less diarrhea than the controls.

Propionibacteria have been investigated as a direct-fed microbial for beef cattle because of their ability to reduce nitrate and nitrite (85). Nitrate from feed is reduced in the rumen to nitrite, which is toxic when absorbed. Nitrite oxidizes ferrous iron in hemoglobin to ferric iron, forming methemoglobin. Nitrate toxicity causes abortions and can be fatal to cattle. A strain of *P. acidipropionici* was found to be capable of reducing high levels of nitrate (15,000 to 20,000 ppm) at

10^5 cfu/ml. The culture produced enzymes necessary for nitrate reduction under both aerobic and anaerobic conditions in the absence of nitrate; however, nitrate reductase activity was induced in the presence of nitrate. The induced cultures were able to reduce 80% of medium nitrate in 6 h, while the noninduced cultures took 16 h to reduce the same amount of nitrate. In addition, P. acidipropionici was able to established itself in the rumen at $>10^5$ cfu/ml. This research lead to the production of BovaPro, a direct-fed microbial produced by LaPorte Biochem International, that contains active P. acidipropionici cultures.

In addition to their use as animal probiotics, propionibacteria have been studied for their potential benefits to human health. A recent study by Perez-Chaia et al. (72) determined that feeding P. acidipropionici increased the intestinal level of propionibacteria and affected the lipid metabolism and immune system of mice. When the mice were daily fed 10^8 bacteria/ml in nonfat milk for one week, P. acidipropionici went from 10^6 to 10^{10} bacteria/g in feces and showed an 8-log increase in the small intestine. Reductions of total cholesterol and serum triglyceride concentration were observed for groups of mice receiving skim milk or skim milk with propionibacteria. The HDL levels were also reduced in both groups, but lower LDL levels were found only in mice fed propionibacteria along with milk. Hypolipemic effect was more noticeable in diets with a high lipid content, and was thought to be due to lower intestinal absorption or high lipid metabolism.

At least three products of propionibacteria fermentation are currently sold commercially (92). Microgard, a fermented milk product produced by Wesman Foods, is used to extend the shelf life of cottage cheese. CAPARVE, a food

preservative produced by PTX Food Corp., is marketed as a "natural mold inhibitor." Upgrade, produced by Microlife Techniques, is also used as a preservative. These products are produced by fermentation of natural materials such as milk and whey and are sold as "value-added" products. Food is preserved by addition of the entire fermentation medium.

Currently commercial production of pure propionic acid is entirely by petrochemical routes (75). Fermentation has not been used commercially primarily because propionibacteria grow slowly and produce relatively low concentrations of organic acids, and the costs of separation and concentration of the acids make recovery difficult and expensive.

However, petroleum is an expensive, nonrenewable resource in high demand in today's market, while fermentation substrates are relatively inexpensive, renewable resources. In addition, use of industrial waste products such as whey, corn steep liquor, and beet molasses as fermentation substrates could decrease disposal costs for these materials.

Further advantages of propionate produced via fermentation include the production of bacteriocins, which could increase the spectrum of antimicrobial activity (34, 56) and the ability to label the product as "natural," which increases a product's appeal to some consumers.

Acid Inhibition and Tolerance

A major factor that limits the production of propionic acid during fermentation is end-product inhibition by the acid (37, 91). Accumulation of end-products causes a continuous decrease in both specific growth rate and the further production of the product (37). Two ways to overcome this end-product inhibition are to make improvements in the fermentation process to avoid product accumulation, or to develop an acid-tolerant variant of the producer strain.

Fermentation processes that have been developed to achieve greater acid yields include fed-batch, cell immobilization, continuous, continuous cell recycle, semi-continuous, and multi-stage systems. This review will focus on acid inhibition and tolerance; the reader is referred to another review for more information about these processes (75).

Bioenergetics

Acid inhibition of microorganisms has been studied in some detail, but the precise mechanism is still not well understood. The study of bioenergetics provides the background necessary to understand how acid molecules affect cells. Bioenergetics as a discipline is a highly directed search for the solution to a single problem: the mechanism by which energy made available by oxidation of substrates or absorption of light could be coupled to "uphill" reactions, such as the synthesis of ATP or the accumulation of ions across a membrane (35, 67).

The largest advance in bioenergetics was Peter Mitchell's chemiosmotic theory (61, 62), which states that the energized state of a membrane is an

electrochemical proton gradient, $\Delta\mu_{H^+}$, that is established by primary proton translocation events that occur during respiration. The $\Delta\mu_{H^+}$ gives rise to the proton motive force of the membrane.

This theory is derived from equilibrium thermodynamics (35, 67). In a closed system, a process will occur if the entropy (or degree of disorder) of the system plus its surroundings increases. The change in entropy (ΔS) cannot be measured and thus, under constant temperature and pressure, it is calculated from the flow of enthalpy (heat, ΔH) across the boundaries of the system. The relationship is

$$\Delta G = \Delta H - T\Delta S \qquad (1)$$

where T is the absolute temperature (Kelvin). The ΔG or Gibb's free energy is the quantitative measure of the net driving force. A process that results in a decrease in free energy proceeds spontaneously (67).

Consider a reaction:

$$aA \rightarrow bB \qquad (2)$$

A mass action ratio, Γ, can be calculated:

$$\Gamma = \frac{[B]^b}{[A]^a} \qquad (3)$$

At equilibrium, Γ is defined as the equilibrium constant which is denoted as K. The ΔG is then determined as:

$$\Delta G = -2.3RT \log \frac{K}{\Gamma} \qquad (4)$$

where R is the gas constant (8.3 J/mol K) and T is the absolute temperature (Kelvin). At equilibrium, ΔG is zero and thus there is no energy available for work. The further the reaction is displaced from equilibrium the larger is the capacity of the reaction to do work (67).

This is the case with ATP. Under cellular conditions (pH 7.0, $[Pi]=10^{-2}$ M) the equilibrium constant for ATP synthesis from ADP and Pi is large ($K=10^5$) while the mass action ratio is small ($\Gamma=10^{-5}$). This ratio produces a ΔG of -57 kJ/mol. This large ΔG is the reason why ATP is a source of cellular energy, not because ATP possess a "high energy" phosphate bond. Theoretically, a cell could use any compound as an energy sink as long as its formation reaction was displaced from equilibrium by 10 orders of magnitude or more (67).

These same principles can be applied to the disequilibrium of an ion or metabolite across a membrane. There are two forces acting on an ion gradient across a membrane, one due to the concentration gradient of the ion and one due to the electrical potential difference between the aqueous phases separated by the membrane (membrane potential, $\Delta \Psi$) (35, 67).

Consider the transfer of 1 mole of anion X^{m+} down an electrical potential of

$\Delta\Psi$ from a concentration $[X^{m+}]_A$ to a concentration $[X^{m+}]_B$. The free energy can

be calculated based on the Nernst equation:

$$\Delta G = -mF\Delta\Psi + 2.3RT\log\frac{[X^{m+}]_B}{[X^{m+}]_A} \qquad (5)$$

where F is the Faraday constant (96.519 J/mol mV).

In the specific case of the proton electrochemical gradient:

$$\Delta\mu_{H^+} = -F\Delta\Psi + 2.3RT\Delta pH \qquad (6)$$

Mitchell defined the proton motive force as:

$$\Delta p = -\frac{\Delta\mu_{H^+}}{F} \qquad (7)$$

$$\Delta p = \Delta\Psi - \frac{2.3RT}{F}\Delta pH \qquad (8)$$

The term $2.3RT/F$ is often designated as z and has the value of 59 mV at 25°C (35,

61, 62, 67).

This force (interior alkaline and negative with respect to the medium) is

generated by primary proton pumps, such as the electron transport chain, which

are closely coupled to metabolism (35, 67). This force can then be utilized and

discharged during the function of the proton-translocating ATP synthase,

H^+/solute symporters and other catalysts of bioenergetic work located in the

membrane. Typical values of the proton motive force and its components, the

electrical potential ($\Delta\Psi$) and the pH gradient (ΔpH), are listed in Table 1 (45).

Fermenting bacteria typically have lower Δp values (<-150 mV) than do

respiring organisms (-150 mV to -200 mV). Kashket (46) stated that Δp plays a

more limited role in anaerobes which can, under special circumstances, dispense

Table 1: Selected bacterial ΔpH, $\Delta\Psi$ and Δp values (data from Kashket [45])

Organism	External pH	zΔpH (mV)	$\Delta\Psi$ (mV)	Δp (mV)
Respiring acidophiles				
Bacillus acidocaldarius	3.0	230	+10	-220
Thermoplasma ferrooxidans	1.0	340	+70	-270
Respiring neutrophiles				
E. coli	7.5	16	-134	-150
Salmonella typhimurium	7.5	0	-162	-162
Staphylococcus aureus	7.0	32	-162	-194
Fermenting cells				
Streptococcus lactis	5.0	110	-52	-162
	6.0	76	-72	-148
S. typhimurium	7.5		-111	-111
S. aureus	7.0	30	-140	-170
Anaerobes				
Clostridium thermoaceticum	6.0	35	-110	-145
C. thermoaceticum (fermenting)	6.0	71	-95	-166

with H^+ and K^+ circulation (36). This ability was demonstrated by *S. faecalis*, which could grow in the presence of gramidicin D, an ionophore that allows the exchange of monovalent cations across the cell membrane. For the organism to grow, the medium needed to be at neutral pH, low in Na^+, and rich in K^+, amino acids and other nutrients. Thus, it appeared that a protonic potential *per se* may not be essential for growth of anaerobes.

Fermenting organisms also differ from aerobes in that they do not maintain a constant internal pH (7, 46, 82). As the bacteria grow they produce acids that lower the external pH. The cells' internal pH decreases with the external pH, although it remains more alkaline than the medium, i.e., the cells maintain a ΔpH. The fermenting anaerobes are able to tolerate fairly wide variations in internal proton concentration.

Internal pH regulation

Although fermentative organisms are capable of growth over a wide range of internal pH, it is still important for the cells to regulate their internal pH because the optimum pH ranges of most enzymes are narrow. Regulation of cytoplasmic pH implies control over the cell membrane's permeability to protons. In general, the cell membrane is relatively impermeable to protons. However, protons are involved in almost every biochemical reaction, and thus are produced both outside and inside the cell (7, 69). It is believed that internal pH homeostasis (i.e. maintenance of a constant internal pH) is achieved by control of the activity of ion transport systems that facilitate proton entry.

In a review on pH homeostasis in bacteria, Booth (7) stated several generalizations. First, it appears that all bacteria do not strive to achieve some "magical" internal pH value. It is recognized that neutrophiles have internal pH values in the range of pH 7.5 to 8.0, while acidophiles are in the range of pH 6.5 to 7.0 and alkalophiles are in the range of pH 8.4 to 9.0. Second, organisms exhibit different capacities to regulate their internal pH. Finally, the mechanism of regulation of cytoplasmic pH is not fully understood.

Possible mechanisms for cytoplasmic pH regulation include cytoplasmic buffers and active transport of H^+ (7, 69). The buffering capacity of all cells, regardless of their type of metabolism and pH range of growth, is relatively similar. Amino acid side chains of proteins are the principal components of the buffer capacity. Values of approximately 50 to 100 nmol of H^+ per pH unit per mg of cell protein are typical for the buffering capacity at internal pH 7.0. *Bacillus subtilis* has been found to have an unusually high buffering capacity of up to 200 nmol of H^+ per pH unit per mg protein. However, there is no evidence that a high buffering capacity increases the pH range for growth of the organism (7).

Controlled transport of protons has long been considered to be the dominant mechanism of regulation of cytoplasmic pH. However, Booth (7) stated that the activity of the primary proton pumps is constrained by the generation of the proton motive force. Given a cytoplasmic buffering capacity of 50 nmol of H^+ per pH unit per mg dry weight and assuming 5×10^8 cells per mg dry weight, the extrusion of approximately 4×10^4 protons, which is enough to generate a Δp of -200 mV, would only raise the internal pH by 0.001 pH units. Thus, the dissipation of excessive membrane potential ($\Delta \Psi$) by either cation influx or anion efflux is

essential for the generation of the pH gradient. For example, the lowering of the internal pH often stimulates H$^+$-ATPase activity, but the extent of the stimulation is constrained by the resulting increased membrane potential. An increase in permeability of the cell membrane to ionic species, such as potassium and sodium, is necessary to allow high rates of ATPase to counter the acidification of the cytoplasm. In bacteria, the uptake of potassium, the major cellular cation, during growth may be a major factor in maintaining an internal pH in the range of growth.

Measurement of the proton motive force

The intracellular pH can be measured by an acid distribution method that is based on the assumption that nondissociated forms of weak acids can diffuse freely through the cell membrane (7, 45, 67, 78). Since the internal and external concentrations equilibrate, distribution of the ionized form becomes a function of the pH gradient across the membrane:

$$\Delta pH = \log \frac{[A^-]_i}{[A^-]_o} \qquad (9)$$

where $[A^-]_i$ is the internal concentration of the anion and $[A^-]_o$ is the external concentration of the anion of the dissociated acid.

The membrane potential ($\Delta\Psi$) is determined by a similar method. It is based on the uptake of lipophilic cations or anions that distribute across the cell membrane in accordance with the Nernst equation:

$$\Delta\Psi = \frac{RT}{nF}\log\frac{[cation]_i}{[cation]_o} \qquad (10)$$

Internal volume of the cell is measured from the difference between total and extracellular volumes in cell pellets. The total volume is measured with ^3H_2O, and the extracellular volume with large, nonmetabolizable molecules such as inulin, dextrin, or sorbitol. The internal volume is then calculated from the ratio of the radio-labeled large molecules associated with the cell subtracted from the ^3H_2O in the cell. The concentration of the probes within the cells can then be calculated (7, 45, 67, 78).

"Uncoupler" theory

Much work has been done using the principles of the chemiosmotic theory to examine the mechanism of acid inhibition and tolerance in microorganisms. One proposed mechanism is that acids may act as solvents that perturb membrane phospholipids. At high concentrations, the acids increase the inward leak of protons so that the proton efflux is not rapid enough to compensate for this leakage and thus the cytoplasm is acidified (46). This action of organic acids had lead to the belief that the acids act as "uncouplers" (4, 37, 46).

The term "uncoupler" was coined by Mitchell in developing the chemiosmotic model of energy coupling (61, 62). Uncouplers are compounds that dissipate the $\Delta\mu_{H^+}$ by translocating H^+ or OH^- across the mitochondrial and cell membranes (14, 61, 62). The protonated species travels across the membrane and releases a proton in response to the pH gradient. The anion is

driven to the external surface of the membrane by the electrical potential. The anion is then protonated and the cycle continues. Membrane-bound ATPase must function to counteract the futile cycle of protons through the membrane; thus, the cell becomes deenergized (4, 14, 61, 62).

According to this model, organic acids would act as "uncouplers" that allowed protons to enter the cell from the medium and thus counteract the action of the proton pump (4, 5, 6, 37). The undissociated form of a weak acid can diffuse freely through lipid-containing membranes, and the concentration of the acid inside a cell can become equilibrated with its external concentration (4, 5, 6, 37, 55, 64, 80). However, because the internal pH is higher than the external pH, acid dissociation will proceed to a larger extent inside the cell (Figure 2). As a result, the anion concentration will be higher in the cytoplasm than in the surrounding medium. This results in a net efflux of anions from the cell. The exit of the charged anion is believed to occur when it collides with an undissociated organic acid molecule partitioned in the membrane. This gives rise to a dimer, HA_2^-, formed by hydrogen bonding within the hydrophobic lipid milieu. Because the species enters as HA and exits as HA_2^-, for every molecule of acid entering the cell one proton is internalized (4, 37).

There are a number of researchers who credit the action of acids to this "uncoupler" theory. Baronofsky *et al.* (4) studied the effect of acetic acid production during the growth of *Clostridium thermoaceticum*. They followed the internal pH of the cells by measuring the *in vivo* ΔpH and $\Delta\Psi$. Growing cells maintained a ΔpH of about 0.6 throughout most of batch growth, while the $\Delta\Psi$ decreased from -140 mV at the beginning of growth when the medium was at pH

7.0 to -80 mV at the end when the pH had declined to 5.0. Corresponding Δp values were -155 mV at pH 7 and -120 mV at pH 5. Below pH 5, the ΔpH and $\Delta \Psi$ collapsed, which suggests that the cells required an internal pH of at least 5.5 to 5.7. The authors stated that the low internal pH inhibited pH-sensitive cellular reactions, which lead to cessation of growth and fermentation.
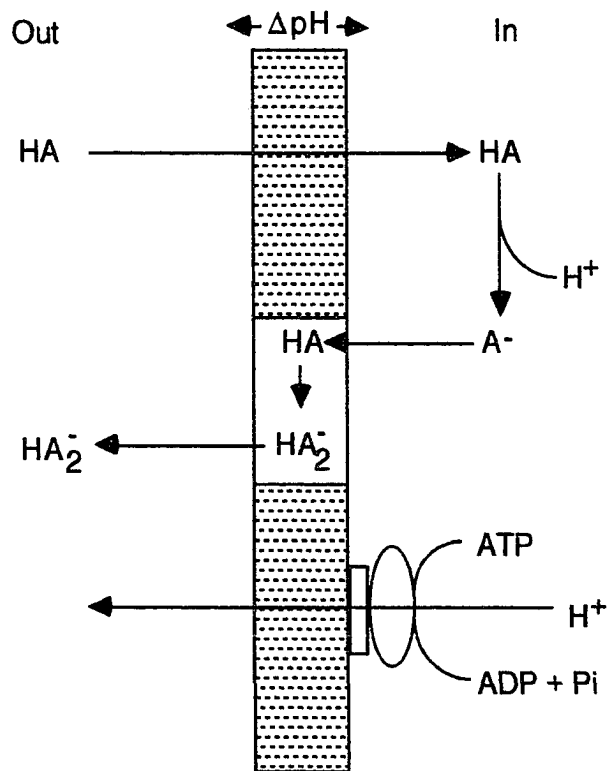

Figure 2: Schematic representation of organic acid movement in bacteria in response to the pH gradient (Adapted from Russell [82]).

When nongrowing cells of C. *thermoaceticum* were resuspended in citrate phosphate buffer at a wide range of pH values, the cells were able to maintain a significantly higher ΔpH and $\Delta\Psi$ than was observed for growing cells (4). The addition of acetic acid caused the ΔpH to dissipate and the $\Delta\Psi$ to decrease. Baronofsky *et al.* proposed that organic acid fermentation end-products acted as uncouplers and short-circuited the ΔpH. Furthermore, the proton motive force of the cell collapsed when metabolism could not supply the ATP required for proton extrusion. This proposed mechanism is based on the assumption that bacteria possess a minimum internal pH for growth and that the cell will strive to maintain this pH by extruding H^+ by means of the membrane-associated H^+-ATPase.

Gatje and Gottschalk (30) examined the effect of increasing undissociated lactic acid and proton concentrations on *Lactobacillus helveticus* in continuous and batch cultures. In continuous culture without pH control, the final pH of the medium was 3.8, with 65 mM undissociated lactic acid present. Batch culture experiments showed growth limitation occurred at 60-70 mM lactic acid. After growth ceased, production of lactic acid continued until a concentration of 100 mM was reached. Thus, an uncoupling of growth and acid production occurred. The authors suggested that the energy gained by lactate production was no longer available for cell growth but it was used for the maintenance of ΔpH.

To determine if the undissociated acid or proton concentration caused the uncoupling, *L. helveticus* was grown in batch culture over a range of pH values with lactic or hydrochloric acid as acidulant (30). At similar pH values the culture behaved similarly in the presence of the different acids. The amount of undissociated lactic acid was considered to be primarily responsible for inhibition

of growth and lactic acid production, since this acid was more inhibitory than hydrochloric acid. The researchers concluded that inhibition was due not only to the acidification of the cytoplasm, but also to other physiological effects of the acid produced.

Low intracellular pH lead to inhibition of growth and metabolic activities in other strains of lactic acid bacteria as well. Nannen and Hutkins (65) measured the ΔpH of several lactic acid bacteria during growth. They found that when *Streptococcus thermophilus* was grown without pH control in three different media the cells maintained a ΔpH of approximately 0.8 to 1.0 pH unit during log phase (medium pH declined from 6.5 to 5.5), but this decreased to 0.5 unit after 10 h of growth (medium pH was 4.5), regardless of the buffering capacity of the medium. Continued incubation for 24 h lead to a collapse of ΔpH.

Lactococcus species also maintained large ΔpH values during batch growth: 0.97 to 1.40 for *L. lactis* subsp. *lactis* C2, and 0.81 to 1.39 for *L. lactis* subsp. *cremoris* HP (65). However, *L. lactis* subsp. *cremoris* HP experienced a rapid decline in ΔpH after about 6 to 8 h of growth (medium pH was 4.6 to 5.0), while *L. lactis* subsp. *lactis* C2 maintained a ΔpH of approximately 0.6 units for up to 72 h. *Lactobacillus casei*, which reportedly grows at the lowest pH values of lactic acid bacteria, maintained an internal pH near 6.0 when the external pH was <4.5 and a large ΔpH (>1.2) when the external pH was <4.0. This may explain why *L. casei* grows at pH values lower than the streptococci. The researchers concluded that since lactose was still present at the end of fermentation for all lactic acid bacteria studied, cessation of growth was due to dissipation of ΔpH, not

carbohydrate depletion. Growth and metabolic activities were inhibited as a

consequence of the deleterious effects of a low intracellular pH.

In a companion study, the H^+-ATPase activity of these lactic acid bacteria

was measured (66). The pH optima for the H^+-ATPase enzymes of S.

thermophilus and L. lactis subsp. cremoris HP were 7.5, while that of L. casei was

5.0. For all species, the H^+-ATPase activity was always greatest when the

cytoplasmic pH was less than the optimum for the enzyme. The H^+-ATPase

activity generally increased as the external pH decreased until pH 5.0 was

reached. Below pH 5.0, the enzyme activities decreased markedly. Among these

organisms, L. casei had the highest basal level of H^+-ATPase. The authors

suggested that the H^+-ATPase enzyme may be involved in the regulation of the

intracellular pH in lactic acid bacteria.

Other researchers have investigated the pH optima of enzymes of L. casei,

with similar results. Bender and Marquis (6) measured the optimal glycolytic pH

and membrane ATPase activity of Lactobacillus casei and Actinomyces viscosus,

two organisms found in dental plaque. The authors found that L. casei, the acid-

tolerant organism, had an optimal glycolytic pH of 6.0 and that a 50% reduction in

glucose degradation occurred at pH 4.2. For A. viscosus, the acid-sensitive

organism, the corresponding pH values were 7.0 and 5.6.

The optimal pH for the ATPase of L. casei was approximately 5.0; that for

the A. viscosus enzyme was pH 7.0 (6). In addition, in the presence of

dicyclohexylcarbodiimide, a specific ATPase inhibitor, the proton permeabilities of

both organisms markedly increased. Bender and Marquis suggested that

ATPases played a role in moving influxing protons back out of the cell.

The authors concluded that acid tolerance could be attributed to greater resistance of the membrane and cellular enzymes to acid damage, higher specific activity of ATPase in the cell membrane, lower pH optimum of ATPase, and greater capacity to expel protons at lower pH (6, 84).

Lactic acid bacteria have been found to increase their ATPase activity in the presence of propionate as well. Perez-Chaia et al. (70, 71, 72) have investigated the effects of propionic acid on lactobacilli. In the presence of a low concentration of propionate (0.7 g/L), Lactobacillus helveticus had a higher growth rate and biomass yield than the control culture. When the concentration of propionate was higher (>1.4 g/L), the growth rate and biomass were reduced, but the fermentative activity was increased, i.e. cells consumed more glucose and produced more lactic acid than did the control cultures. Increasing the glucose concentration counteracted these effects. Glucose provided the cells with more metabolic energy to counteract the inhibition of propionate. In contrast, L. casei was not inhibited by propionate regardless of the glucose concentration.

The membrane-bound ATPase activity of cultures grown in the presence of propionate was higher than for control cultures (70). The pH optima and K_m values for ATP were identical for the increased and basal ATPase activities for both organisms. Thus, it appeared that the same enzyme was amplified, rather than that a new, more active enzyme was induced. Substrate consumption of resting cells was increased by propionate. In the presence of an ATPase inhibitor, dicyclohexylcarbodiimide, this effect reverted, which indicated that the increase of fermentative activity was related to the H^+-ATPase activity.

By increasing the fermentative activity, the cells were able to extrude protons from the cytoplasm by means of the H^+-ATPase. This enzyme utilizes glycolytically generated ATP to expel protons accumulated by the cell as a result of lactic acid production and exposure to propionate. Growth rate and biomass production decreased when metabolism could not supply ATP required for proton extrusion. The tolerance of *L. casei* to propionate may be a result of its high basal level of H^+-ATPase (66).

In a review of the bioenergetics of lactic acid bacteria, Kashket (46) stated that the minimum pH values for growth and fermentation differ among the lactic acid bacteria probably because of the slightly different complement of their enzymes and transport carriers. Cessation of growth and fermentation occur when the internal pH reaches a given value, which is characteristic of the organism. Internal enzyme activity can only tolerate a pH above a critical value, and this threshold pH depends on the organism. It is not clear whether these cells regulate the internal pH through systems that respond to perturbations in internal pH by restoring the pH to the original value, or whether the internal pH results from all the H^+-translocating activities taking place at the time without being under the control of a specific pH-sensing regulator.

In the presence of large concentrations of fermentation end-products, the cells eventually are no longer able to expel protons rapidly enough to keep the cytoplasm alkaline with respect to the medium (46). The uncoupling effect of permeant organic acids and the chaotropic effect of solvents are responsible for the increased proton leakage into the cell.

Similar effects of organic acids have been found in other bacteria as well. Luli and Strohl (55) correlated the accumulation of acetate with reduced production of recombinant protein during recombinant E. coli fermentations. The toxic effects of acetate were demonstrated at pH values at or below the pK_a and even at neutral pH when sufficient amounts of acetate had accumulated in the medium. The authors noted that homeostatic mechanisms (mechanisms to maintain a constant internal pH) of E. coli required energy to adjust to a decrease in intracellular pH. If not enough energy was available the proton motive force collapsed and growth and fermentation ceased.

Anion accumulation theory

Other researchers question the idea that organic acids act as "uncouplers." Although the undissociated form of the acid is able to diffuse through the membrane, the anion cannot. Thus, the acid would not continue the futile cycle of transporting protons across the membrane. Russell (82) states "the expansion of 'uncoupling' to include any type of metabolic inhibition would create a term with little biochemical meaning."

Russell (82) suggests that the intracellular release of protons by organic acids is overemphasized. For example, consider a 1 M increase in intracellular acid concentration for a bacterium in which the H^+/ATP stoichiometry of the membrane-bound ATPase is 3:1 and the intracellular volume is 3 μl/mg protein. Based on these assumptions, 1.0 μmol ATP/mg protein would be required to expel the protons brought in by the acid. Even anaerobic bacteria with moderate growth rates can produce more than 50 μmol ATP/mg protein.

It was observed that some bacteria allowed their internal pH to drop as the extracellular pH decreased (80). Cells of *Streptococcus bovis* grown in the presence of 100 mM acetate allowed their internal pH to decrease to 5.4 before growth was inhibited. Acetate distributed across the membrane in accordance with the Henderson-Hasselbalch equation (on which the measurement of ΔpH is based). Because the ΔpH remained nearly constant, the acetate anion gradient across the membrane remained the same (approximately 10-fold) regardless of the external pH. If *S. bovis* had maintained a constant internal pH, the acetate gradient would have been almost 1500-fold, resulting in an internal concentration of greater than 5 M. Thus, the bacterium's ability to tolerate increasingly acidic conditions was linked to its ability to decrease its internal pH.

When *Streptococcus bovis* and *Lactococcus lactis* were grown with an excess of glucose, lactic acid accumulation caused a decrease in extracellular pH (15). The *S. bovis* culture grew in medium at pH values as low as 4.9, but *L. lactis* was unable to grow below pH 5.3. Both bacteria decreased their internal pH as a function of external pH; the ΔpH was relatively constant. Neither culture showed a significant decline in $\Delta\Psi$ until the medium was below pH 4.8. The glycolytic rate of *S. bovis* decreased as the intracellular pH decreased, but intracellular ATP levels did not decrease. Thus, it appeared that metabolic energy was not the critical aspect of pH sensitivity. Cook and Russell (15) postulated that biosynthesis was pH-sensitive. However, it appeared that metabolic energy was a key factor in the pH-sensitivity of *L. lactis*; the gylcolytic rate and the intracellular ATP concentration decreased as the medium pH declined.

Russell (79, 80, 81, 82) concluded that the critical aspect of a particular bacterium's pH sensitivity may vary. However, fermenting bacteria must decrease their internal pH and maintain a low ΔpH. A high ΔpH would cause a large and toxic accumulation of fermentation acid anions. He suggested that the organic acid toxicity was more closely related with intracellular pH regulation and anion accumulation than with "uncoupling" per se.

Other researchers agree that acid inhibition is due to anion accumulation. Brink and Konings (9) found that the internal pH of Streptococcus cremoris, when grown in batch culture without pH control, remained constant over only a short period and then decreased gradually with external pH. Directly after inoculation no ΔpH could be detected; the ΔpH gradually formed during culture growth and acidification of the medium. The maximal value of ZΔpH reached -35 mV when the external pH was 5.7, while $\Delta\Psi$ remained nearly constant (-85 mV to -90 mV) during growth. Total Δp increased from -90 mV to -122 mV. At the end of growth both components dissipated completely. Internal lactate concentration decreased during growth, while external concentration increased, which corresponded to the lowering of the internal and external pH values. When growth stopped, the internal and external lactate concentrations equilibrated. Growth proceeded for about 6 h and then stopped abruptly after all carbon source was depleted. Even though the external pH decreased from 6.8 to 5.3, the acidification of the medium did not stop growth; when lactose was added growth resumed.

In a study examining the intracellular pH response of Leuconostoc mesenteroides and Lactobacillus plantarum to external pH changes created by acid production or by addition of lactic or acetic acids to the medium, McDonald et

al. (60) found that the internal pH of both bacteria declined with exposure to acidic pH. Growth of *L. mesenteroides* stopped when its internal pH reached 5.4 to 5.7 while growth of *L. plantarum* stopped at an internal pH of 4.6 to 4.8. Variation in growth medium composition or pH did not alter the growth-limiting internal pH. The *L. plantarum* was more acid-tolerant and was able to maintain a ΔpH even when external pH reached 3.0 with 160 mM sodium acetate or lactate. In contrast, the ΔpH of *L. mesenteroides* was zero at pH 4.0 in the presence of acetate and at pH 5.0 in the presence of lactate. The low growth-limiting internal pH and ability to maintain a pH gradient at high organic acid concentration contributed to *L. plantarum*'s acid tolerance.

These researchers stated that lactic acid might affect pH homeostasis through a mechanism which is not solely dependent upon the undissociated acid molecule or H^+ concentration (60). At all pH values studied, lactic acid was more effective than acetic acid at decreasing the internal pH of *L. mesenteroides* even though the undissociated concentration of acetic acid (136 mM) was higher than that of lactic acid (9 mM). Thus, the authors suggested that lactic acid inhibition was caused by the anion attacking a specific target and not just by acidification of the cytoplasm.

Other researchers agree that organic acid inhibition is due to more than just low internal pH. Salmond *et al.* (83) investigated the effects of organic acids on the growth and intracellular pH of *E. coli*. They grew *E. coli* in the presence of cinnamic, propionic, benzoic or sorbic acids. At higher external pH values (>5.0), a high concentration of acid was necessary to cause complete inhibition of the culture. For all four acids, growth was not inhibited until the internal pH was

below 7.1. Severe inhibition occurred once the internal pH dropped below 6.8. In an effort to determine which form of the acid (undissociated or dissociated) caused the inhibition, the effect of the ratio of benzoic acid and benzoate anion was studied. Different ratios were achieved by varying the undissociated acid concentration at a constant pH or by changing the pH of the medium at a constant acid concentration. Growth inhibition strongly correlated with the concentration of undissociated acid, regardless of how it was achieved.

Salmond et al. (83) determined that the acids did not exert their effects simply as a function of their excessive accumulation which would cause acidification of the cytoplasm. If this were the case, the pattern of inhibition would reflect the pKa of the acids. Despite the fact that these acids have similar pK_a values (propionic acid 4.87, sorbic acid 4.77, benzoic acid 4.20, and cinnamic acid 4.44 [22]), they displayed significant differences in their effectiveness as growth inhibitors. Cinnamic acid was found to be the most inhibitory followed by benzoic, sorbic, and propionic acids. It was observed that the predicted intracellular concentration of the dissociated weak acids did not correlate with either the pK or the severity of growth inhibition. The authors concluded that growth inhibition by organic acids had two components: a specific inhibition due to an unidentified metabolic function by the undissociated acid, and a generalized inhibition by the acidification of the cytoplasm.

Shift in fermentation pattern

Fermentative organisms obtain less energy from substrates than do respiring organisms; thus, many fermentative organisms adapt to low pH through

mechanisms that are not energy-consuming, such as shifting their fermentation pattern to producing neutral products such as butanol, ethanol, and acetoin. Tseng et al. (87) studied the effects of a shift from pH 5.5 to 7.5 on a steady-state Lactobacillus plantarum anaerobic chemostat culture. The Δp decreased 50% (from -106 mV to -55 mV) as a result of the depletion of the transmembrane proton gradient. The internal pH rose from 6.6 to 7.3, while the $\Delta \Psi$ partially compensated for the lowering of the ΔpH by increasing from -42 mV to -63 mV. Intracellular ATP displayed an immediate 20% increase, but then decreased quickly. The authors suggested that the transient increase may have been due to L. plantarum shifting to producing some acetate at alkaline pH, which generated an additional ATP by acetate kinase. The overall decrease in ATP concentration may have been caused by the export of protons or ATP used to drive the uptake of nutrients. The H^+/lactate stoichiometry changed from 0.7 to 1.8 while the H^+/acetate value increase from 0.9 to 1.9 at pH 7.5. The authors stated that the increase in H^+/product stoichiometric ratio under alkaline conditions demonstrated that L. plantarum co-excreted more protons with end products to maintain intracellular pH homeostasis and generate proton gradients.

The maintenance of pH homeostasis of L. plantarum was examined during growth in the presence of glucose and pyruvate (86). The energy derived from the metabolism of glucose generated a constant Δp of about -120 mV regardless of medium pH. The ΔpH increased with the decreasing external pH while the $\Delta \Psi$ decreased correspondingly, so that the Δp remained constant. In medium at low pH, energized cells rapidly transported and accumulated pyruvate, but did not do so when the cells were deenergized by nigericin. This confirmed that pyruvate

was actively transported. The internal pH of cells that accumulated pyruvate immediately decreased from pH 6.3 to 5.5 due to the cotransported protons. The conversion of pyruvate to acetoin, instead of to acidic end-products, caused the internal pH to increase gradually.

Fermentation patterns of *Sarcina ventriculi* were investigated during glucose metabolism in unbuffered batch cultures (31). The culture shifted from hydrogen-acetate production to ethanol production as the medium pH declined from 7.0 to 3.3. At a constant pH of 3.0, acetate production ceased when the concentration of acetate in the medium reached 40 mM, while at pH 7 acetate production continued throughout the entire growth time course. As the pH of the medium declined from 7.0 to 3.0, the ΔpH increased from 0.1 to 1.3 and the $\Delta\Psi$ decreased from -67 mV to -38 mV; the magnitude of Δp increased as the medium pH decreased. The addition of acetic acid, but not hydrogen or ethanol, inhibited growth and resulted in Δp dissipation and internal acetic acid accumulation. The authors suggested that the ability of *S. ventriculi* to shift to ethanol production and continue to ferment glucose while cytoplasmic pH values were low adapted this organism for growth at low pH.

The proton motive force was involved in the regulation of the transition from acetogenic to solventogenic fermentation in *Clostridium acetobutylicum* as well (42). The extracellular pH of the culture was varied from pH 6.5 to 4.5 and the ΔpH, $\Delta\Psi$ and fermentation products were measured. The internal pH dropped from 6.7 to 6.0 when the external pH changed from 6.5 to 5.5, but there was no further decline in internal pH even when the medium pH decreased to 4.5. The $\Delta\Psi$ decreased as the external pH decreased; at pH 6.5, $\Delta\Psi$ was -90 mV and at pH

4.5 no $\Delta\Psi$ was detected. The corresponding Δp values were -106 mV at pH 6.5

and -102 mV at pH 4.5. The ability of C. acetobutylicum to maintain a high

internal pH even at pH 4.5 points to a very efficient deacidification system. This

system was presumed to be responsible for the shift from acetogenesis to

solventogenesis when the medium pH dropped below 5.5.

The shift to solvent formation appeared to occur between pH 5.0 and 5.5

(42). Acetone and butanol were the predominant products at low pH but were

present in only trace amounts at high pH, while butyrate and acetate were the

major products formed at high pH. The researchers noted that hydrogenase,

which is responsible for the disposal of excess hydrogen as hydrogen gas, was

pH-sensitive. The loss of this activity may result in more reducing power

becoming available for solvent formation. In addition, the enzyme acyl coenzyme

A transferase, which converts fatty acids to the corresponding coenzyme A

derivative, showed increased activity at low pH. Solvent formation appeared to

allow C. acetobutylicum to maintain internal pH homeostasis.

Acidophiles

An alternative approach to studying the mechanism of acid inhibition is to

examine organisms that tolerate high levels of acid. Acidophiles, i.e. organisms

whose optimal pH is generally between 2 and 4, have been the subject of several

studies (1, 14, 33). Respiring acidophiles maintain a large Δp, which consists of a

large ΔpH and a small $\Delta\Psi$. The $\Delta\Psi$ is reversed from that of other organisms

(nonacidophiles), i.e. positive inside the cell. The $\Delta\Psi$ most likely plays a role in

the maintenance and perhaps even generation of ΔpH. These organisms can

tolerate acidic media because they respire rapidly, thereby ejecting protons. In addition, the cells do not produce organic membrane-permeable fermentation end-products (48).

In a study of sensitivity to anions and organic acids of the acidophile *Thiobacillus ferrooxidans*, Alexander *et al.* (1) suggested that the toxicity of weak acids could be attributed to their accumulation in the cell matrix in response to the pH difference between the cytoplasm and the supporting medium. This accumulation led to cytoplasmic acidification that resulted in inhibition of the respiratory chain and cessation of growth.

Goulbourne *et. al* (33) measured the proton motive force of active and starved cells of PW2, a heterotrophic, mesophilic acidophile. The cells were starved in a mineral salts medium lacking citrate and magnesium. The starved cells possessed no respiration, no cellular ATP and no ATPase activity, yet they maintained a large ΔpH. This organism was able to maintain a passive ΔpH by the buildup of the counterforce, $\Delta\Psi$, in the same magnitude, but opposite polarity (interior positive). The role of this electrical potential force was to prevent a net H^+ influx and thus help to maintain the cytoplasmic pH.

In activated cells metabolic energy was available, and respiration-dependent H^+ extrusion offset the $\Delta\Psi$ (33). Eventually a steady state was achieved in which H^+ influx was equal to H^+ efflux. The addition of 1.3 mM azide to these cells caused a net H^+ influx of 14.4 nmol per mg of protein, which should be sufficient to lower the internal pH to 2. However, this concentration had no effect on the cytoplasmic pH. The cells must have resisted acidification by their cytoplasmic buffering capacity. The authors concluded that the mechanism that

linked H^+ influx with the rise in $\Delta \Psi$ as well as the cytoplasmic buffering capacity played a central role in the acidophile's mode of existence.

Some believe that ΔpH is maintained in acidophiles by Gibbs-Donnan equilibrium (14, 48). According to this mechanism, H^+ entry is prevented by a high intracellular concentration of cations to which the membrane is impermeable. Thus, even when treated with an uncoupler, the cell is able to maintain the ΔpH by a mechanism that does not require an immediate source of metabolic energy.

Acid tolerance response

It has been found that some organisms are able to increase their resistance to extreme acid conditions through previous exposure to mildly acidic conditions. Examination of this response may offer insight into acid tolerance. Foster and Hall (27) discovered that when logarithmically growing *Salmonella typhimurium* cells (pH 7.6) were shifted to pH 5.8 by adding hydrochloric acid to the medium for one culture doubling time (preshock), the cells were 100 to 1,000 times more resistant to subsequent strong acid challenge (pH 3.3) than were unadapted cells shifted directly from 7.6 to 3.3 (acid shock). This response was called the acidification tolerance response (ATR).

By measuring the internal pH of adapted and unadapted cultures at pH 3.3, it was found that the ATR system produced an inducible pH-homeostatic function (28). The adapted culture was able to maintain an internal pH of 5.2 to 5.4 while the internal pH of the unadapted culture was 4.2 to 4.6. In addition, the H^+-ATPase was found to play an important role in ATR (27, 28). Mutants lacking this activity were unable to display an ATR and were extremely acid-sensitive.

The ATR required the synthesis of preshock proteins and appeared to be a specific defense mechanism for acid, since no cross-protection was noted for hydrogen peroxide, SOS (the multifunctional DNA damage repair system), or heat shock (27). However, other researchers found that adapted cultures of S. typhimurium had an increased tolerance towards heat, salt, an activated lactoperoxidase system, and the surface-active agent crystal violet (53). These researchers found that acid adaptation increased the cell surface hydrophobicity.

The analysis of two-dimensional SDS-PAGE autoradiographs revealed six repressed and 12 induced membrane proteins in adapted cultures. Leyer and Johnson (53) found that the lipopolysaccharide component of the membranes appeared to be unaltered and thus, the induced outer membrane proteins were thought to be responsible for the change in surface properties.

During acid shock, S. typhimurium induced the synthesis of 37 proteins and repressed 15 (29). All but four were distinct from the preshock ATR proteins. Acid shock alone did not protect the culture against acidic conditions. Inhibiting protein synthesis with chloramphenicol prior to preshock or acid shock revealed that acid shock proteins did not appear to contribute to acid survival in medium even at pH 4.3, however, preshock proteins were necessary for resistance. These observations indicated that the induction of acid shock and preshock ATR proteins were separate processes requiring separate signals. The organism required both systems to survive extreme acid conditions. Foster (29) proposed a two-stage process that allowed the organism to phase in acid tolerance as the environmental pH became progressively more acidic.

A recent study showed that stationary-phase cells of *S. typhimurium* had

two additional acid tolerance responses (52). One was a pH-dependent system

that was distinct from log-phase ATR. Stationary-phase cells with a final pH of 4.3

survived a subsequent 4-h pH 3.0 challenge at least 1000 times better than

stationary-phase cells that had a final pH of 7.3. The stationary-phase ATR

induced fewer proteins than did log-phase ATR. The other system of acid

resistance was not induced at low pH, but appeared to be part of a general stress

resistance induced by stationary phase. It was determined that although all three

systems were distinct from each other, together they afforded maximum acid

resistance for *S. typhimurium*.

Production of acid-tolerant organisms

Many researchers are interested in increasing end-product tolerance of

microorganisms. These tolerant strains could be important to several industrial

fermentations for production of fuel additives (butanol and ethanol) and organic

acids (lactic and propionic acids). Lin and Blaschek (54) used serial enrichment

to obtain a derivative of *Clostridium acetobutylicum* that grew at concentrations of

butanol that prevented growth of the parent strain. At 15 g/L of butanol, the wild-

type strain demonstrated a negative growth rate while the mutant (SA-1) grew at a

rate that was 66% of that of the uninhibited control. When the parent and SA-1

strain were grown in batch fermentation with 6% extruded corn broth, SA-1 had a

significantly faster generation time than the parent strain (32.8 min versus 57.9

min), a 9- to 12-h earlier onset of butanol production, better carbohydrate

utilization (88.1% versus 82.3%) and a higher final butanol concentration (8.6 g/L

versus 6.8 g/L). In addition, SA-1 demonstrated higher conversion efficiency to butanol than did the wild-type strain.

Jimenez and Benitez (44) formed hybrids between naturally occurring and laboratory wine yeast strains to increase genetic variability and improve ethanol tolerance of the strains. The hybrids were cultivated in pH-controlled continuous culture with increasing ethanol concentration. The fastest growing, most ethanol-tolerant strains were selected by this system.

In another experiment, *Streptococcus mutans* was shown to increase its acid tolerance when grown in continuous culture at low pH (5). Cells harvested from the acidified cultures had a lower pH minimum for glycolysis as well as increased ATPase activity. However, this adaptation was found to be reversible over a number of generations, and thus did not seem to be due to selection of variants in the population with greater constitutive acid resistance. The authors suggested that increased ATPase activity was the major factor in the cultures' increased acid tolerance.

Woskow and Glatz (91) developed an acid-tolerant strain of *Propionibacterium acidipropionici* by a serial enrichment process. This strain (P200910) showed significantly higher specific growth rates than its parent strain (P9) at 1 to 7% propionic acid. However, in laboratory media without propionic acid, P200910 had a longer lag time and grew to a lower final cell density than did P9. In batch fermentation, P200910 produced more propionic and acetic acid per gram biomass than P9 (0.91 versus 0.50 for propionic acid and 0.21 versus 0.13 for acetic acid). The variant exhibited a propionic acid production pattern

typical of non-growth-associated product formation, while the parent followed a typical growth-associated pattern.

When both strains were used in fed-batch semi-continuous fermentations, much higher final concentrations of biomass and organic acids were obtained for both strains than in simple batch fermentations (91). After 5 days of incubation, P9 had produced 26 g/L of propionic acid and 0.41 g of propionic acid per g of biomass, while P200910 produced 38 g/L and 0.81 g of propionic acid per g of biomass. Rates of propionic acid production decreased for both strains after this time. However, P200910 continued to use glucose at a high rate throughout the fermentation. These observations led the authors to suggest that the acid-tolerant strain may expend energy to rid itself of excess acid.

The variant did not differ from its parental strain in morphology, gram reaction, fermentation of sucrose, maltose and mannitol, reduction of nitrate or pigment production (91). However, changes in lipid composition of the variant from more branched-chain fatty acids to straight-chain fatty acids did occur. The authors suggested such changes may have resulted in a less fluid, less permeable membrane.

The precise method by which short-chain organic acids inhibit microbial growth is still unresolved. Studies discussed here were based on different hypotheses and it appears that no single mechanism applies to all organisms. Further examination and characterization of acid-tolerant variants and their parental strains will lead to a greater understanding of the mechanism of acid inhibition.

RESPONSE OF CULTURES OF *PROPIONIBACTERIUM* TO ACID AND LOW PH: TOLERANCE AND INHIBITION

Jill L. Rehberger and Bonita A. Glatz

Abstract

Propionibacterium strains were screened for acid production and final pH achieved on glucose, fructose, and maltose as primary carbon source. On average, the *P. acidipropionici* strains produced more acid and achieved lower final pH values than did the other species. Six strains, which consisted of three *P. acidipropionici*, one *P. jensenii*, and two *P. thoenii* strains, were identified as potential acid- and low-pH-tolerant strains. The lowest pH values at which the strains could initiate growth and remain viable were determined for the six strains with lactic, hydrochloric, and propionic acid as the acidulants. For all three acids, the *P. jensenii* and *P. thoenii* strains initiated growth and survived at lower pH values than did the *P. acidipropionici* strains. Thus, the ability to produce large amounts of acid or achieve low final pH values was not directly related to a strain's ability to initiate growth or survive in low-pH conditions. In addition, the strains were not inhibited by increased lactate concentrations (up to 180 mM) at neutral pH.

46

Introduction

The propionibacteria are gram-positive, nonmotile, pleomorphic rods that are anaerobic to aerotolerant [8] and produce propionic acid, acetic acid, and carbon dioxide from the metabolism of sugar and/or lactate [25]. They have been isolated from soil, silage, rumen, raw milk, Swiss cheese, and other dairy products [21, 25].

Propionibacteria have traditionally been used in the dairy industry as starter cultures. In addition, the propionibacteria can be used to produce valuable products such as propionic acid, which is an important preservative for foods and grains [13, 21]. Propionate inhibits growth of molds, bacteria, and yeasts; it is biostatic in dilute aqueous systems and biocidal at higher concentrations [13].

Currently commercial production of pure propionic acid is chiefly by petrochemical routes. Fermentation has not been used commercially primarily because propionibacteria grow slowly and produce relatively low concentrations of organic acids. Also, the costs of separation and concentration of the acids make recovery difficult and expensive [21].

A major factor that limits the production of propionic acid during fermentation is end-product inhibition by the acid [11, 25]. Accumulation of end products causes a continuous decrease in both specific growth rate and the further production of the product [11]. To overcome this end-product inhibition,

either improvements in the fermentation process to avoid product accumulation can be made, or an acid-tolerant variant of the producer strain can be developed.

A strain of *Propionibacterium* that could tolerate high concentrations of organic acids and low pH could be of economic importance as a more productive strain for industrial fermentations or as part of a silage inoculum or probiotic feed additive. One objective of this work was to identify strains that produced high amounts of acid and can grow at low pH (pH 5.0 or lower) on various carbon sources. A second objective was to determine the lowest pH (adjusted with lactic, propionic, or hydrochloric acid) at which growth occurred as well as the pH at which significant loss of viability occurred.

Materials and Methods

Strains and culture maintenance

Propionibacterium strains that had not been previously screened for acid production as well as strains previously identified as good acid producers [1] were obtained from the culture collection of the Department of Food Science and Human Nutrition at Iowa State University. Strains were classified according to Bergey's Manual of Systematic Bacteriology [8]. The strains were grown in sodium lactate broth (NLB) which contained 1% trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD), 1% yeast extract (Difco, Detroit, MI), 1% sodium lactate 60% syrup (Fisher Scientific Co., Pittsburgh, PA) and 0.6% Tween 80 (Fisher). Stock cultures were maintained at -80°C in NLB supplemented with

20% glycerol. Working cultures were maintained on sodium lactate agar plates (NLA; NLB + 1.5% agar, Difco), grown for 5 days at 32°C in an anaerobic environment in GasPak jars (BBL) with the GasPak system, stored at 4°C, and transferred at approximately one-month intervals.

Isolated colonies from working plates were inoculated into screw-capped test tubes containing 10 ml NLB and incubated at 32°C for 48 h. At least 3 sequential transfers were made by transferring 0.1 ml of culture to a test tube containing 10 ml NLB and incubating at 32°C for 24 h. The cells were harvested in the exponential phase (absorbance of approximately 0.6 at 600 nm) for use in experiments.

Culture screen

Cultures were spotted as 5-μl drops (approximately 10^6 cells) onto bromocresol agar (BCP agar) to test for acid production as measured by pH change [22]. The plates were incubated at 32°C for 5 to 7 days in the GasPak system. Acid production was semiquantitatively determined by measuring the diameter of the zone of yellow around the colony. To test for tolerance to lactate at both neutral and acidic conditions, cultures were also inoculated onto NLA supplemented with different concentrations of sodium lactate (2 to 3.5%) at pH 7.0 or adjusted to pH 5.5 or 5.0 with lactic acid (Fisher) as the acidulant. Plates were incubated at 32°C for 5 to 28 days in the GasPak system.

The best acid-producing strains identified in the above screening experiments were grown (1% inoculum) in duplicate in 15-ml screw-capped test tubes with 10 ml modified NLB in which the sodium lactate was replaced by 1%

glucose, maltose, or fructose. The tubes were incubated for 96 h at 32°C, the final pH measured (Corning Science Products, Corning, NY), and propionic and acetic acid concentrations determined by HPLC.

Minimum and lethal pH determinations

Cultures were grown in 15-ml screw-capped test tubes containing 10 ml NLB adjusted to pH values ranging from 6.5 to 3.75 with lactic, propionic, or hydrochloric acid (Fisher). The experiment was conducted by using a Randomized Block Design containing 2 blocks, each of which consisted of a separate batch of medium, with 2 replicates in each block [24]. Test tubes were inoculated with approximately 10^6 or 10^4 cells/ml. The cultures were incubated at 32°C for 7 days and examined each day for the presence of growth, as indicated by visible turbidity. If no growth appeared after 48 h, a 0.1-ml sample of the culture was used to inoculate a fresh tube of NLB at pH 7.0. These cultures were incubated at 32°C for 7 days and examined each day for growth.

Growth curves

Strains P9, P127, and P200910 were grown in 250-ml screw-capped Erlenmeyer flasks containing 100 ml of NLB and various concentrations of lactic acid adjusted to both neutral and acidic pH values. The cultures were incubated at 32°C and sampled at timed intervals. At each sampling time, absorbance, pH, and concentrations of lactate, propionic, and acetic acid were determined. Specific growth rates (μ) during exponential growth were determined from the slope of the straight line portion of the semilogarithmic plot of absorbance versus time [15].

Analysis of products

Lactate, propionate, and acetate were separated by a high-performance liquid chromatography (HPLC) system that consisted of a model 501 pump (Waters, Division of Millipore, Milford, MA) and an HPX-87H column (BioRad, Richmond, CA) operated at 65°C with 0.012N H_2SO_4 (pH 2.0) as the mobile phase at a flow rate of 0.8 ml min^{-1} and an injection volume of 20 µl. Peaks were detected with a Waters differential refractometer (model R401). Samples for analysis were centrifuged to remove the cells, filtered through 0.2-µm filters, and stored at -20°C before analysis. A Maxima 820 software program (Waters) was used to analyze the data and plot the chromatograms. The product concentration was calculated by comparing the peak areas with those of external standards (Aldrich Chemical Co., Milwaukee, WI).

Statistical analysis

An analysis of variance was performed by using the General Linear Model of SAS [23]. Differences with $P<0.0001$ were considered significant only if they were at least 0.1 pH unit. This pH difference was considered significant because the strains responded to pH values that differed by 0.1 pH unit, i.e. strains could grow at pH 5.0 but not at pH 4.9.

Results and Discussion

Strain selection

The initial objective of this work was to select the most acid- and pH-tolerant strains in our culture collection. Strains were selected based on their ability to metabolize all three carbon sources, their tolerance to 3.5% lactate, and the amount of acid produced. Acid production was estimated by the size of the zone of yellow around the colony on BCP agar. All strains were able to grow in the presence of 3.5% lactate and on pH 5.5 agar plates (data not shown). However, no strains were able to grow on pH 5.0 agar, even after a 4-week incubation in GasPak jars. Seventeen strains, including seven strains previously identified as good acid producers on maltose [2], were chosen for further study primarily based on the acid zone size on BCP agar.

Propionic and acetic acid production

The ability of propionibacteria to produce propionic and acetic acids with maltose, fructose, or glucose as the carbon source was tested by measuring the acids produced and pH achieved after 96 h in broth culture (Table 1). In general, the final pH was lowest when glucose was the carbon source (pH 4.28 +/- 0.18). With fructose as carbon source, the average final pH was higher (4.39 +/- 0.24); on maltose it was higher still (pH 4.53 +/- 0.38). The final pH differed the most among strains grown in maltose broth, as indicated by the largest standard deviation (0.19 pH unit versus 0.09 pH unit for glucose and 0.12 pH unit for

fructose). This indicates that strains of propionibacteria differ significantly in their ability to transport and/or hydrolyze maltose.

The average propionic:acetic acid ratios were approximately 4:1 for cultures grown on glucose and fructose and 3:1 for those grown on maltose (Table 1). Significant differences among the strains in the amounts and ratios of acids produced are consistent with previous observations that *P. acidipropionici* produced significantly different concentrations of propionic and acetic acids when grown on lactate, glucose, and lactose [18]. Variations in the ratios of acids produced by cultures of propionibacteria have been credited to the reactions of the citric acid cycle [6, 25], to aspartate metabolism [5, 6], and to polysaccharide production [7]. The utilization of aspartate varies among species and strains and shifts the balance from propionate production to acetate and carbon dioxide formation to maintain the redox balance of the cell [5, 6]. The formation of polysaccharides during glucose and lactose fermentation by propionibacteria alters the acid ratio by increasing the formation of acetate and carbon dioxide without any associated propionate production [7]. Thus, some of these strains may have produced different acid ratios because of these different reactions.

Strains of *P. acidipropionici* seemed to be the most acid-tolerant; 14 of the 17 selected strains were members of this species. These strains also produced higher amounts of propionic and acetic acids on all three carbon sources than did strains of *P. thoenii* and *P. jensenii*. Previous work in our laboratory found *P. acidipropionici* strains were the best acid producers on sodium lactate and lactose as well [2].

Among the strains studied, the ability to produce the most acid on a particular carbon source did not necessarily correlate with attaining the lowest final pH at the end of growth. Strain P9 and P86 reached the lowest pH with glucose as the carbon source (pH 4.17 and 4.18, respectively), but strain P200910, a propionate-tolerant variant of P9 developed in our laboratory [27], produced the most propionic and acetic acids (8.31 and 2.75 g l^{-1}, respectively). With fructose as the carbon source, P9 reached the lowest final pH (4.29), but P86 produced the most propionic acid (5.68 g l^{-1}), and P131 produced the most acetic acid (1.52 g l^{-1}). In maltose broth, strain P200910 reached the lowest pH (4.25) and produced the most propionic acid (5.31 g l^{-1}), but P127 produced the most acetic acid (1.78 g l^{-1}). The final pH achieved may depend on the culture's ability to conserve nitrogen-containing compounds in the medium that contribute to the buffering capacity [2]. Propionibacteria have been shown to metabolize several amino acids, especially aspartate, alanine, serine, and glycine [5, 6, 16]. However, these investigators observed that the specific activities of the enzymes involved in amino acid degradation (e.g. aspartase and alanine dehydrogenase) differed significantly among strains studied. In addition, some strains did not degrade amino acids.

Six strains (P9, P20, P126, P127, P155, and P200910) were selected for further study based on their low average final pH on all carbon sources, the amounts of acids produced, and other significant characteristics (i.e. P126 and P127 produce bacteriocins [10, 19] and P200910 is a propionate-tolerant variant of P9). The final pH was considered most important because low pH seemed to be the most inhibitory factor, i.e. no strains could grow on solid medium acidified

to pH 5.0. In addition, the original goal was to select a strain that would be useful as a silage inoculant and thus must be tolerant to high lactate and low pH. Each strain chosen reached an average pH <4.4.

In an effort to isolate pH-tolerant variants, strains were grown in NLB acidified to pH 5.0 with lactic acid. The cultures were incubated until visible turbidity appeared and then were transferred (1% inoculum) to another test tube containing pH 5.0 NLB. However, after 3 to 4 such transfers the strains were no longer able to grow. The strains were also streaked across pH gradient plates and incubated at 32°C in GasPak jars for 7 to 28 days. It was hoped that the most tolerant cells in the population would be selected and pushed to greater tolerance; however, the strains would not grow at pH values lower than pH 4.75 even after 28 days.

In another attempt to select the strain that could best tolerate low pH, the strains were inoculated as a mixed culture in a fermenter at pH 5.0. The intention was to start fermentation as a batch culture at pH 5.0 and then begin slow medium addition with gradual decrease in pH. However, the strains did not grow in batch culture after 48 hours, (i.e. no change in absorbance). The same results were obtained three times. Viable counts were essentially unchanged after 48 hours. Thus, it appears that the cells were able to survive even though they did not divide and grow. Previous researchers had observed that the pH optima of enzymes are narrow. This allows internal enzymes to be active only at a pH above a certain critical value with this threshold pH value depending on the organism [3, 13]. Our observation that there is a difference between the pH value at which cells could multiply and the value at which cells were killed may suggest that the biosynthetic

enzymes of the cells were more pH-sensitive than enzyme(s) essential for cell survival.

Minimum growth and lethal pH

To investigate further this disparity between lethal pH and pH for growth, the minimum growth pH was determined as the lowest pH at which the culture developed visible turbidity. The lethal pH, the highest pH at which significant loss of culture viability occurred, was determined as the pH value at which the cells could not be recovered at optimum conditions (i.e. pH 7.0). The minimum growth pH and lethal pH values were determined for each strain in broth acidified with lactic, propionic, or hydrochloric acid, inoculated with either 10^4 or 10^6 cells/ml (Figures 1 and 2).

There were significant differences among the abilities of the strains to grow and survive in each acid and at each inoculum level; no single strain had the lowest minimum growth pH and lethal pH values for all three acids at both inoculum levels (Figures 1 and 2). However, strain P127 could grow at the lowest pH values with all three acids at the higher inoculum level: pH 4.95 with lactic acid, pH 4.73 with hydrochloric acid, and pH 4.94 with propionic acid (Figure 1). Also, strain P200910 seemed to be the most sensitive to pH when inoculated at 10^4 cells/ml; it had the highest lethal and minimum growth pH values with all three acids (Figure 2).

When averaged over all strains, inoculum level had the most significant effect on minimum growth pH values of the cultures. On average when cultures were inoculated at 10^6 cells ml^{-1}, they grew at pH values that were 0.34 pH unit

lower than when inoculated at 10^4 cells ml^{-1} (Figure 1). In addition, cultures were able to survive at pH values that were 0.13 pH units lower when inoculated at 10^6 cells/ml versus 10^4 cells ml^{-1} (Figure 2). This difference was greatest for NLB acidified with propionic acid. However, the effect of inoculum level differed between the strains (Figure 3). Inoculum size had a greater effect for *P. acidipropionici* strains than for *P. jensenii* or *P. thoenii* strains. Previous researchers [2] found that inoculum size significantly influenced biomass and acid production as well. In addition, larger inocula have been found to increase the "adaptability" of the propionibacteria and result in a shorter lag phase and better growth [26].

The nature of the acid also had a significant effect on the minimum growth and lethal pH values. Cultures grown in broth with hydrochloric acid as the acidulant were able to grow at pH values that were 0.21 pH unit lower than for cultures grown in the presence of organic acids. Cultures could survive in broth acidified with hydrochloric acid to a value 0.34 pH unit below the lethal pH values of broth containing lactic acid or propionic acid as the acidulant. This is consistent with previous observations that organic acids are more inhibitory than are inorganic acids [9]. The antimicrobial action of organic acids has been attributed both to a generalized inhibition due to low pH and a specific inhibition due to the action of the anion, while inorganic acid inhibition is solely due to low pH [9].

Propionic acid was the most inhibitory of the three acids. The average minimum growth pH was 5.29 and the average lethal pH was 4.31 across all strains for this acid. In comparison, the average minimum growth pH values were 5.13 and 5.00 and the lethal pH values were 4.13 and 3.87 for lactic and

hydrochloric acids, respectively. Propionic acid has been found to be more effective than lactic acid in inhibiting salmonellae [4] and acid-tolerant yeasts that cause food spoilage [20].

Although the *P. acidipropionici* strains (P9, P155, and P200910) produced lower final pH values and on average more acid than the *P. thoenii* (P20 and P127) and *P. jensenii* (P126) strains in glucose, fructose, and maltose broth (Table 2), they did not initiate growth or survive at lower pH values (Figures 1 and 2). The average minimum growth pH values of the *P. acidipropionici* strains were 0.17 pH unit higher than those of the other strains. The ability to achieve a low final pH or produce large amounts of propionic acid may not be directly related to the ability to initiate growth at low pH or in the presence of propionic or other acids.

The difference between tolerance of the acid produced by a culture and the ability to initiate growth under similar conditions of acid concentration and low pH is illustrated further in Tables 2 and 3. When inoculated at 10^6 cells/ml into medium acidified with propionic acid, strains could not initiate growth below approximately pH 5.0, which corresponded to 25 mM propionic acid (Table 3). However, all of these strains reached final pH values <4.4, and produced propionic acid in concentrations ranging from 41 to 112 mM (Table 2). This may suggest that as a culture grows it adapts to its environment and thus can tolerate conditions in which it could not initiate growth. Late-log and stationary phase cells of *E. coli* O157:H7 were more acid-tolerant than mid-log cells [1]. In addition, stationary phase cells are more resistant to a variety of chemical and physical

challenges than are cells in other phases of growth. This is due to lower enzyme activity and thicker cell walls during stationary phase [16].

When the medium is acidified with an organic acid, inhibition may be due primarily to the concentration of the anion or to the hydrogen ion. To differentiate between these, all strains were grown in pH 7.0 NLB with increased lactate concentrations from 90 to 180 mM and propionate concentrations from 50 to 250 mM. All strains were able to grow in the presence of 180 mM lactate (data not shown); this was equivalent to the concentration of lactate used to adjust the medium to pH 4.0, at which growth was prevented. Thus, the strains were inhibited by the hydrogen ion concentration of the medium, not the lactate ion.

However, strains P20, P126, and P127 were more sensitive to the propionate anion than were the P. acidipropionici strains. Strains P20, P126, and P127 were inhibited by propionate concentrations greater than 150 mM, while strains P9, P155, and P200910 were able to grow in 250 mM propionate (data not shown). These results suggest that there is a significant difference between tolerance to lactate and propionate; this agrees with previous observations that propionic acid was more inhibitory to these strains than was lactic acid (Figures 1 and 2). The inhibition appears to be due to the specific action of the propionate anion, at least for strains P20, P126, and P127. Furthermore, the results confirm our conclusion that the ability to tolerate or produce high concentrations of propionate is not directly related to the ability to tolerate or initiate growth at low pH.

Effect of lactic acid on propionic and acetic acid yields

To determine if the lactic acid concentrations at acidic or neutral pH values affected the propionic and acetic acid yields (g acid produced per g substrate consumed), strains P9, P127, and P200910 were inoculated into broth containing increased lactic acid concentrations. The pH values were 7.0, 5.0 (the minimum growth pH for these strains), 4.75, and 4.25 (the lethal pH for these strains). The corresponding lactate concentrations were 55, 70, 90, and 120 mM, respectively.

As expected, none of the strains were able to grow at pH 4.75 or 4.25. Although the strains could survive at pH 4.75, they did not produce any measurable propionic or acetic acid. Thus, the ability to survive was not contingent on acid production. This suggests that these strains are able to remain viable even when they are not actively metabolizing.

Propionic acid yield decreased with increasing lactate concentrations for all strains (Table 4). At all lactate concentrations, strain P127 had the highest propionic acid yields while P200910 had the lowest yields. Low pH did not have a significant effect on propionic or acetic acid yields for P9 or P127. Previous research indicated that propionic acid yield of *P. acidipropionici* increased with decreasing pH but that acetic acid yields were not affected by pH during growth on lactose and glucose [12, 18]. The authors found that at low pH (pH 4.5-5.0) the culture produced less succinate and pyruvate; with less carbon diverted to other products, more propionic acid could be produced. In the current study we found no significant levels of succinate at any of the lactate concentrations studied, regardless of the pH value. This is consistent with previous observations of *P. acidipropionici* fermentation of lactate [18].

60

As previously observed for propionibacteria [18], the specific growth rates

(μ) were significantly slower at pH 5.0 than at neutral pH (Table 4). Low pH had

the most significant effect on P200910, which did not grow at pH 4.93. Strain

P200910 appeared to have a distinct pH threshold; it was able to grow at pH 5.08

(Figure 1) but did not grow in medium that was 0.15 pH unit lower (Table 4).

These results agree with the observation that biosynthetic enzymes have narrow

pH optima [3, 13].

Increased lactate concentrations at neutral pH did not have a significant

effect on the specific growth rates of the cultures; all cultures grew at similar

growth rates. This indicates that inhibition at low pH was not due to the lactate

anion, but to proton concentration.

The initial goal of this study was to identify potential acid- and low-pH-

tolerant variants which could be used as silage inocula or probiotic feed additives.

We proposed that strains that produced large amounts of acids and obtained a

low final pH would be the best strains to select. However, there appeared to be a

distinct difference between ability to tolerate low pH (hydrogen ions) versus high

concentrations of the acid anion. Thus, isolation and/or development of an acid-

and low-pH-tolerant variant seems unlikely; tolerance to low pH and acid anions

appeared to be two different mechanisms. Indeed, the propionate-tolerant variant

of P9, P200910, had been isolated after repeated exposure to increasing

propionate concentrations at neutral pH [27], but it showed the least tolerance to

low pH. Strains P20, P126, and P127 are potential strains for low-pH-tolerant

variants while strains P9 and P155 have potential as acid-tolerant variants which

could be used as more productive strains for industrial fermentations.

References

1.   Arnold, K.W., and C.W. Kaspar. 1995. Starvation- and stationary-phase-induced-acid tolerance in *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 61:2037-2039.

2.   Babuchowski, A., E.G. Hammond, and B.A. Glatz. 1993. Survey of propionibacteria for ability to produce propionic and acetic acids. J. Food Prot. 56:493-496, 527.

3.   Booth, I.R. 1985. Regulation of cytoplasmic pH in bacteria. Microbiol. Rev. 49:359-378.

4.   Cherrington, C.C., V. Allen, and M. Hinton. 1992. The influence of temperature and organic matter on the bactericidal activity of short-chain organic acids on salmonellas. J. Appl. Bacteriol. 72:500-503.

5.   Crow, V.L. 1986. Metabolism of aspartate by *Propionibacterium freudenreichii* subsp. *shermanii*: effect on lactate fermentation. Appl. Environ. Microbiol. 52:359-365.

6.   Crow, V.L. 1987. Citrate cycle intermediates in the metabolism of aspartate and lactate by *Propionibacterium freudenreichii* subsp. *shermanii*. Appl. Environ. Microbiol. 53:2600-2602.

7.   Crow, V.L. 1988. Polysaccharide production by propionibacteria during lactose fermentation. Appl. Environ. Microbiol. 54:1892-1895.

8.   Cummins, C.S. and J.L. Johnson. 1986. Genus 1. *Propionibacterium*., p.1346-1353 *In* P.H.A. Sneath, N.S. Mair and J.G. Holt (ed.), Bergey's manual of systemic bacteriology, vol 2. Williams and Wilkins, Baltimore.

9.   Dziezak, J.D. 1986. Preservatives: antimicrobial agents. Food Technol. 35(9):104-111.

10. Grinstead, D.A., and S.F. Barefoot. 1992. Jenseniin G, a heat-stable bacteriocin produced by *Propionibacterium jensenii* P126. Appl. Environ. Microbiol. 58:215-220.

11. Herrero, A.A. 1983. End-product inhibition in anaerobic fermentations. Trends Biotechnol. 1:49-53.

12. Hsu, S.T., and S.T. Yang. 1991. Propionic acid fermentation of lactose by *Propionibacterium acidipropionici*: effects of pH. Biotechnol. Bioeng. 38:571-578.

13. Huitson, J.J. 1968. Cereals preservation with propionic acid. Proc. Biochem. 3:31-32.

14. Kashket, E.R. 1987. Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. FEMS Microbiol. Rev. 46:233-244.

15. Koch, A.L. 1994. Growth measurement, p.248-277. In P. Gerhardt (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.

16. Kolter, R., D.A. Siegele, and A.Tormo. 1993. The stationary phase of the bacterial life cycle. Annu. Rev. Microbiol. 47:855-874.

17. Langsrud, T., T. Sorhaug, and G.E. Vegarud. 1995. Protein degradation and amino acid metabolism by propionibacteria. Lait 75:325-330.

18. Lewis, V.P., and S.T. Yang. 1992. Propionic acid fermentation by Propionibacterium acidipropionici: effect of growth substrate. Appl. Microbiol. Biotechnol. 37:437-442.

19. Lyon, W.J., and B.A. Glatz. 1991. Partial purification and characterization of a bacteriocin produced by Propionibacterium thoenii. Appl. Environ. Microbiol. 57:701-706.

20. Moon, N.J. 1983. Inhibition of the growth of acid-tolerant yeasts by acetate, lactate and propionate and their synergistic mixtures. J. Appl. Bacteriol. 55:551-556.

21. Playne, M.J. 1985. Propionic and butyric acids, p.731-759. In M. Moo-Young (ed.), Comprehensive biotechnology, vol 3. Pergamon Press, Oxford.

22. Rehberger, T.G., and B.A. Glatz. 1990. Characterization of Propionibacterium plasmids. Appl. Environ. Microbiol. 56:864-871.

23. SAS Institute, Inc, 1985. SAS© user's guide: statistics. SAS Inst. Inc., Raleigh, NC.

24. Steel, R.G.D., and J.H. Torrie. 1980. Principles and procedures of statistics: a biometrical approach. McGraw-Hill, Inc., New York.

25. Wood, H. 1981. Metabolic cycles in the fermentation by propionic acid bacteria, p.255-287 In R.W. Eastabrook and P. Srere (ed.), Current topics in cellular regulation, Vol. 18. Academic Press, New York.

26. Wood, H.G., E.L. Tatum, and W.H. Peterson. 1937. Growth factors for bacteria. IV. An acidic ether-soluble factor essential for growth of propionic acid bacteria. J. Bacteriol. 33:227-342.

27. Woskow, S.A., and B.A. Glatz. 1991. Propionic acid production by a propionic acid-tolerant strain of *Propionibacterium acidipropionici* in batch and semicontinuous fermentation. Appl. Environ. Microbiol. 57:2821-2828.

Table 1. Acid production and final pH achieved by cultures of propionibacteria grown on various substrates for 96 h. Values are the average of duplicate cultures with standard error values <0.12.

Species	Strain	Glucose pH	Glucose P.A.[a]	Glucose A.A.[b]	Fructose pH	Fructose P.A.[a]	Fructose A.A.[b]	Maltose pH	Maltose P.A.[a]	Maltose A.A.[b]
P. acidipropionici	P2	4.30	5.10	1.01	4.31	5.58	1.50	4.67	2.76	1.12
	P3	4.37	4.49	0.81	4.37	5.31	1.34	4.88	2.42	0.90
	P5	4.38	2.11	0.60	4.81	2.12	0.60	4.72	2.30	1.03
	P9	4.17	4.31	0.91	4.29	5.16	1.27	4.26	4.15	1.29
	P50	4.47	3.31	1.22	4.57	3.40	1.31	4.55	2.84	0.98
	P78	4.44	4.09	1.32	4.33	4.89	1.12	4.63	2.77	1.02
	P86	4.18	6.39	1.28	4.31	5.68	1.38	4.74	3.71	1.25
	P125	4.28	5.29	1.10	4.40	5.20	1.26	4.46	3.41	1.16
	P131	4.21	4.92	1.27	4.35	4.65	1.52	4.54	3.24	1.12
	P133	4.28	6.13	1.26	4.38	5.52	1.42	4.82	2.36	0.92
	P134	4.29	4.70	1.12	4.41	4.78	1.32	4.53	3.51	1.22
	P155	4.22	4.75	0.92	4.33	5.57	1.05	4.34	4.25	1.24
	P156	4.22	4.69	0.95	4.32	5.28	1.14	4.44	4.69	0.95
	P200910	4.21	8.31	2.75	4.35	5.27	1.14	4.25	5.31	1.50
P. thoenii	P20	4.21	5.47	1.37	4.40	5.02	1.36	4.46	3.06	1.17
	P127	4.26	3.82	0.95	4.35	4.46	1.30	4.37	4.57	1.78
P. jensenii	P126	4.26	3.79	0.93	4.41	4.01	1.09	4.39	3.93	1.56
	Average	4.28	4.80	1.16	4.39	4.82	1.24	4.53	3.49	1.19
	St. Dev.	0.09	1.36	0.46	0.12	0.92	0.23	0.19	0.89	0.24

[a] P.A. = propionic acid in g l^{-1}
[b] A.A. = acetic acid in g l^{-1}

Table 2: Final pH and concentration of propionic acid (mM) produced in broth culture with various carbon sources when inoculated at 10^6 cells ml^{-1}.

Strain	Glucose pH	Glucose P.A.[a]	Fructose pH	Fructose P.A.[a]	Maltose pH	Maltose P.A.[a]
P9	4.17	58	4.29	70	4.26	56
P20	4.21	74	4.40	68	4.46	41
P126	4.26	51	4.41	54	4.39	53
P127	4.26	52	4.35	60	4.37	62
P155	4.22	64	4.33	75	4.34	57
P200910	4.21	112	4.35	71	4.25	72

[a] P.A. = propionic acid in mM

Table 3: Minimum growth and lethal pH values and corresponding concentrations of propionic and lactic acids (mM) for cultures inoculated at 10^6 cells ml^{-1}.

Strain	Propionic acid Minimum pH	Propionic acid Minimum mM	Propionic acid Lethal pH	Propionic acid Lethal mM	Lactic acid Minimum pH	Lactic acid Minimum mM	Lactic acid Lethal pH	Lactic acid Lethal mM
P9	5.07	25	4.27	160	5.08	70	4.04	137
P20	5.07	25	4.15	229	4.95	73	3.92	145
P126	5.07	25	4.21	192	5.08	70	3.92	145
P127	4.94	31	4.26	165	4.95	73	4.10	135
P155	5.07	25	4.32	138	4.95	73	4.10	135
P200910	5.07	25	4.15	229	5.08	70	4.10	135

Table 4.   Yield (g acid produced/g lactate consumed) of propionic and acetic acids of cultures grown in media with various lactate concentration at different pH values. Values are the average of duplicate cultures with standard error values <0.15.

Strain	Lactate (mM)	Initial pH	$\mu^a(h^{-1})$	$Y_p{}^b$ (g/g)	$Y_a{}^c$ (g/g)	Lowest pH	Final pH
P9	55	6.88	0.15	0.55	0.31	5.58	5.78
	70	6.81	0.15	0.56	0.21	5.59	5.87
	70	4.94	0.044	0.51	0.26	4.90	5.27
	90	6.74	0.15	0.55	0.30	5.55	5.76
	120	6.45	0.15	0.52	0.27	5.48	5.77
P127	55	6.88	0.13	0.50	0.31	5.53	5.84
	70	6.82	0.12	0.51	0.31	5.54	5.88
	70	4.95	0.053	0.54	0.29	4.78	5.27
	90	6.74	0.12	0.51	0.26	5.50	5.78
	120	6.27	0.12	0.55	0.29	5.34	5.79
P200910	55	6.87	0.15	0.48	0.33	5.34	5.71
	70	6.80	0.11	0.50	0.34	5.29	5.71
	70	4.93	-0.011	--	--	--	--
	90	6.71	0.14	0.49	0.37	5.34	5.73
	120	6.50	0.13	0.50	0.43	5.28	5.69

[a] μ = specific growth rate
[b] Y_p = propionic acid yield
[c] Y_a = acetic acid yield

A: 10^4 cells/ml

B: 10^6 cells/ml
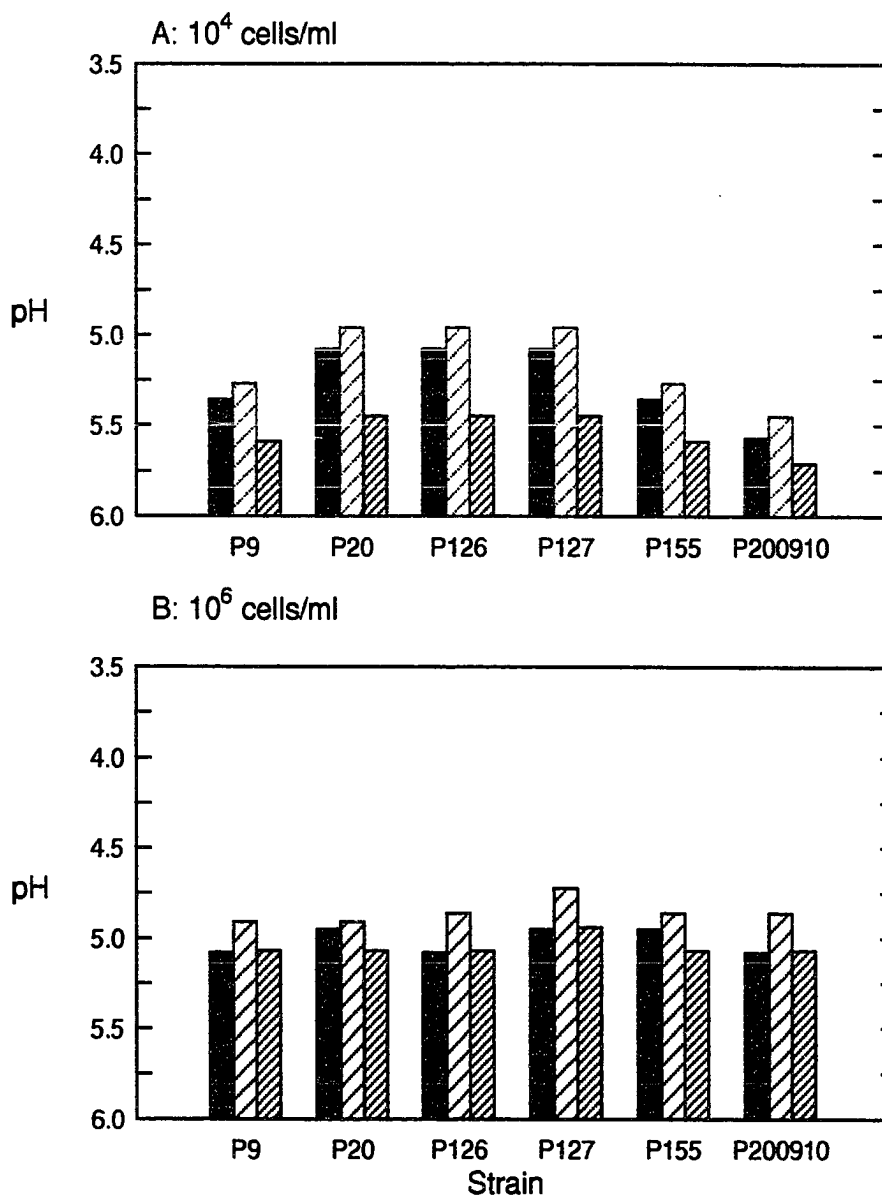
Figure 1: Comparison of minimum pH values at which growth was observed in broth acidified with lactic, hydrochloric, or propionic acid. Inoculum was 10^4 [A] or 10^6 [B] cells ml^{-1}. Inoculum level and nature of acidulant (inorganic vs organic) were found to be significant (P<0.0001). Symbols: � lactic acid; ▨ hydrochloric acid; ▨ propionic acid.
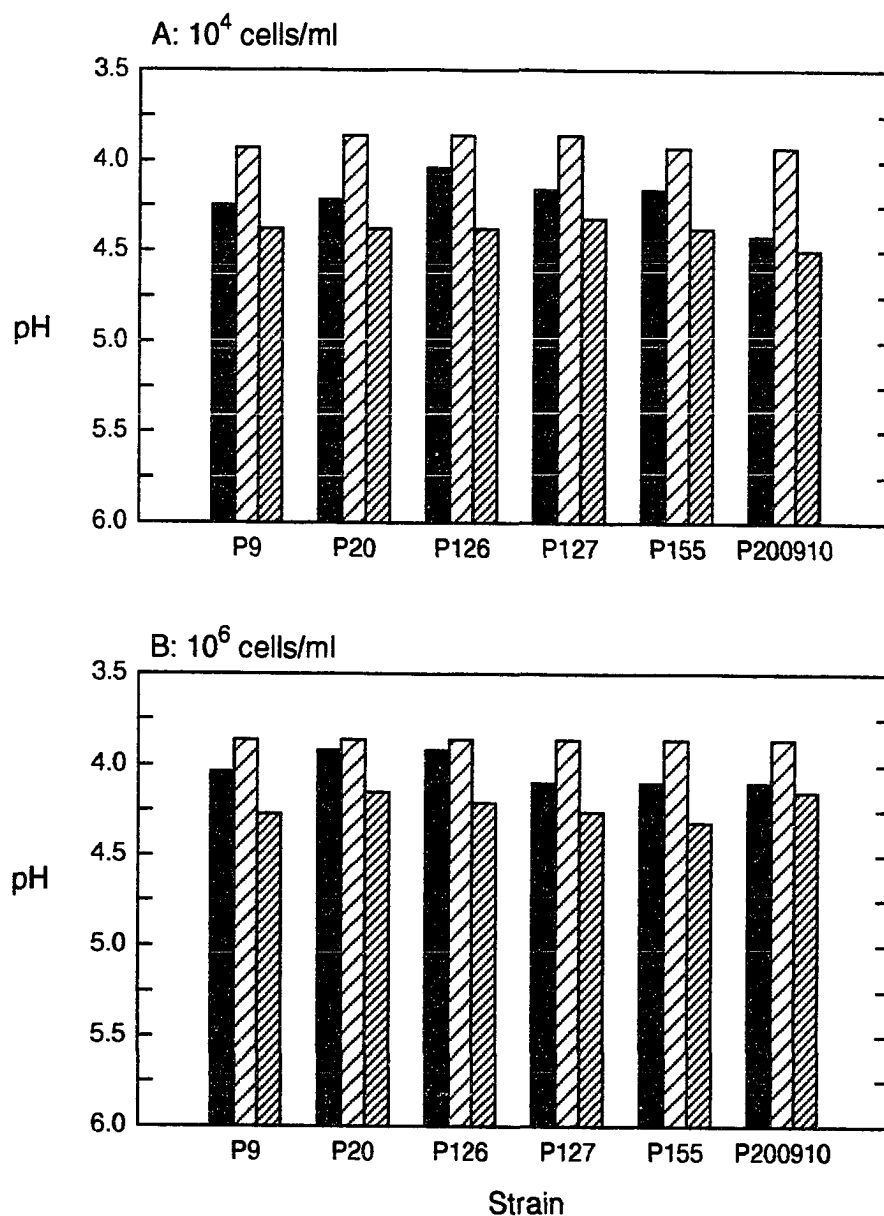
Figure 2: Comparison of lethal pH values in broth acidified with lactic, hydrochloric, or propionic acid. Inoculum was 10^4 [A] or 10^6 [B] cells ml^{-1}. Inoculum level and nature of acidulant (inorganic vs organic) were found to be significant ($P<0.0001$). Symbols: ▉ lactic acid; ▨ hydrochloric acid ▨ propionic acid.

Figure 3: Comparison of minimum growth and lethal pH values for strains of *Propionibacterium* inoculated at 10^4 cells ml^{-1} and 10^6 cells ml^{-1} in broth acidified with lactic, hydrochloric, or propionic acid. Symbols: □, lactic acid at 10^4 cells ml^{-1}; △, HCl at 10^4 cells ml^{-1}; O, propionic acid at 10^4 cells ml^{-1}; ■ lactic acid at 10^6 cells ml^{-1}; ▲, HCl at 10^6 cells ml^{-1}; and ●, propionic acid at 10^6 cells ml^{-1}.

Minimum Growth pH

Lethal pH
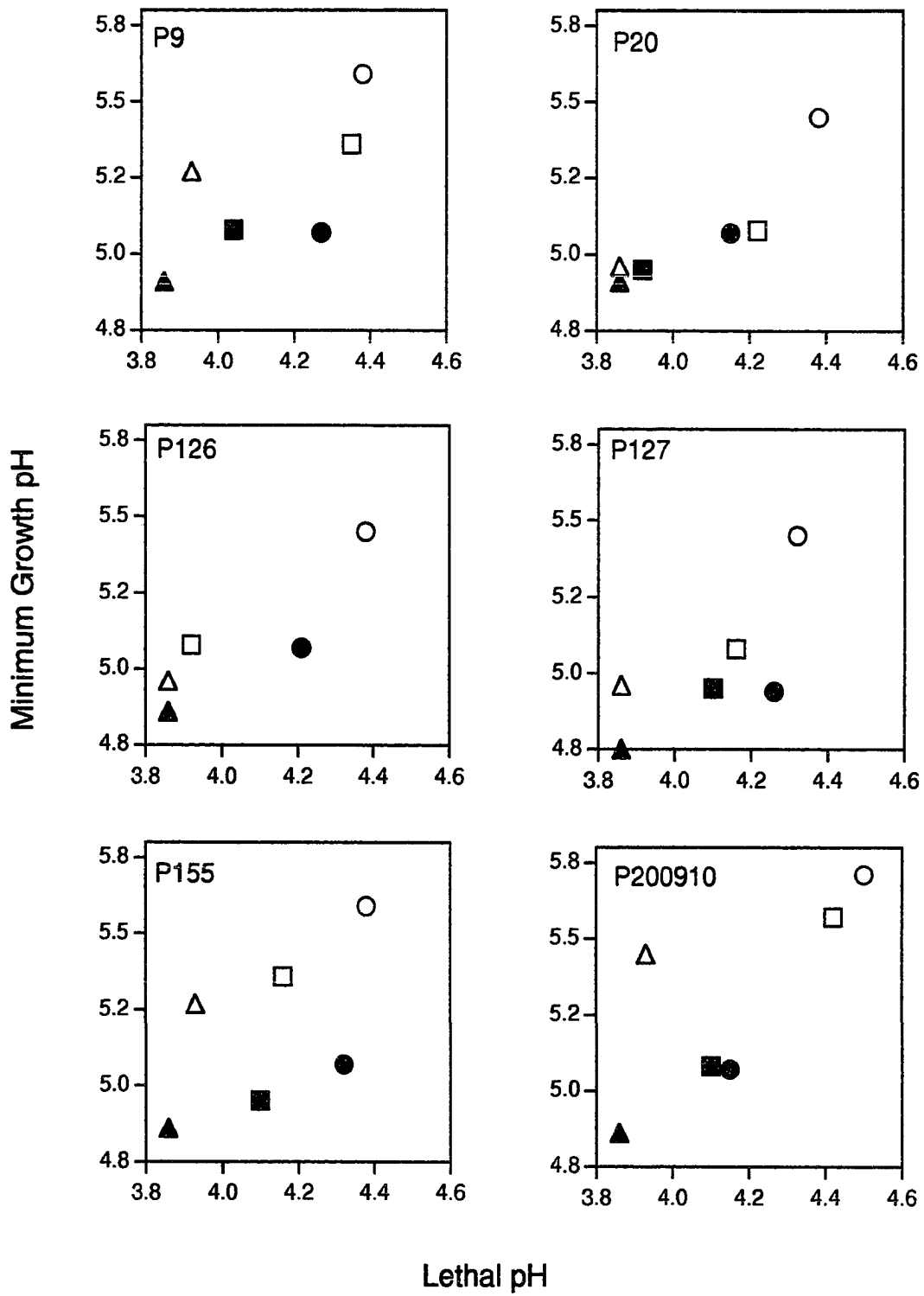
THE EFFECT OF LOW PH, ACID, AND GROWTH ON THE PROTON MOTIVE FORCE OF *PROPIONIBACTERIUM THOENII* P127 IN BATCH CULTURE

Jill L. Rehberger and Bonita A. Glatz

Abstract

The transmembrane electrical potential ($\Delta\Psi$) and pH gradient (ΔpH) of cultures of *P. thoenii* P127 were measured during batch growth started at pH 7 or pH 5 without pH control. The cells generated a Δp during growth, reaching a maximum of -169 mV when inoculated at pH 7 and -201 mV when inoculated at pH 5. The internal pH of cells of both cultures decreased as the external pH decreased. When grown at controlled pH, cells reached a maximum Δp of -173 mV at 144 h at pH 7, and a maximum Δp of -181 mV at 240 h at pH 5. The Δp at pH 7 was comprised of only $\Delta\Psi$, i.e. the internal pH remained constant at pH 7. At pH 5, however, the Δp consisted of both ΔpH and $\Delta\Psi$. The internal pH decreased slowly throughout the fermentation from pH 6.06 at 48 h until it reached pH 5.03 at 930 h, at which time the ΔpH collapsed; the critical internal pH threshold appeared to be pH 5.8. At pH 5 the specific growth rate (μ) was slower than at pH 7 (0.037 h^{-1} vs. 0.13 h^{-1}); ATP concentrations at mid-log phase were lower (2.05

mM vs. 3.28 mM); and propionic and acetic acid yields from lactate were lower

(0.58 and 0.23 vs. 0.60 and 0.28 for propionic and acetic acid, respectively). The

external pH did not affect the overall Δp values, but did affect the individual

components of Δp, i.e. $\Delta \Psi$ and ΔpH.

Introduction

Propionibacteria are fermentative organisms that produce propionic and

acetic acids from the metabolism of lactate. Although they are primarily used in

the dairy industry as starter cultures for Swiss-type cheeses (10), propionibacteria

are also used industrially as silage inoculum (6, 25), as a probiotic agent (18) and

for production of vitamin B_{12} and propionic acid (22). However, currently

commercial production of propionic acid is entirely by petrochemical routes.

Fermentation has not been used industrially because propionibacteria grow

slowly and produce organic acids in relatively low concentrations, and the costs of

separation and concentration of the acids make recovery difficult and expensive

(22). If higher concentrations of propionic acid could be obtained, the economics

of the fermentation could be improved.

A major factor that limits the production of propionic acid during

fermentation is end-product inhibition by the acid (9). Organic acids inhibit

microorganisms by entering the cell in the undissociated form and then

dissociating within the cell; this causes acidification of the cytoplasm and collapse

of the proton motive force (Δp) (9, 14, 21, 33). Some researchers believe that

organic acids act as "uncouplers" because they allow protons to enter the cell from the medium. The membrane-bound ATPase must function to counteract the futile cycle of protons through the membrane and thus the cell becomes deenergized (1, 2, 14, 19). Other researchers believe that inhibition is due to anion accumulation and that the intracellular release of protons by organic acids is overemphasized (4, 30, 31, 32, 33). Russell (4, 33) stated that the critical aspect of a particular bacterium's pH sensitivity may vary, but that organic acid toxicity was more closely related to intracellular pH regulation and anion accumulation than to "uncoupling" per se.

Little is known about the physiological response of propionibacteria to low pH. Hsu and Yang (11) found that the optimum pH for cell growth of P. acidipropionici was from pH 6.0 to 7.1 and that the growth rate decreased at acidic pH values. However, propionic acid yields were higher at low pH because the cells diverted less energy to production of succinate and pyruvate.

The objectives of this study were to determine effect of low pH, growth, and acid production on the proton motive force of P. thoenii P127. Previous attempts to isolate acid- and/or pH-tolerant variants of Propionibacterium led to the discovery that propionibacteria are very sensitive to low pH and cannot grow below pH 5.0 (26). However, they are tolerant to relatively high concentrations of lactic acid at neutral pH (up to 3.5%). To our knowledge, this is the first report of the measurement of the proton motive force of propionibacteria.

Materials and Methods

Organism and culture conditions

Propionibacterium thoenii P127 was obtained from the culture collection of the Department of Food Science and Human Nutrition at Iowa State University. Growth medium was sodium lactate broth (NLB) which contained 1% trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD), 1% yeast extract (Difco, Detroit, MI), 1% sodium lactate 60% syrup (Fisher Scientific Co., Pittsburgh, PA) and 0.6% Tween 80 (Fisher). For experiments conducted at lowered pH values, NLB was acidified to the desired pH with lactic acid. Inocula used in all experiments were grown in NLB incubated at 32°C for 24 h followed by at least two sequential transfers of 1% inocula into NLB.

Cells for transmission electron microscopic analysis were prepared by growing P127 in 250-ml screw-capped Erlenmyer flasks containing 100 ml of NLB at pH 7 or pH 5. The cultures were incubated at 32°C until mid-logarithmic growth (24 h for pH 7 and 72 h for pH 5). To avoid a low final pH because of acid production, the pH 7 culture was centrifuged (9,000 x g for 15 min at 4°C) and resuspended in fresh pH 7 NLB at 20 h. The final pH was 6.2.

Fermentation conditions

Batch fermentations without pH control were performed in a 2-L Erlenmeyer flask which contained 1.8 L NLB at pH 7 (55 mM lactate) or pH 5 (70

mM lactate). Samples of approximately 50 ml were taken every 12 to 24 h for analyses. To avoid large changes in head space that would result from sampling a single flask, six replicate fermentations were conducted and sampled at different times. Each sample time was duplicated; all fermentations were sampled at 24 h.

Batch fermentations with pH control were conducted in a 2-L bench-top fermenter (Biostat M, B. Braun Biotech. Inc., Allentown, PA) with a working volume of 1.8 L. The temperature was controlled at 32°C, the agitation was 150 rpm, and the pH was controlled at pH 7 or pH 5 +/- 0.1 pH unit with 3 N NaOH. Samples were taken at timed intervals for analyses. At 18, 24, and 36 h, 60-ml samples were taken from the fermenter and replaced with 60 ml of fresh NLB. At other sample times, smaller volumes (approximately 25 ml) were taken and were not replaced with fresh NLB.

Proton motive force determination

Estimation of the proton gradient (ΔpH) and the electrical potential ($\Delta\Psi$) was based on distribution of [^{14}C]salicylic acid and [^{3}H]tetraphenylphosphonium bromide (TPP^{+}), respectively (13, 21, 29). The accumulation of salicylate and TPP^{+} was determined by the silicon oil centrifugation technique derived from Rottenberg (29) and Tseng et al. (36). Briefly, the method was as follows.

Samples were centrifuged at 9000 x g for 15 min at 4°C and resuspended in the spent NLB to an absorbance of 1.0 at 600 nm. Aliquots of 1 ml were placed into microcentrifuge tubes and [^{14}C]salicylate (2.5 μM, 0.125 μCi; Dupont NEN, Boston, MA), [^{14}C]PEG (3.9 μM, 0.25 μCi; Amersham Corp., Arlington Heights, IL), [^{3}H]tetraphenlyphosphonium bromide (2.25 μM, 0.5 μCi; Amersham), or ^{3}H$_2$O

Γ

(1.25 μCi; Amersham) was added to duplicate tubes. After a 15-min incubation at room temperature, 0.5 ml of silicone fluid, density = 1.025 g ml^{-1} (50:50 mixture of Fluid 550 and Fluid 556; Dow Corning Corp., Midland, MI) was layered on top of each sample and the tubes were centrifuged at 25°C at 13,000 x g for 3 min. A 100-μl sample of supernatant was pipetted into a scintillation vial (Research Products International Corp., Mount Prospect, IL), 4 ml of 30% Scintiverse (Fisher) were added, and the vial was thoroughly mixed with a vortex mixer. The remaining supernatant was carefully removed with a pipet and cotton swab, and the oil was drained from the tubes. The bottom of the tube, containing the pellet, was cut and allowed to fall directly into a scintillation vial that contained 1 ml of 0.3N perchloric acid. The vial was thoroughly mixed by vortex to dissolve the pellet, 4 ml of 30% Scintiverse were added, and the vial was mixed again. Radioactivity was measured with a Packard Liquid Scintillation Analyzer Model 1900 TR (Packard, Meriden, CT) for 5 min in the DPM mode. Corrections for nonspecific binding of the probes were based on the extent of probe accumulation in cells treated with 20 μM valinomycin (Sigma, St. Louis, MO) and 5 μM nigericin (Sigma) for 60 min.

Calculations

The internal volume of the cells was calculated as outlined by Kashket (12) from the difference between the total (^{3}H$_2$O permeable) and extracellular (^{14}C-PEG-nonpermeable) volume of pellets. The internal volume of the cells in the pH 7 fermentations was calculated as 0.96 +/- 0.13 μl mg^{-1} dry weight; for pH 5 fermentations the internal volume was calculated as 0.51 +/- 0.068 μl mg^{-1} dry

weight. The calculated internal volumes were the averages of at least six different measurements determined at several different phases of culture growth. Although the volumes decreased slightly as the culture went into stationary phase, these differences were not significant and thus one value was used in the calculations of ΔpH and $\Delta\Psi$.

The ΔpH was calculated from the distribution ratio of [^{14}C]salicylic acid by using the Henderson-Hasselbalch equation (13, 21, 29):

$$\Delta pH = \log\frac{[\text{salicylic acid}]_{in}}{[\text{salicylic acid}]_{out}}$$

The [salicylic acid]$_{in}$ was determined from the DPM in the pellets divided by the cell volume, while the [salicylic acid]$_{out}$ was determined from the DPM in the supernatant (21, 29). The $\Delta\Psi$ was calculated in a similar manner (13, 21, 29):

$$\Delta\Psi = -Z\log\frac{[\text{TPP}^+]_{in}}{[\text{TPP}^+]_{out}}$$

Where $z = 59$ mV at 25°C. The proton motive force was calculated as (13, 21, 29):

$$\Delta p = \Delta\Psi - Z\Delta pH$$

In this paper, negative signs will be used for $\Delta\Psi$ (i.e. negative inside) and for Δp (negative and alkaline inside compared to the external medium). In discussions comparing the $\Delta\Psi$ and ΔpH, $Z\Delta$pH is used because multiplication by z converts the ΔpH to the same units (millivolts) as $\Delta\Psi$.

Transmission Electron Microscopy (TEM)

Cell preparation and electron microscopy were performed at the Bessey Microscopy Facility at Iowa State University. Mid-logarithmic cells were fixed for

1 h at room temperature in 3% glutaraldehyde (Electron Microscopy Sciences (EMS), Fort Washington, PA) and 2% paraformaldehyde (EMS) in phosphate buffer adjusted to either pH 7 or pH 5. Cells were washed three times in phosphate buffer adjusted to pH 7 or pH 5 and post fixed with 1% osmium tetroxide (EMS) for 30 min. The cells were washed and *en bloc* stained with 2% aqueous uranyl acetate overnight at 4°C, washed, and dehydrated in a graded ethanol series into pure acetone. The cells were embedded in epon resin (EMS) and thin sectioned (50 to 60 nm) on a Reichert Ultracut S ultramicrotome (Leica, Vienna, Austria). Thin sections were placed on copper grids and stained with 3% methanolic uranyl acetate Reynolds lead stain (27) and viewed on the JEOL 1200EXII transmission electron microscope (JOEL, Peabody, MA) at 30,000 x magnification.

Analysis of products

Lactate, propionate, and acetate were separated by a high-performance liquid chromatography (HPLC) system equipped with a model 501 pump (Waters, Division of Millipore, Milford, MA) and an HPX-87H column (BioRad, Richmond, CA) operated at 65°C with 0.012 N H_2SO_4 (pH 2.0) as the mobile phase at a flow rate of 0.8 ml min^{-1} and an injection volume of 20 µl. Peaks were detected with a Waters differential refractometer (model R401). Samples for analysis were centrifuged to remove the cells, filtered through 0.2 µm filters, and stored at -20°C before analysis. A Maxima 820 software program (Waters) was used to analyze the data and plot the chromatograms. Product concentrations were calculated by

comparing the peak areas with those of external standards (Aldrich Chemical Co., Milwaukee, WI).

For ATP determination, samples from the fermenter were prepared according to the method of Strobel and Russell (35). Samples consisted of cells resuspended in spent medium to an absorbance of 2.0 at 600 nm (4.36 mg dry weight ml^{-1}). The ATP was measured by the firefly luciferase method (17). The supernatant was diluted 100-fold with 40 mM Tris buffer (Fisher) that contained 2 mM EDTA (Fisher), 10 mM $MgCl_2$ (Fisher) and 0.1% bovine serum albumin (Sigma) (pH 7.75). The luciferase reaction was initiated by mixing 100 µl of the diluted sample with 100 µl of luciferine-luciferase assay mix according to the recommendations of the supplier (Sigma). Light intensity was measured with a Packard Model 1900 TR Liquid Scintillation Analyzer using ATP as the standard (20).

An analysis of variance was performed using the General Linear Model of SAS (34). Differences with $P < 0.01$ were considered significant.

Results and Discussion

Fermentations without pH control

No information concerning the proton motive force and internal pH was previously available for propionibacteria and it was not known how these parameters would respond to growth and acidic pH. Previous studies determined that *P. thoenii* P127 was sensitive to low pH and was not able to grow below pH 5

(26). Measuring the cellular response at pH 7 and pH 5 may indicate the critical aspect(s) of low pH inhibition and tolerance. To determine how culture growth and acidification of the medium affected the cellular proton motive force and internal pH of this strain, P127 was grown at starting pH values of pH 7 and pH 5 in batch culture without pH control (Figure 1). The Δp of the culture inoculated at pH 7 increased throughout exponential growth from -118 mV at 12 h to -134 mV at 36 h and reached a maximum at 48 h of -169 mV. The Δp then decreased to a minimum of -53 mV at 192 h. For cultures inoculated at pH 5, the Δp increased from -99 mV at 48 h to the maximum value of -201 at 144 h. There was a decrease in Δp at 192 h to -137 mV.

As previously observed in bacteria, the relative contribution of $\Delta\Psi$ and ΔpH to Δp are dependent on the pH of the medium (2, 12). Throughout the fermentation, the Δp of the culture inoculated at pH 7 consisted of a large $\Delta\Psi$ and a small $z\Delta$pH. While for the cultures inoculated at pH 5, the $z\Delta$pH was the major component of the Δp for the first 72 h. After this time, the $\Delta\Psi$ was the major component. The $z\Delta$pH of the pH 5 culture was larger than that of the pH 7 culture at all sample times. This resulted in a larger Δp for the culture inoculated at pH 5.

During logarithmic growth, the internal pH (external pH + ΔpH) of the pH 7 culture without pH control decreased as the external pH decreased, until the internal pH reached approximately 6.4, (external pH was approximately 5.5) (Figure 2). The internal pH then remained relatively constant until the ΔpH collapsed at 192 h. The culture started at pH 5 maintained a relatively constant internal pH of approximately 6.0 throughout the growth phase while the external pH decreased only slightly to 4.8. The ΔpH began to decrease at 192 h, which

resulted in an internal pH of 5.8. The decrease in internal pH with the external pH is consistent with observations made with other fermentative microorganisms including *Clostridium thermoaceticum* (1), *Streptococcus cremoris* (3), *Sarcina ventriculi* (8), and various lactic acid bacteria (4, 14, 19).

Fermentations with pH control

To measure the response of P127 to acid production at neutral and low pH, the strain was grown in batch culture at pH 7 and 5 with pH control (Figures 3 and 4). As previously observed in propionibacteria (11, 16), the specific growth rate was significantly slower at the lower pH; μ was 0.13 h^{-1} at pH 7 and 0.037 h^{-1} at pH 5. However, the pH 5 cultures grew to a higher total cell count: 2.1 x 10^9 cfu ml^{-1} versus 9.1 x 10^8 cfu ml^{-1} at pH 7.

At pH 7, acid production followed a growth-associated pattern. Lactate was depleted by 72 h; propionic acid concentration was highest (6.17 g l^{-1}) at 192 h while acetic acid was highest (3.07 g l^{-1}) at 144 h. Acid production at pH 5 was significantly slower; propionic and acetic acid concentrations were highest at 666 h (6.21 g l^{-1} and 3.36 g l^{-1}, respectively) while the culture was in death phase. Lactate consumption rate decreased at 240 h; lactate was not depleted until 552 h. This is consistent with previous observations that when *P. acidipropionici* was grown at pH 5, acid production and lactose consumption continued after cells had entered stationary phase (11). Propionic and acetic acid yields (gram acid produced per gram lactate consumed) were slightly higher at pH 7: 0.60 and 0.28, respectively and 0.58 and 0.23 for pH 5, respectively.

The ATP concentration in the pH 7 culture decreased with lactate consumption and propionic and acetic acid production during logarithmic growth, but increased at the beginning of stationary phase (from 1.43 mM at 72 h to 2.97 mM at 96 h). The ATP concentration remained elevated through 144 h and then decreased dramatically at 240 h to 0.46 mM. This transient increase occurred in all three pH 7 fermentations conducted and may have been due to metabolism of amino acids or other growth factors in the medium. Strains of propionibacteria have been found to utilize amino acids, especially alanine, aspartate, glycine, and serine (5, 15). Alanine was not metabolized until after lactate had been depleted (5). This shift in metabolism may have supplied additional ATP to the cells.

At pH 5, the ATP concentration decreased along with lactate consumption and propionic and acetic acid production from 2.05 mM at 72 h to 0.38 mM at 552 h, and increased only slightly at 168 h. For both pH 7 and 5 fermentations, the cells entered death phase when ATP levels were below approximately 0.5 mM. Previous researchers found that in the presence of propionic and lactic acids, *L. helveticus* increased its H^+-ATPase activity (24). This enzyme utilized glycolytically generated ATP to expel protons accumulated by the cell as a result of lactic acid production and/or exposure to propionic acid. In addition, growth rate and biomass production decreased when metabolism could not supply ATP required for proton extrusion (23).

During log-phase growth at pH 7, the proton motive force potential increased from -87 mV at 18 h to -137 mV at 36 h and reached a maximum at the beginning of stationary phase (144 h) of -173 mV; after this time the Δp began to be depleted.

However, with extended incubation the Δp began to increase again, from -119 mV at 480 h to -158 mV at 1500 h. There was also an increase in acetic acid concentration and in viable counts, from 3.04 g l^{-1} and 2.9 x 10^7 cfu ml^{-1} at 480 h to 3.43 g l^{-1} and 8.1 x 10^7 cfu ml^{-1} at 1500 h. These data may suggest that the culture had undergone some metabolic change which may have induced the production of new enzymes. An SDS-PAGE of intracellular proteins obtained from cells at 1500 h and from logarithmically growing cells contained an additional band in cells at 1500 h at approximately 25 kDa (Appendix B). The culture may have synthesized enzymes necessary for the utilization of the amino acids remaining in the medium. The protein might also correspond to a protein synthesized during stationary phase.

At pH 5, the proton motive force increased from -67 mV at 48 h to -113 mV at 120 h; at the beginning of stationary phase (240 h) it reached a maximum of -181 mV. Throughout stationary phase, the Δp steadily decreased to a minimum at 810 h (-40 mV) and was collapsed at 930 h.

Throughout the entire pH 7 batch fermentation, the Δp consisted entirely of the $\Delta \Psi$, i.e. there was no measurable pH gradient. The internal pH remained constant at approximately 7.0. Others have reported that *Streptococcus cremoris* also had no measurable ΔpH when grown in batch culture at pH 7; the ΔpH did not develop until the external pH decreased with acid production (12).

In contrast, for the first 96 h of the pH 5 fermentation the Δp was comprised of only the $z\Delta pH$ component, i.e. there was no measurable $\Delta \Psi$. At all sample times, the $\Delta \Psi$ of the pH 5 culture was lower than that of the pH 7 culture. At mid log-phase, the $\Delta \Psi$ of the pH 7 culture was -132 mV, while at pH 5 the $\Delta \Psi$ was

zero. In contrast, the $z\Delta pH$ of the pH 5 culture was -63 mV and zero at pH 5. These results are consistent with previous observations that the activity of proton pumps is constrained by the generation of the proton motive force; to generate a pH gradient, excessive membrane potential must be dissipated (2).

The internal pH of the pH 5 culture remained relatively constant at the beginning of the fermentation (6.06 to 5.93), but began to decrease gradually after 96 h (Table 1). The ability of a culture to tolerate acidic conditions has been attributed to its ability to lower its internal pH with the pH of the medium in the presence of weak organic acids (4, 30, 31, 32, 33). Weak organic acids will diffuse across the membrane in accordance with the pH gradient and dissociate at the more alkaline internal pH. The impermeable anion will accumulate based on the Henderson-Hasselbalch equation (33). During fermentation, P127 decreased its internal pH with acid production and thus the propionic and acetic acid gradient across the membrane decreased (Table 2). If P127 had maintained a constant internal pH, the gradient would have remained the same and would have resulted in higher internal concentrations of propionic and acetic acids.

The ΔpH decreased from 0.80 to 0.68 pH unit from 336 to 456 h, which resulted in a decrease in internal pH from 5.8 to 5.6. At the same time the cells entered death phase and the rate of lactate consumption decreased, even though the lactate had not been depleted. This may suggest that an internal pH of 5.8 may be the critical pH threshold for strain P127. Most internal enzymes have a narrow range in which they are active (2, 14); pH 5.8 may be the lower limit for enzymes in this strain. Similar values were measured for *Leuconostoc mesenteroides* (19). McDonald *et al.* (19) demonstrated that growth of

Leuconostoc mesenteroides terminated when its internal pH reached 5.4 to 5.7 at an external pH of 4.0.

Although the external propionic and acetic acid concentrations were similar for both pH 7 and pH 5 fermentations, at pH 7 the acids were in the dissociated form and thus could not penetrate the cell membrane. In contrast, at pH 5 about half of the total amount of the acids was in the undissociated form (Table 2). This form could re-enter the cell and acidify the cytoplasm (4, 14, 19, 33). The lowering of the internal pH after 96 h may result from the increase in undissociated acetic and propionic acid concentrations.

The ATP concentrations for the pH 5 culture were significantly lower than for the pH 7 culture; this may be due to the reverse action of the H^+-ATPase which consumes ATP to expel protons in order to maintain an alkaline internal pH with respect to the medium (1, 2, 21). We had expected the pH 5 culture to reach lower total cell numbers than the pH 7 culture because less energy was available at the lower pH for biosynthesis of macromolecules and cell division. The culture at pH 5 also might have rapidly consumed the lactate in order to keep up with the demand for ATP. Neither of these anticipated results was observed, however. Results similar to ours were observed with *L. lactis* by Cook and Russell (4). They found that *L. lactis* decreased its intracellular pH, glycolytic rate, and intracellular ATP concentration when the external pH became acidic, and concluded that energy availability for growth was a key factor in the pH sensitivity of this organism. Thus, it appears that energy availability may also play an important role in pH sensitivity of P127.

During the pH 5 fermentation, a base pump malfunction caused the pH of the medium to increase to pH 5.23 at 168 h. The ΔpH measured at this time was significantly lower (0.34 pH unit) than the ΔpH measured at the previous (120 h) and subsequent (240 h) sample times (0.84 and 0.80 pH unit, respectively) (Table 1). The decrease in ΔpH at 168 h indicated both a lower internal pH (5.57) and a lower acid gradient across the membrane (1.8 for propionic acid and 1.9 for acetic acid). The lower acid gradient would result in less acid transversing the membrane and accumulating inside. There was also an increase in ATP from 0.88 mM at 120 h to 1.18 mM at 168 h (Figure 4). This transient increase may have occurred because there was a lower demand for ATP due to less accumulation of protons with incoming acid.

Effect of acidic pH on cell wall and membrane

To determine the effect of growth at low pH on the cell wall and membrane of strain P127, cells grown at pH 7 and pH 5 were examined by transmission electron microscopy. The electron micrographs show that cells grown at pH 7 (Figure 5A and 5B) had a more uniformly dense cell wall than did cells grown at pH 5 (Figure 5C and 5D). The walls of cells grown at pH 5 appeared to be thicker than were those of cells grown at pH 7. They also seemed to lack definitive structure (i.e. no dark band at the outer and inner edge of the cell wall and no indication of the bilayer of the membrane).

These differences might be due to changes occurring at pH 5 in the murein structure, fatty acid composition of the membranes, composition or amount of extrapolysaccharides produced, or possibly in uptake of the microscopy stains

(uranyl acetate and lead citrate). Teichoic acids have been found to play a role in the expansion and contraction of cell walls that occurred with alterations in pH and ionic strength (28). There may also have been a difference in the amount or composition of the teichoic acids found in the cell walls at the different pH values.

Comparison of $\Delta\Psi$ and Δp values

To determine whether it was the actual value of the external pH or the presence or absence of pH control that contributed the most to the variance of Δp and $\Delta\Psi$ values, a comparison was made of these values during growth phase only of cultures grown with and without pH control. Because cultures grew more slowly at pH 5 than at pH 7, absorbances at different sample times were compared to the highest absorbance attained by that culture. The Δp and $\Delta\Psi$ values were plotted against these standardized absorbance values. A linear regression was done separately for each pH and treatment (with and without pH control) using the General Linear Model of SAS (34). The statistically predicted lines are shown in Figure 6. The Δp values differed significantly between cultures with and without pH control, but did not significantly differ between pH 7 and pH 5. At mid-growth, the average Δp for cultures without pH control was 31% larger than the Δp of the pH-controlled cultures. This difference was due to larger $Z\Delta pH$ values in both pH 7 and 5 fermentations without pH control.

In contrast, the $\Delta\Psi$ values differed significantly between pH 7 and pH 5, but did not differ between the cultures with and without pH control. At mid-growth, the average $\Delta\Psi$ for both cultures inoculated at pH 7 was 52% higher than the average

$\Delta\Psi$ for both pH 5 cultures. These results suggest that pH did not affect the overall Δp values, but did affect the individual components, i.e. $\Delta\Psi$ and ΔpH.

The Δp and $\Delta\Psi$ of the cultures with the same initial pH responded to growth in the same manner, i.e. showed the same change in potential (Δp or $\Delta\Psi$) with change in growth. This response (shown by the slopes of the lines) differed between cultures inoculated at pH 7 and pH 5, although this difference only comprised 8% of the total variance of the $\Delta\Psi$ and Δp values.

It appeared that the Δp and $\Delta\Psi$ were generated during growth at approximately the same rate in pH 7 and pH 5 cultures. Although pH significantly affected the specific growth rates and ATP concentrations of strain P127, pH did not affect the generation and magnitude of the Δp. However, pH affected the components that comprised the total Δp; pH 5 cultures had larger ΔpH and smaller $\Delta\Psi$ components than did cultures at pH 7.

Acknowledgments

89

References

1. **Baronofsky, J.J., W.J.A. Schreurs, and E.R. Kashket.** 1984. Uncoupling by acetic acid limits growth of and acetogenesis by *Clostridium thermoaceticum.* Appl. Environ. Microbiol. **48**:1134-1138.

2. **Booth, I.R.** 1985. Regulation of cytoplasmic pH in bacteria. Microbiol. Rev. **49**:359-378.

3. **Brink, B.T., and W.N. Konings.** 1982. Electrochemical proton gradient and lactate concentration gradient in *Streptococcus cremoris* cells grown in batch culture. J. Bacteriol. **152**:682-686.

4. **Cook, G.M., and J.B. Russell.** 1994. The effect of extracellular pH and lactic acid on pH homeostasis in *Lactococcus lactis* and *Streptococcus bovis.* Curr. Microbiol. **28**:165-168.

5. **Crow, V.L.** 1987. Properties of alanine dehydrogenase and aspartase from *Propionibacterium freudenreichii* subsp. *shermanii.* Appl. Environ. Microbiol. **53**: 1885-1892.

6. **Flores-Galarza, R.A., B.A. Glatz, C.J. Bern and L.D. Van Fossen.** 1985. Preservation of high moisture corn by microbial fermentation. J. Food Prot. **48**:407-411.

7. **Gatje, G., and G. Gottschalk.** 1991. Limitation of growth and lactic acid production in batch and continuous cultures of *Lactobacillus helveticus.* Appl. Microbiol. Biotechnol. **34**:446-449.

8. **Goodwin, S., and J.G. Zeikus.** 1987. Physiological adaptation of anaerobic bacteria to low pH: metabolic control of proton motive force in *Sarcina ventriculi.* J. Bacteriol. **169**:2150-2157.

9. **Herrero, A.A.** 1983. End-product inhibition in anaerobic fermentations. Trends Biotech. **1**:49-53.

10. **Hettinga, D.H., and G.W. Reinbold.** 1972. The propionic-acid bacteria. A review. II. Metabolism. J. Milk Food Technol. **35**:358-372.

11. **Hsu, S.T., and S.T. Yang.** 1991. Propionic acid fermentation of lactose by *Propionibacterium acidipropionici*: effects of pH. Biotechnol. Bioeng. **38**:571-578.

12. **Kashket, E.R., A.G. Blanchard, and W.C. Metzger.** 1980. Proton motive force during growth of *Streptococcus lactis* cells. J. Bacteriol. **143**:128-134.

13. **Kashket, E.R.** 1985. The proton motive force in bacteria: a critical assessment of methods. Ann. Rev. Microbiol. **39**:219-242.

14. **Kashket, E.R.** 1987. Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. FEMS Microbiol. Rev. **46**:233-244.

15. **Langsrud, T., T. Sorhaug, and G.E. Vegarud.** 1995. Protein degradation and amino acid metabolism by propionibacteria. Lait **75**:325-330.

16. **Lewis, V.P., and S.T. Yang. 1992.** Propionic acid fermentation by *Propionibacterium acidipropionici*: effect of growth substrate. Appl. Environ. Microbiol. **37**:437-442.

17. **Lundin, A., and A. Thore.** 1975. Comparison of methods for extraction of bacterial adenine nucleotides determined by firefly assay. Appl. Environ. Microbiol. **30**:713-721.

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

19. **McDonald, L.C., H.P. Fleming, and M.H. Hassan.** 1990. Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*. Appl. Environ. Microbiol. **56**:2120-2124.

20. **Nguyen, V.T., M. Morange, and O. Bensaude.** 1988. Firefly luciferase luminescence assays using scintillation counters for quantitation in transfected animal cells. Anal. Chem. **171**:404-408.

21. **Nicholls, D.G., and S.J. Ferguson.** 1992. Bioenergetics 2. Academic Press Inc., San Diego.

22. **Playne, M.J.** 1985. Propionic and butyric acids, p.731-759. *In* M. Moo-Young (ed.), Comprehensive biotechnology, vol. 3. Pergamon Press, Oxford.

23. **Perez-Chaia, A., A.M.S. de Saad, A.A.P. de Reuiz Holgado, and G. Oliver.** 1994. Influence of propionate on growth and fermentative activity of lactobacilli. J. Appl. Bacteriol. **77**:134-139.

24. **Perez-Chaia, A., A.M.S. de Saad, A.A.P. de Reulz Holgado, and G. Oliver.** 1995. Proton-ATPase activity in cells of lactobacilli grown in the presence of propionate. J. Appl. Bacteriol. **77**: 37-41.

F

25. **Raeker, M.O., C.J. Bern, L.A. Johnson, and B.A. Glatz.** 1992. Preservation of high-moisture maize by various propionate treatments. Cereal Chem. **69**:66-69.

26. **Rehberger, J.L.** 1996. Response of *Propionibacterium* to acid and low pH: tolerance and inhibition. Ph.D. dissertation, Iowa State University, Ames, IA.

27. **Reynolds, E.S.** 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17**:208-212.

28. **Rogers, H.J.** 1983. Bacterial cell structure. Van Nostrand Reinhold (UK) Co. Ltd., Berkshire, England.

29. **Rottenberg, H.** 1979. Measurement of membrane potential and ΔpH in cells, organelles, and vesicles. Meth. Enzymol. **55**:547-569.

30. **Russell, J.B. 1987.** Effect of extracellular pH on growth and proton motive force of *Bacteroides succinogenes*, a cellulolytic ruminal bacterium. Appl. Environ. Microbiol. **53**:2379-2383.

31. **Russell J.B.** 1991. Resistance of *Streptococcus bovis* to acetic acid at low pH: relationship between intracellular pH and anion accumulation. Appl. Environ. Microbiol. **57**:255-259.

32. **Russell, J.B.** 1991. Intracellular pH of acid-tolerant ruminal bacteria. Appl. Environ. Microbiol. **57**:3383-3384.

33. **Russell, J.B.** 1992. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. J. Appl. Bacteriol. **73**:363-370.

34. **SAS Institute, Inc.** 1985. SAS© user's guide: statistics. SAS Inst. Inc., Raleigh, NC.

35. **Strobel, H.J., and J.B. Russell.** 1989. Non-proton-motive-force-dependent sodium efflux from the ruminal bacterium *Streptococcus bovis*: Bound versus free pools. Appl. Environ. Microbiol. **55**:2664-2668.

36. **Tseng, C.P., J.L. Tsau, and T.J. Montville.** 1991. Bioenergetic consequences of catabolic shifts by *Lactobacillus plantarum* in response to shifts in environmental oxygen and pH in chemostat cultures. J. Bacteriol. **173**:4411-4416.

Table 1: pH and acid gradients of strain P127 at controlled pH 5.0.

Time (h)	pH out[a]	ΔpH	pH in[b]
48	4.92	1.14	6.06
72	4.83	1.08	5.91
96	4.96	0.97	5.93
120	5.06	0.80	5.86
168	5.23	0.34	5.57
240	4.90	0.84	5.74
336	4.99	0.80	5.79
456	4.93	0.68	5.61
552	4.98	0.60	5.58
666	4.90	0.36	5.26
810	4.90	0.14	5.04
930	5.03	0	5.03

[a] external pH
[b] internal pH (ΔpH + external pH)

Table 2: Concentration of dissociated and undissociated propionic and acetic acid and their gradients across the cell membrane in batch fermentation controlled at pH 5.

Time (h)	pH	Propionic acid (mM)				Acetic acid (mM)			
		Total [a]	A- [b]	HA [b]	In/out [c]	Total [a]	A- [b]	HA [b]	In/out [c]
48	4.92	24	13	11	7.8	0	0	0	0
72	4.83	33	16	17	6.3	0	0	0	0
96	4.96	39	22	17	5.6	0	0	0	0
120	5.06	49	30	19	4.2	31	21	10	4.5
168	5.23	66	46	20	1.8	42	31	11	1.9
240	4.90	72	37	35	4.1	45	26	19	4.4
336	4.99	75	43	32	4.0	49	30	19	4.3
456	4.93	79	42	37	3.0	53	31	22	3.2
552	4.98	83	47	36	2.7	57	34	23	2.9
666	4.90	84	43	41	1.7	59	33	26	1.8
810	4.90	80	41	39	1.2	56	31	25	1.2
930	5.03	79	47	32	1.0	58	37	21	1.0

[a] Total acid concentration as measured by HPLC
[b] A- = undissociated, HA = undissociated, both calculated from the Henderson-Hasselbalch equation, pK_a propionic acid 4.87, pK_a acetic acid 4.76
[c] Total acid gradient across the membrane calculated from the Henderson-Hasselbalch equation and the internal and external pH values.

Figure 1: Growth and the components of the proton motive force for cultures of *P. thoenii* P127 inoculated at pH 7 (a) and pH 5 (b) without pH control. Symbols: ▼, absorbance; ●, Δp; ▢, $\Delta \Psi$; and ◆, $Z\Delta pH$.
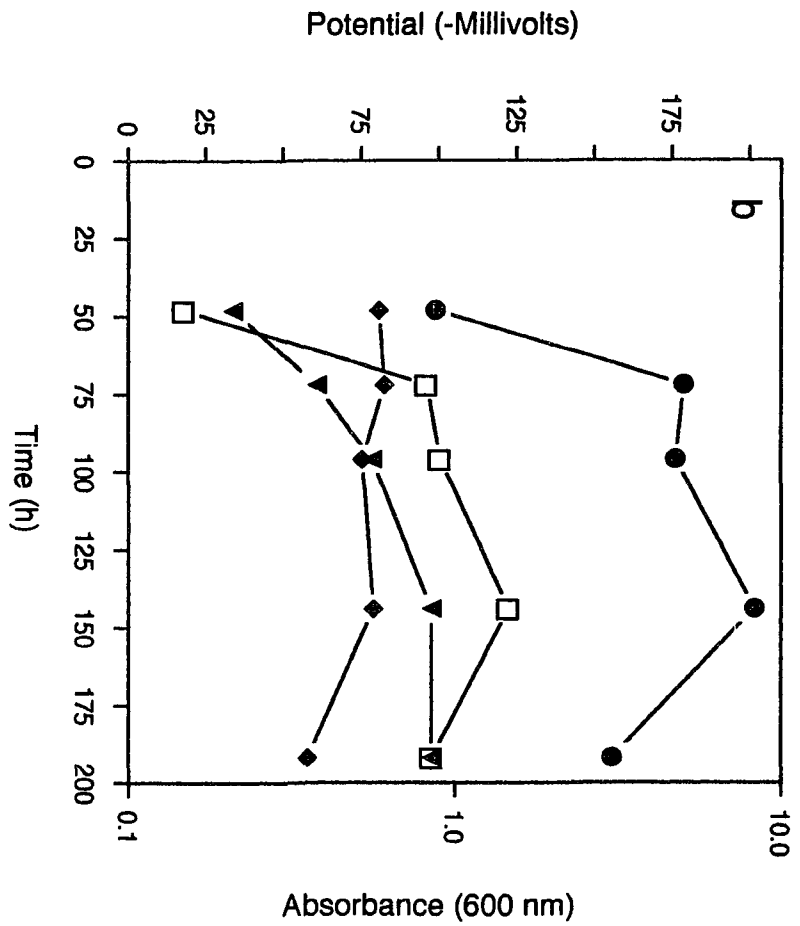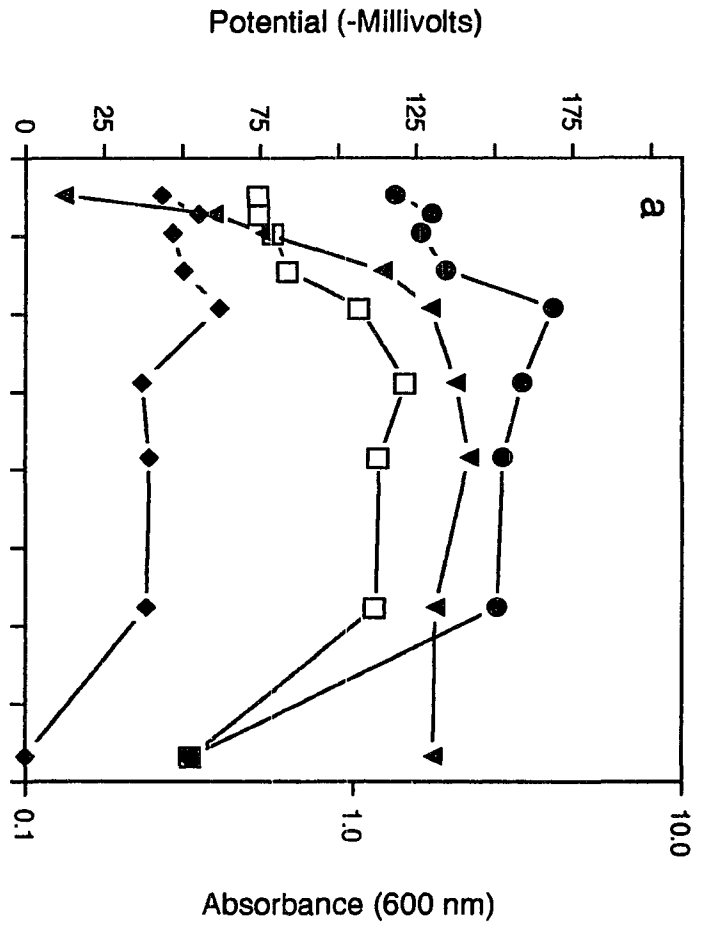
Figure 2: Internal and external pH values for P127 cultures inoculated at pH 7 and pH 5 without pH control. Symbols: O, external pH at pH 7; □, internal pH at pH 7; ●, external pH at pH 5; and ■, internal pH at pH 5.
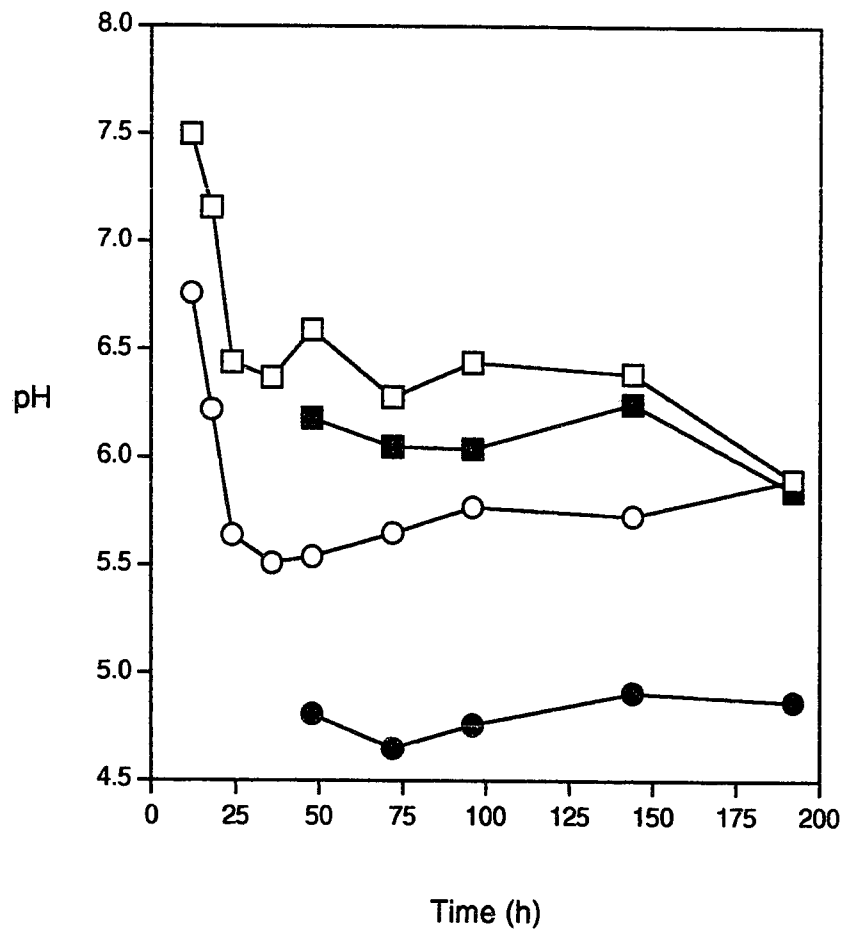
Figure 3: Growth, product formation, and culture characteristics of strain P127 at controlled pH 7.0. Symbols: ▼, absorbance; ✳, viable counts; +, ATP; ▲, lactic acid; O, propionic acid; △, acetic acid; and ●, Δp or ΔΨ.
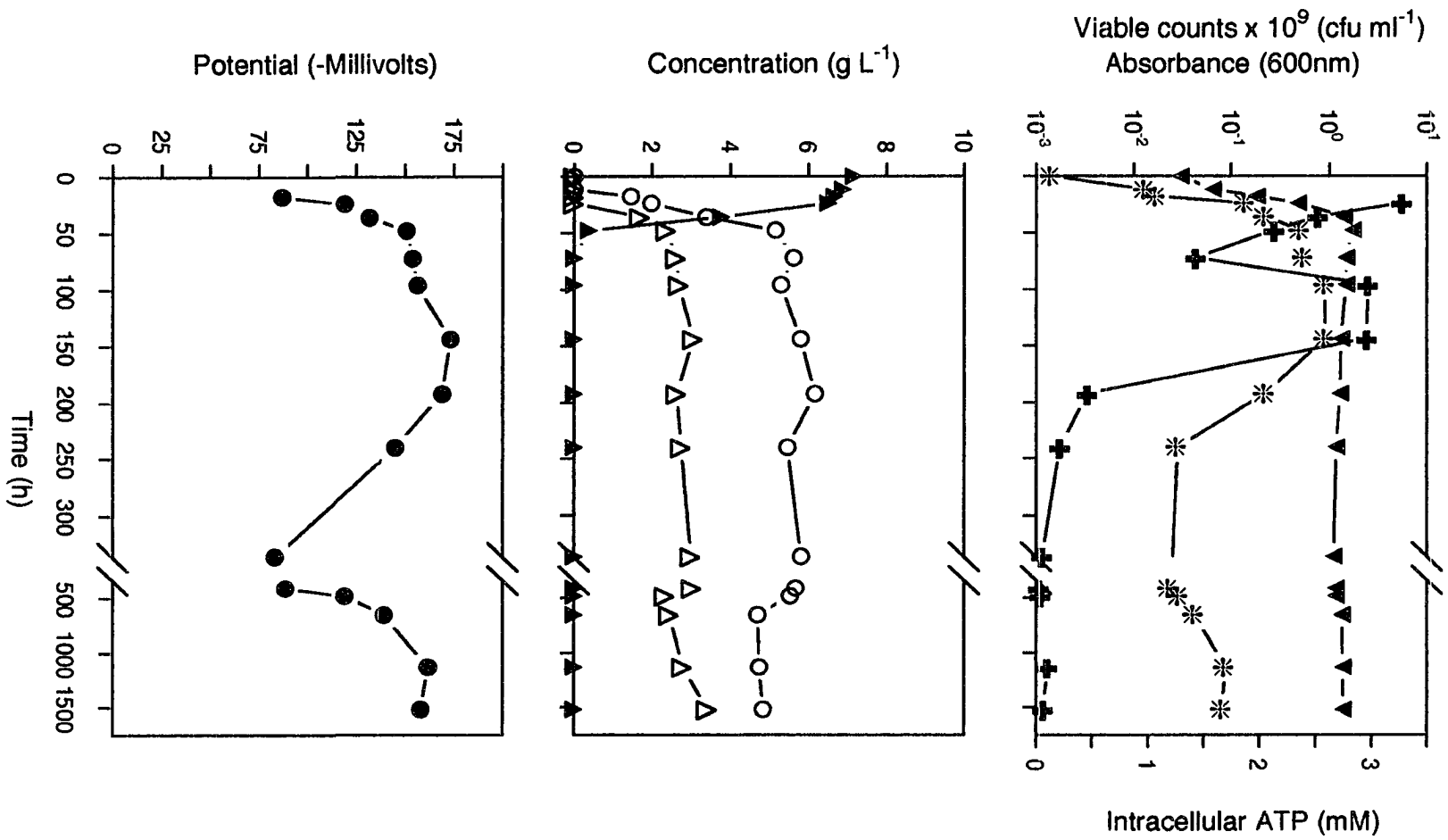
Figure 4: Growth, product formation, and culture characteristics of strain P127 at
controlled pH 5.0. Symbols: ▼, absorbance; ✳, viable counts; ✚, ATP; ▲,
lactic acid; O, propionic acid; Δ, acetic acid; ●, Δp; □, ΔΨ; and ◆, ZΔpH.
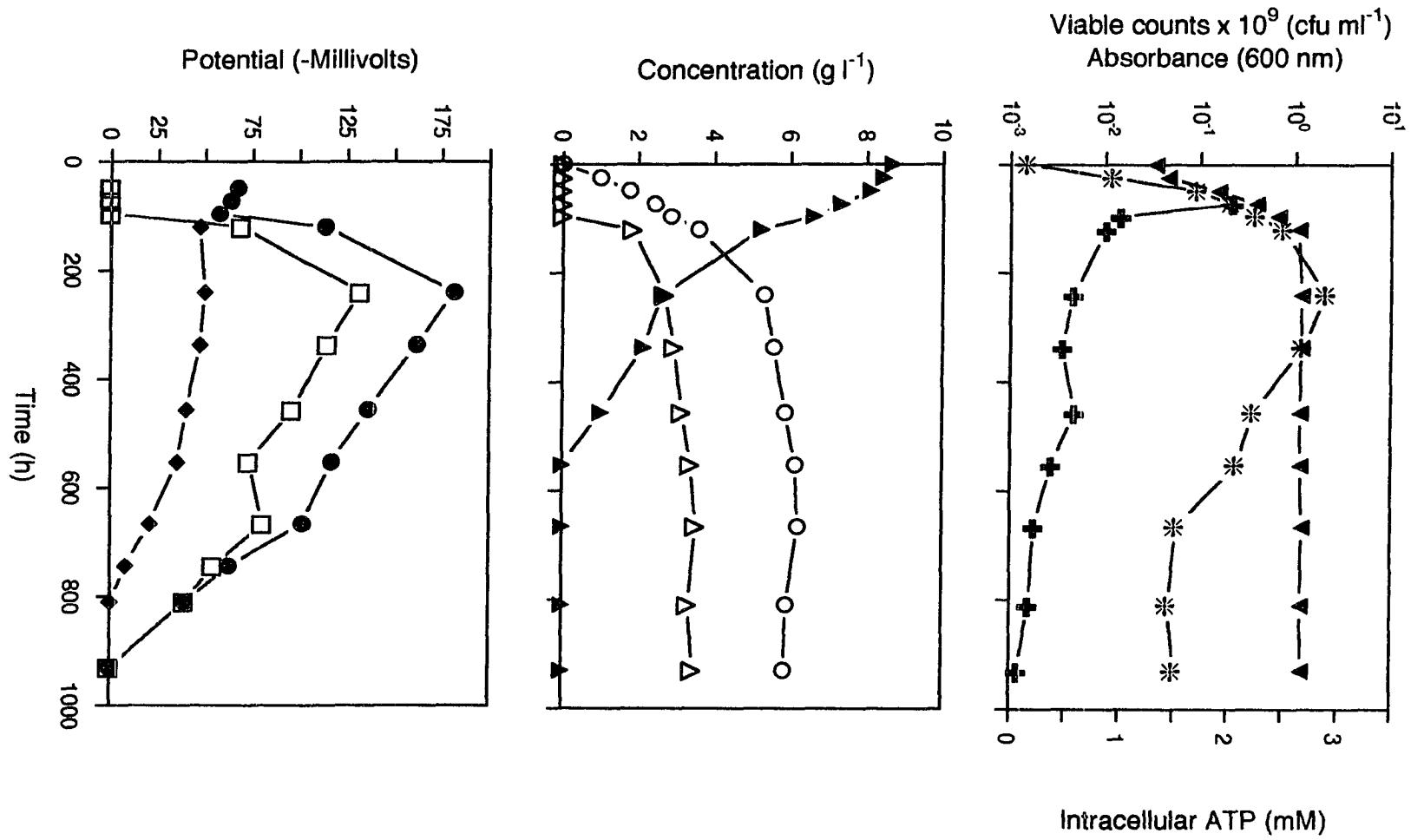
Figure 5: Transmission electron micrographs of thin sections of strain P127 grown at pH 7 (A and B) and pH 5 (C and D). Arrows, cell walls and membrane. Bars A and C, 300 nm; B and D, 150 nm.
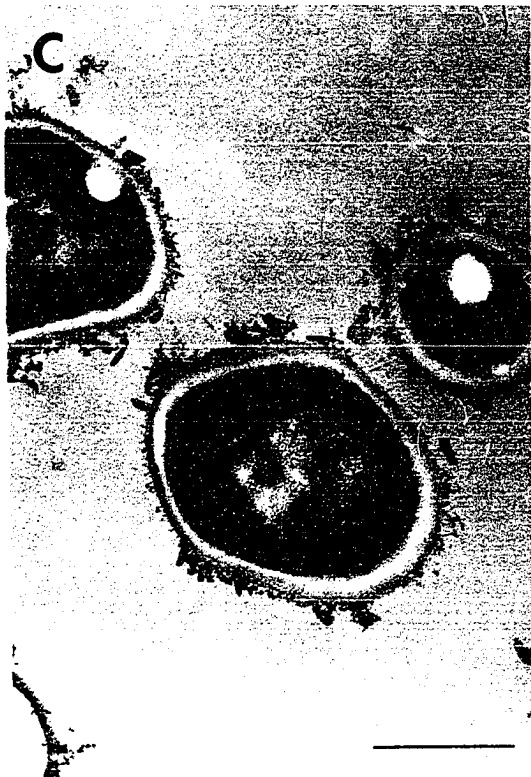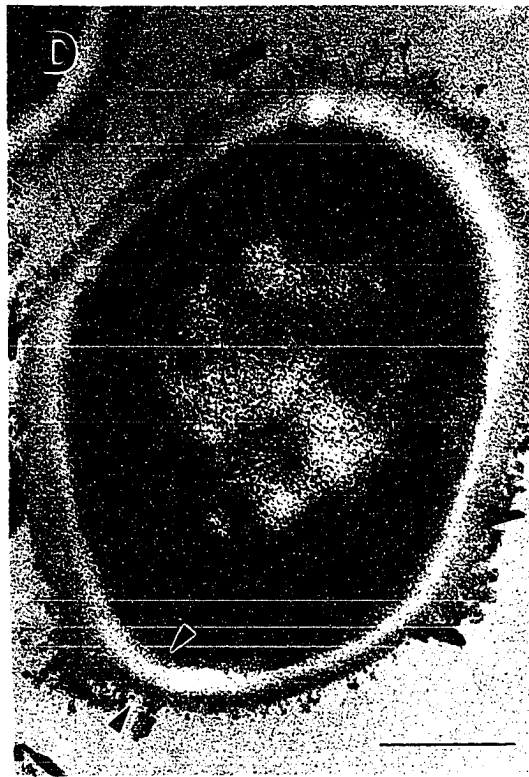
Figure 6: Response of Δp (a) and ΔΨ (b) during the growth phase of batch fermentations with and without pH control. Lines were generated from a linear regression done separately for each pH and fermentation condition. The % highest absorbance was determined from absorbance at the sample time compared to the highest absorbance attained by that culture. The Δp values (a) differed between the cultures with and without pH control (P<0.01). The ΔΨ values (b) differed between cultures grown at different pH values (P<0.01). Symbols: O, pH 7 with pH control; □, pH 7 without pH control; ●, pH 5 with pH control; and ■, pH 5 without pH control.
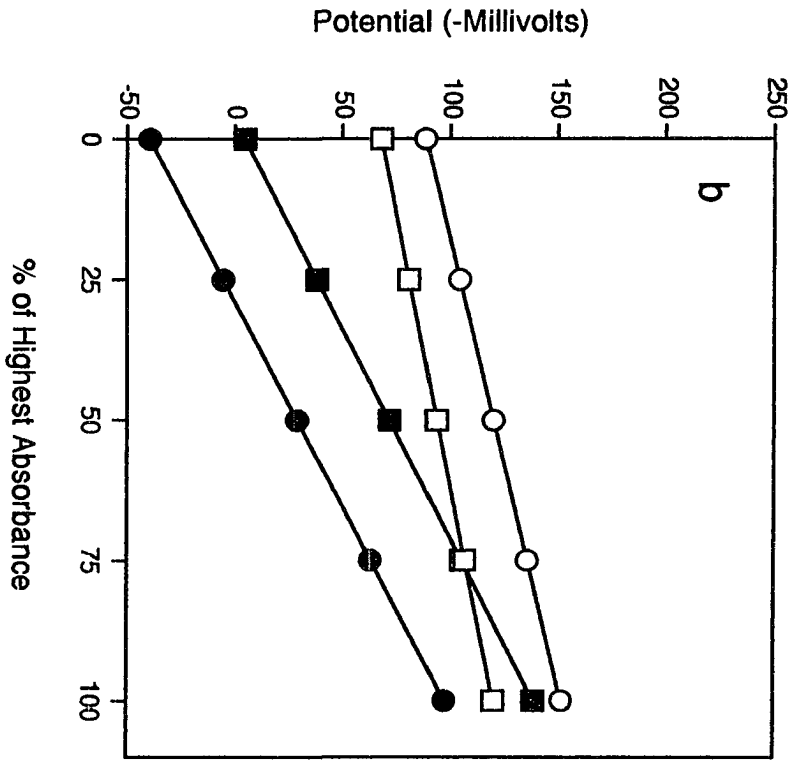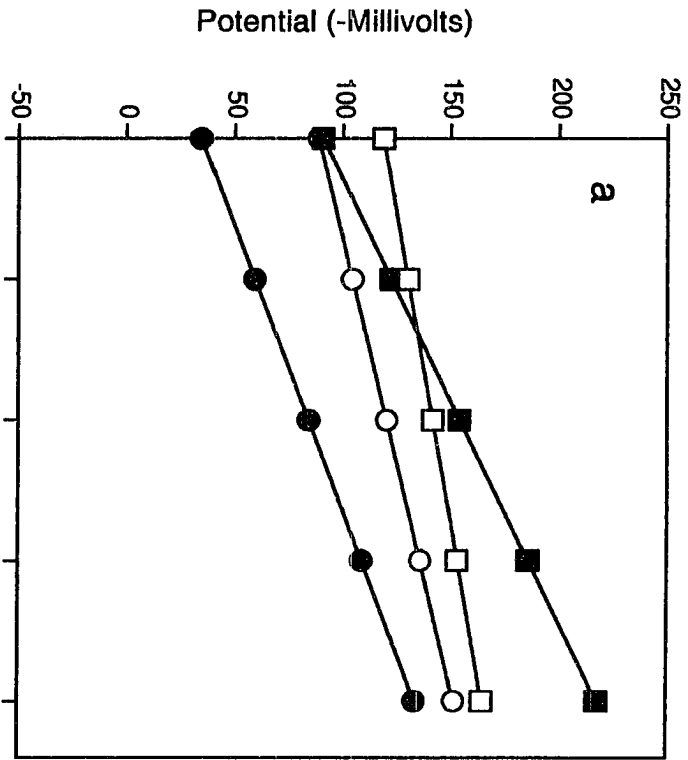
RESPONSE OF *PROPIONIBACTERIUM THOENII* P127 TO ACID EXPOSURE: ADAPTATION AND MANIPULATION OF THE COMPONENTS OF THE PROTON MOTIVE FORCE

Jill L. Rehberger and Bonita A. Glatz

Abstract

Logarithmically growing cells of *Propionibacterium thoenii* P127 that were exposed to mild acid conditions (pH 5.5) for one doubling were respectively 100 and 1000 times more resistant after 90 and 120 min of exposure to lethal acid conditions (pH 3.5) than were cells shifted directly from neutral pH to pH 3.5. This acid tolerance response required protein synthesis. The adapted (pH 5.5) cells were able to maintain a Δp (proton motive force) after 120 min of exposure to pH 3.5, while the Δp of unadapted (pH 7.0) cells had collapsed after 120 min at pH 3.5. When logarithmically growing cells were exposed to increased lactate concentrations at acidic pH, the $Z\Delta pH$ (pH gradient) component of the Δp increased from -12 mV at pH 6.8 to -62 mV at pH 5.5. There was a concomitant decrease in $\Delta\Psi$ (electrical potential) from -99 mV at pH 6.8 to 0 mV at pH 5.5. The $\Delta\Psi$ then increased from -20 mV at pH 5.0 to -108 mV at pH 4.0, while the $Z\Delta pH$ decreased from -48 mV to -5 mV. The cells were able to maintain a near neutral

internal pH when external pH values decreased from 7.0 to 6.1; the internal pH

then decreased with further decrease in external pH. Cells exposed to high lactic

acid concentrations neutralized at pH 7.0 had Δp values between

-114 mV and -120 mV; the Δp was comprised almost entirely of $\Delta \Psi$. These results

suggest that proton concentration rather than the lactate anion had the greatest

influence on Δp. The cells may manipulate the components of the Δp to maintain

a large transmembrane potential. In addition, P127 cannot grow at pH values

below 5.0. This may suggest that the cells' inability to maintain a large enough

$Z\Delta pH$ is responsible for growth inhibition.

Introduction

Propionibacteria are fermentative organisms that produce propionic and

acetic acid from the metabolism of lactate and/or carbohydrates. The primary

industrial use of these organisms is as a starter culture for Swiss-type cheeses

(9), but they are also used as a silage inoculum (4), probiotic agent (14), and in

the production of vitamin B_{12} and propionic acid (19). The propionibacteria are

relatively sensitive to low pH and generally will not grow below pH 5.0 (20). A

strain that is tolerant to low pH could be of economic importance as an improved

silage inoculum or probiotic feed additive. In addition, growth at low pH may

facilitate downstream processes to remove product acids from fermentation broth.

It has been found that previous exposure to mildly acidic conditions

enables some organisms to increase their resistance to extreme acid conditions.

Foster and Hall (5, 6) discovered that when logarithmically growing *Salmonella typhimurium* cells at neutral pH were shifted to pH 5.8 for one doubling, the cells were 100 to 1000 times more resistant to subsequent strong acid challenge (pH 3.3) than were cells that were shifted directly from neutral pH to pH 3.3. This response was called the acid tolerance response (ATR). The adapted cells survived lethal acid conditions because of an enhanced ability to maintain an internal pH above 5.0. Unadapted cells had internal pH values below 5.0, and rapidly lost viability. The authors concluded that the ATR system enhanced pH-homeostatic mechanisms and that acid-induced death was the direct result of lowered internal pH.

The objectives of this work were to determine if cells of *Propionibacterium thoenii* P127 became more resistant to lethal acidic pH when first exposed to mildly acidic conditions; to determine the effect of acid adaptation on the components of the proton motive force; and to determine the effect of exposure to lactic acid at acidic and neutral pH on the proton motive force of logarithmically growing P127 cells.

Materials and Methods

Organism and culture conditions

Propionibacterium thoenii P127 was obtained from the culture collection of the Department of Food Science and Human Nutrition at Iowa State University. Growth medium was sodium lactate broth (NLB) which contained 1% trypticase

soy broth (BBL Microbiology Systems, Cockeysville, MD), 1% yeast extract (Difco, Detroit, MI), 1% sodium lactate 60% syrup (Fisher Scientific Co., Pittsburgh, PA), and 0.6% Tween 80 (Fisher). For experiments conducted at lowered pH values, NLB was acidified to the desired pH with lactic acid. Inocula used in all experiments had been previously transferred at least three times (1% inoculum) in NLB with incubation at 32°C for 24 h between transfers.

Acid challenge experiments

The measurement of the acid tolerance response was based on the methods of Foster and Hall (5). Strain P127 was inoculated (1%) into 300 ml of NLB (pH 7) in a 500-ml screw-capped Erlenmeyer flask and incubated at 32°C until mid-log phase (absorbance of 0.3-0.4 at 600 nm). The culture was divided into 100-ml aliquots, each of which was centrifuged at 9000 x g at 4°C for 15 min and resuspended in 100 ml of NLB at pH 7, 5.9, or 5.5. These cultures were incubated at 32°C for one doubling (absorbance of 0.6-0.8 at 600 nm); approximate doubling times were 5, 7, and 9 h, respectively. The cultures were then centrifuged (9000 x g at 4°C for 15 min) and resuspended in NLB adjusted to pH 3.5. This pH had been previously determined to be lethal to P127 (19). Samples were taken at 15- to 30-minute intervals, diluted, and plated onto nonselective medium (NLA: NLB + 1.5% agar). The plates were incubated at 32°C in GasPak jars (BBL) with the GasPak anaerobic generating system for 5-7 days. The ΔpH and ΔΨ of the cultures were measured at sample times 0, 60, and 120 min.

Acid exposure experiments

Continuous fermentation was conducted in a BioFlo 3000 bioreactor (New Brunswick Scientific, Edison, NJ) with a working volume of 840 ml. The dilution rate was 0.14 h^{-1} with yeast extract (0.1%) as the limiting nutrient. The temperature was controlled at 32°C, the agitation was 125 rpm, and the pH was controlled at pH 7 with 3 N NaOH. Samples were taken at various time points with at least one retention time between sampling times. The cells were centrifuged (9000 x g at 4°C for 15 min) and resuspended either in fresh growth medium acidified with lactic acid, or in neutralized (pH 7) medium with increased lactate concentrations (55 to 120 mM). Resuspended cells were incubated for 30 min at 25°C before samples were taken for $\Delta \Psi$ and ΔpH measurements. The cells were exposed to each lactic acid concentration and pH at least twice with 2 replicates at each exposure time.

Proton motive force determination

Estimation of the proton gradient (ΔpH) and the transmembrane electrical potential ($\Delta \Psi$) was based on distribution of [^{14}C]salicylic acid (2.5 μM, 0.125 μCi; Dupont NEN, Boston, MA) and [^{3}H]tetraphenylphosphonium bromide (TPP^{+}, 2.25 μM, 0.5 μCi; Amersham Corp., Arlington Heights, IL), respectively (11, 17, 18, 21). The accumulation of salicylate and TPP^{+} was determined by the silicon oil centrifugation technique (20). Corrections for nonspecific binding of the probes were based on the extent of probe accumulation in cells treated with 20 μM valinomycin (Sigma Chemical, Co., St. Louis, MO) and 5 μM nigericin (Sigma) for 60 min. For internal volume measurements, cells were incubated with ^{3}H$_{2}$O

(1.25 μCi; Amersham) and [^{14}C]polyethylene glycol (PEG, 3.9 μM, 0.25 μCi; Amersham).

Calculations

The internal volumes were calculated as outlined by Kashket (11) from the difference between the total (^{3}H$_2$O permeable) and extracellular (^{14}C-PEG nonpermeable) volume of pellets. The internal volume for the cells grown at pH 7 was calculated as 0.96 +/- 0.13 μl mg^{-1} dry weight; the internal volume of the pH 5.5 culture was calculated as 0.65 +/- 0.039 μl mg^{-1} dry weight.

The ΔpH was calculated from the distribution ratio of [^{14}C]salicylic acid using the Henderson-Hasselbalch equation (11, 17, 18, 21):

$$\Delta pH = \log \frac{[\text{salicylic acid}]_{in}}{[\text{salicylic acid}]_{out}}$$

When the pH of the medium was less than the pK$_a$ (2.97) + 1.0 (i.e. less than 3.97) a correction factor was necessary (16, 18). In these cases, the ΔpH was calculated by the following equation:

$$\Delta pH = \log \left\{ \left(\frac{\text{salicylic acid}_{in}}{\text{salicylic acid}_{out}} \right) \left(10^{pKa - pH \, out} + 1 \right) - 10^{pKa - pH \, out} \right\}$$

The $\Delta\Psi$ was calculated in a similar manner (11, 17, 18, 21):

$$\Delta\Psi = -Z \log \frac{[TPP^+]_{in}}{[TPP^+]_{out}}$$

Where z = 59 mV at 25°C. The proton motive force was calculated as (11, 17, 18, 21):

$$\Delta p = \Delta\Psi - Z\Delta pH$$

By convention, negative signs are used for $\Delta\Psi$ (negative inside) and Δp (negative and alkaline inside as compared to the external medium). In discussions comparing the $\Delta\Psi$ and ΔpH, $Z\Delta pH$ is used because multiplication by Z converts the ΔpH to the same units (millivolts) as $\Delta\Psi$.

Results and Discussion

Acid adaptation

To determine if *P. thoenii* P127 exhibited an acid tolerance response, the viability of P127 in NLB at pH 3.5 was compared after prior growth at pH 7, 5.9, and 5.5. We choose pH 5.9 and 5.5 as the adaptive pH values because they were mild acid stress conditions for P127. At values below pH 5.5, growth was more severely inhibited (20). In addition, previous researchers (5, 6, 7) had found that pH 5.8 to 6.0 induced the acid tolerance response in *S. typhimurium*.

The results (Figure 1) show that although all cultures experienced a loss of viability, the adapted cultures were better protected from acid death relative to the pH 7 culture. The doubling at pH 5.9 provided some protection, but not as much as did the growth at pH 5.5. This suggests that different levels of adaptation may occur as the cells are exposed to greater acid stress. Foster and Hall (5) found that the induction of ATR in *S. typhimurium* was not an all-or-nothing phenomenon; adaptation at pH values from 6.3 to 5.7 resulted in a graded ATR.

Because of the higher survival rate (100 times) of cultures exposed to pH 5.5 vs. pH 5.9, pH 5.5 was chosen as the adaptation pH for subsequent

experiments. Prior exposure to pH 5.5 allowed 1000 times higher survival rate after 120 min of exposure to pH 3.5 compared to the unadapted (pH 7) culture.

To determine if adaptation would protect the cells from an even greater acid challenge, both adapted and unadapted cultures were resuspended in NLB adjusted to pH 3.0 and pH 2.5 (Table 1). Both cultures experienced a one-log reduction in viable counts immediately after exposure to pH 3.0; after 20 min the viable counts for both cultures were below the detection limit. In addition, viable cells were below the detection limit for both cultures immediately after exposure to pH 2.5.

Foster and Hall (5) offered two possible explanations for the development of acid tolerance: growth at mild acid stress could have resulted in a physiological alteration in the cell which made it less susceptible to acid damage, or acid tolerance could be a molecular process that required genetic regulation and synthesis of new proteins. Protein synthesis was determined to be involved in the induction of the ATR system in S. typhimurium (5, 13).

To determine if protein synthesis was necessary for acid adaptation of P127, chloramphenicol (100 μg ml^{-1}) was added to the original culture 2.5 h prior to the point of adaptation; chloramphenicol was also present during the adaptation period. Choramphenicol was previously determined to be nonlethal to P127 at 100 μg ml^{-1} (data not shown). The adapted (pH 5.5) culture showed 27% survival after 60 min of exposure to pH 3.5 (Table 2). In contrast, in the presence of chloramphenicol the culture was much more susceptible to acid damage, with <0.01% survival. Unadapted cultures with and without chloramphenicol experienced a significant loss of viability after 60 min of pH 3.5 exposure (Table

2). The results indicate that protein synthesis is required for acid adaptation to occur in P127.

Proton motive force during acid challenge

Acid damage caused rapid loss of viability in *S. typhimurium* cells when the internal pH dropped below 5.0 (6). The researchers concluded that adapted cells survived the acid challenge (pH 3.3) better than did unadapted cells because of their enhanced ability to maintain an internal pH above 5.0. To determine if acid adaptation affected the internal pH homeostatic mechanisms of P127, the ΔpH and $\Delta\Psi$ were measured after 0, 60, and 120 min of acid challenge (pH 3.5) for both adapted and unadapted cultures (Table 3). The unadapted cells were able to maintain a Δp at time 0 (-49 mV) and 60 min (-61 mV) but not at 120 min. In contrast, the adapted cells were able to maintain a large Δp throughout the acid challenge: -76, -85, and -72 mV at 0, 60, and 120 min, respectively.

The internal pH (external pH + ΔpH) value of the cells of the unadapted culture were higher at time 0 (pH 3.93) than was that of the adapted cells (pH 3.58) (Table 4). After 120 min at pH 3.5, the ΔpH of the unadapted cells had collapsed. In contrast, the ΔpH of adapted cells increased after 60 min of acid challenge. The transient low ΔpH immediately after exposure to pH 3.5 may result from slow response of the primary proton pumps, by whose action the cell regulates its internal pH. Previous researchers have found that upon shifting *E. coli* cells from pH 7.2 to more alkaline conditions, a transient decrease in ΔpH and an increase in $\Delta\Psi$ were seen before steady state values were established by the pH homeostatic mechanisms (18).

The adapted P127 cells may have survived at pH 3.5 better than the

unadapted cells because of their ability to maintain a ΔpH. The internal pH values

of *P. thoenii* were lower than those measured previously in *S. typhimurium*; this

may be because fermenting bacteria such as the propionibacteria can tolerate

higher internal proton concentrations than can other bacteria (12).

Acid exposure

To determine the effect of acid on the proton motive force of P127,

logarithmically growing cells were harvested from continuous culture and

exposed to NLB acidified to pH values ranging from 7.0 to 4.0 with lactic acid.

Continuous culture was used so that the cells were in the same physiological

state at different sampling times; this should reduce error due to differences in

growth stage.

The ZΔpH increased from -12 mV at pH 6.8 to -62 mV at pH 5.5 (Figure 2a).

In the same cultures, the $\Delta\Psi$ values decreased from -99 mV to 0. This is consistent

with previous observations in bacteria that the contribution of $\Delta\Psi$ and ZΔpH to Δp

are dependent on the pH of the medium (2, 12). The $\Delta\Psi$ was the major

component at neutral pH values, while the ZΔpH was the major component at

acidic pH values. The $\Delta\Psi$ then increased from -20 mV at pH 5.0 to -108 mV at pH

4.0, while the ZΔpH decreased from -48 mV to - 5 mV. The large decrease in ΔpH

below pH 5.0 suggests that the cell membranes might have lost their

impermeability to protons and thus were not able to maintain a pH gradient in the

presence of high concentrations of protons. The concomitant increase in $\Delta\Psi$ with

the decrease in ΔpH is consistent with observations in other bacteria (2, 12).

Researchers have determined that changes in the proton gradient were compensated for by corresponding changes in the electrical gradient; by this mechanism a constant Δp could be maintained (2, 12, 18)

To investigate further the cells' ability to increase one component of the Δp when the other decreases, the ΔpH was measured while the $\Delta\Psi$ was reduced with valinomycin (20 μM). Valinomycin is an ionophore that catalyzes the electrical uniport of cations (especially K^+) and depletes the $\Delta\Psi$ (17). Conversely, the $\Delta\Psi$ was measured while the ΔpH was reduced with nigericin (5 μM), an ionophore that carries out the antiport of K^+ for H^+ and dissipates the ΔpH in an electroneutral manner (17). When the ΔpH was depleted, the $\Delta\Psi$ increased for cultures at acidic and neutral pH; the most dramatic increase occurred at pH 5 (Table 5). In contrast, the ΔpH dramatically increased at neutral pH when the $\Delta\Psi$ had been dissipated.

These results suggest that the cells manipulate the components of the Δp to maintain a large transmembrane potential. When the major component of the Δp was reduced, i.e. ΔpH at pH 5 and $\Delta\Psi$ at pH 7, the cells dramatically increased the remaining component. However, when the secondary component of the Δp was reduced, i.e. $\Delta\Psi$ at pH 5 and ΔpH at pH 7, there was only a small increase in the remaining component. This confirms the importance of the ΔpH at low pH and $\Delta\Psi$ at neutral pH in the maintenance of Δp.

Internal pH values

Cells exposed to media with decreasing pH values were able to maintain a near neutral internal pH when the external pH ranged from 6.8 to 6.1 (Table 6).

The internal pH then decreased with further decrease in external pH. Previous observations with other fermentative organisms including *Clostridium thermoaceticum* (1), *Sarcina ventriculi* (8), and lactic acid bacteria (3, 12, 15, 16, 23) demonstrated that the internal pH of these organisms decreased as the external pH declined.

The most dramatic change in internal pH, from 5.9 to 4.7, occurred when the pH of the medium decreased from 5.1 to 4.5. Previously we found that strain P127 could not initiate growth in medium acidified below pH 5.0 with lactic acid (20) and considered pH 5.0 to be the critical external pH threshold for this strain. The large decline in internal pH that occurred at external pH values below 5.0 suggests that the cells' inability to maintain a large enough ΔpH at these conditions is responsible for growth inhibition. Furthermore, we had observed that the critical internal pH threshold for P127 was 5.8 (20). The internal pH values measured in this study are in agreement with this value.

We previously observed that strain P127 remained viable but could not grow at pH 4.5; viability was lost at pH 4.1 (20). In the current study we measured an internal pH of 4.7 when the external pH was 4.5. This internal pH did not allow the cells to grow, but was not lethal. At external pH 4.0, the ΔpH of the cells was very small, and the internal pH was 4.1 These results suggest that the cells need to maintain an internal pH above 4.1 to remain viable.

Effect of lactate

The Δp values for cells exposed to increased lactate at neutral pH ranged from -111 mV to -126 mV and were primarily composed of $\Delta\Psi$ (Figure 2). The

cells maintained a neutral internal pH at all lactate concentrations (Table 6). In addition, the $\Delta \Psi$ and ΔpH values measured in the presence of nigericin and valinomycin were not significantly affected by lactate concentrations (Table 5). These results suggest that the cells were primarily affected by the proton concentration and not by the lactate anion.

This study has shown that strain P127 undergoes some adaptation when grown at pH 5.5. We propose that protein synthesis and the ability to maintain a ΔpH are necessary for this adaptation. Researchers have shown that the synthesis of outer membrane proteins in *S. typhimurium* was involved in acid adaptation (13). These proteins may have increased the cell membrane's impermeability to protons, which would allow the cells to survive better at lethal acidic pH conditions. Investigation of the proteins that were synthesized by P127 during growth at pH 5.5 may help to elucidate the adaptation process.

Growth inhibition of strain P127 below pH 5 appears to be due to its inability to maintain its internal pH at or above pH 5.8. Similar values have been found for lactic acid bacteria (15, 16, 22). Nannen and Hutkins (16) found that growth and metabolic inhibition of *S. thermophilus, L. lactis* ssp. *lactis*, and *L. lactococcus* ssp. *cremoris* occurred below an external pH of 5.0 as a result of low internal pH (between pH 5.0 and 5.5). However, the acid-tolerant organism *L. casei* was able to maintain an internal pH between 5.1 and 6.4, even at external pH values as low as 3.8. Thus, for P127 to increase its acid tolerance, it would need to increase its ability to maintain a large pH gradient. This would involve several changes in the cell: increases in membrane impermeability to protons, increases in activity of the proton pumps, or decreases in the pH optima of internal

enzymes and proton pumps. Thus, isolation and/or development of a variant of

P127 that could grow below pH 5 involves several mechanisms and would likely

be difficult.

Acknowledgments

References

1. **Baronofsky, J.J., W.J.A. Schreurs, and E.R. Kashket.** 1984. Uncoupling by acetic acid limits growth of and acetogenesis by *Clostridium thermoaceticum*. Appl. Environ. Microbiol. **48**:1134-1138.

2. **Booth, I.R.** 1985. Regulation of cytoplasmic pH in bacteria. Microbiol. Rev. **49**:359-378.

3. **Brink, B.T., and W.N. Konings.** 1982. Electrochemical proton gradient and lactate concentration gradient in *Streptococcus cremoris* cells grown in batch culture. J. Bacteriol. **152**:682-686.

4. **Flores-Galarza, R.A., B.A. Glatz, C.J. Bern and L.D. Van Fossen.** 1985. Preservation of high moisture corn by microbial fermentation. J. Food Prot. **48**:407-411.

5. **Foster, J.W., and H.K. Hall.** 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. J. Bacteriol. **172**:771-778.

6. **Foster, J.W., and H.K. Hall.** 1991. Inducible pH homeostasis and the acid tolerance response of *Salmonella typhimurium*. J. Bacteriol. **173**:5129-5135.

7. **Foster, J.W.** 1991. *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. J. Bacteriol. **173**:6896-6902.

8. **Goodwin, S., and J.G. Zeikus.** 1987. Physiological adaptation of anaerobic bacteria to low pH: metabolic control of proton motive force in *Sarcina ventriculi*. J. Bacteriol. **169**:2150-2157.

9. **Hettinga, D.H., and G.W. Reinbold.** 1972. The propionic-acid bacteria-a review. II. Metabolism. J. Milk Food Technol. **35**:358-372.

10. **Kashket, E.R., A.G. Blanchard, and W.C. Metzger.** 1980. Proton motive force during growth of Streptococcus lactis cells. J. Bacteriol. **143**:128-134.

11. **Kashket, E.R.** 1985. The proton motive force in bacteria: a critical assessment of methods. Ann. Rev. Microbiol. **39**:219-242.

12. **Kashket, E.R.** 1987. Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. FEMS Microbiol. Rev. **46**:233-244.

13. **Leyer, G.J. and E.A. Johnson.** 1993. Acid adaptation induces cross-protection against environmental stresses in Salmonella typhimurium. Appl. Environ. Microbiol. **59**:1842-1847.

14. **Mantere-Alhonen, S.** 1995. Propionibacteria used as probiotic-a review. Lait **75**:447-452.

15. **McDonald, L.C., H.P. Fleming, and M.H. Hassan.** 1990. Acid tolerance of Leuconostoc mesenteroides and Lactobacillus plantarum. Appl. Environ. Microbiol. **56**:2120-2124.

16. **Nannen, N.L., and R.W. Hutkins.** 1991. Intracellular pH effects in lactic acid bacteria. J. Dairy Sci. **74**:741-746.

17. **Nicholls, D.G., and S.J. Ferguson.** 1992. Bioenergetics 2. Academic Press Inc., San Diego.

18. **Padan, E., and S. Schuldiner.** 1986. Intracellular pH regulation in bacterial cells. Meth. Enzymol. **125**:337-365.

19. **Playne, M.J.** 1985. Propionic and butyric acids, p.731-759. In M. Moo-Young (ed.), Comprehensive biotechnology, vol. 3. Pergamon Press, Oxford.

20. **Rehberger, J.L.** 1996. Response of Propionibacterium to acid and low pH: tolerance and inhibition. Ph.D. dissertation, Iowa State University. Ames, IA.

21. **Rottenberg, H.** 1979. Measurement of membrane potential and ΔpH in cells, organelles, and vesicles. Meth. Enzymol. **55**:547-569.

22. **Russell, J.B.** 1991. Intracellular pH of acid-tolerant ruminal bacteria. Appl. Environ. Microbiol. **57**:3383-3384.

23. **Russell, J.B.** 1992. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. J. Appl. Bacteriol. **73**:363-370.

Table 1: Viable counts (cfu ml^{-1}) of unadapted (pH 7) and adapted (pH 5.5) P127 cultures after exposure to pH 3.0 and pH 2.5.

Culture	pH 3.0				pH 2.5		
	Original[a]	0 min	10 min	20 min	Original[a]	0 min	10 min
pH 7.0	4.7×10^7	3.2×10^6	$<10^1$	$<10^1$	4.7×10^7	$<10^1$	$<10^1$
pH 5.5	2.3×10^8	4.5×10^7	1.5×10^3	$<10^1$	1.4×10^8	$<10^1$	$<10^1$

[a] Viable cell counts in cfu ml^{-1} of culture prior to acid challenge

Table 2: Effect of growth with chloramphenicol (100 µg ml^{-1}) on acid adaptation of P127. Percent survival was determined 60 min after resuspending unadapted (pH 7.0) and adapted (pH 5.5) cultures in NLB acidified to pH 3.5 with lactic acid. Values are averages of 3 experiments.

Culture	Chloramphenicol	% Survival
Unadapted	-	0.04
Unadapted	+	<0.01
Adapted	-	26.6
Adapted	+	<0.01

Table 3: Components of the proton motive force for unadapted (U) and adapted (A) cells during acid challenge at pH 3.5.

Time (min)	Δp (mV) U[a]	Δp (mV) A[b]	$\Delta\Psi$ (mV) U[a]	$\Delta\Psi$ (mV) A[b]	$Z\Delta pH$ (mV) U[a]	$Z\Delta pH$ (mV) A[b]
0	-49	-76	-24	-65	-25	-11
60	-61	-85	-39	-50	-22	-35
120	0	-72	0	-31	0	-41

[a] unadapted (pH 7.0) cells
[b] adapted (pH 5.5) cells

Table 4: Internal and external pH values for unadapted and adapted cells during acid challenge at pH 3.5.

Time (min)	Unadapted pH out[a]	Unadapted ΔpH	Unadapted pH in[b]	Adapted pH out[a]	Adapted ΔpH	Adapted pH in[b]
0	3.50	0.43	3.93	3.40	0.18	3.58
60	3.50	0.37	3.87	3.50	0.60	4.10
120	3.60	0	3.60	3.50	0.70	4.20

[a] pH out = external pH
[b] pH in = internal pH (external pH + ΔpH)

Table 5: Comparison of the $\Delta\Psi$ and $Z\Delta pH$ values at different pH and lactate concentrations for cells of P127 treated with nigericin (5 μM) or valinomycin (20 μM) to dissipate the ΔpH or $\Delta\Psi$.

pH	Lactate (mM)	Nigericin	Valinomycin	$\Delta\Psi$ (mV)	$Z\Delta pH$ (mV)
6.83	55	+	-	-134	-
6.96	70	+	-	-132	-
5.07	70	+	-	-128	-
6.83	55	-	+	-	-55
6.96	70	-	+	-	-48
5.07	70	-	+	-	-53
6.78	55	-	-	-99	-12
6.96	70	-	-	-115	-11
5.06	70	-	-	-20	-48

Table 6: External and internal pH values for cells exposed to increased lactate at acidic and neutral pH values.

Lactate (mM)	Acidic pH			Neutral pH		
	pH out[a]	ΔpH	pH in[b]	pH out[a]	ΔpH	pH in[b]
55	6.78	0.21	6.99	6.78	0.20	6.98
56	6.45	0.74	7.19	6.96	0.11	7.07
59	6.06	0.94	7.00	7.00	0.05	7.05
61	5.52	1.05	6.57	6.94	0.10	7.04
70	5.06	0.82	5.88	6.96	0.18	7.14
83	4.48	0.25	4.73	6.96	0.09	7.05
120	4.00	0.09	4.09	6.90	0.31	7.21

[a] pH out = external pH
[b] pH in = internal pH (external pH + ΔpH)
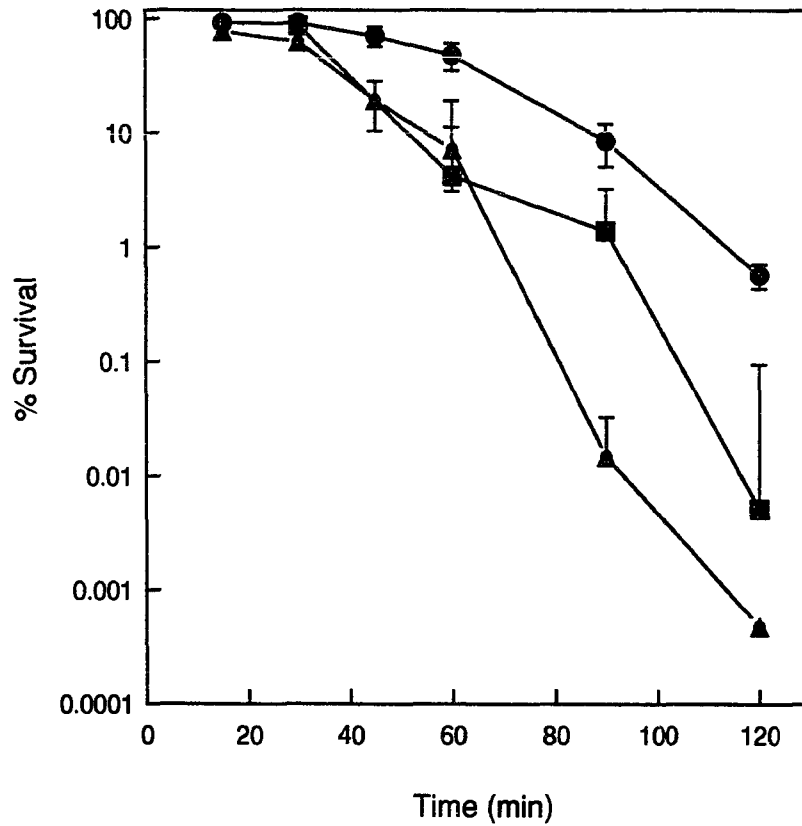
Figure 1: Survival of cultures of P127 with time of exposure to pH 3.5.
Survivors were enumerated on nonselective medium. Values
are the average of 5 experiments with standard error values shown.
Symbols: ▲   unadapted culture grown at pH 7,   ■   culture
adapted at pH 5.9, and   ●   culture adapted at pH 5.5.
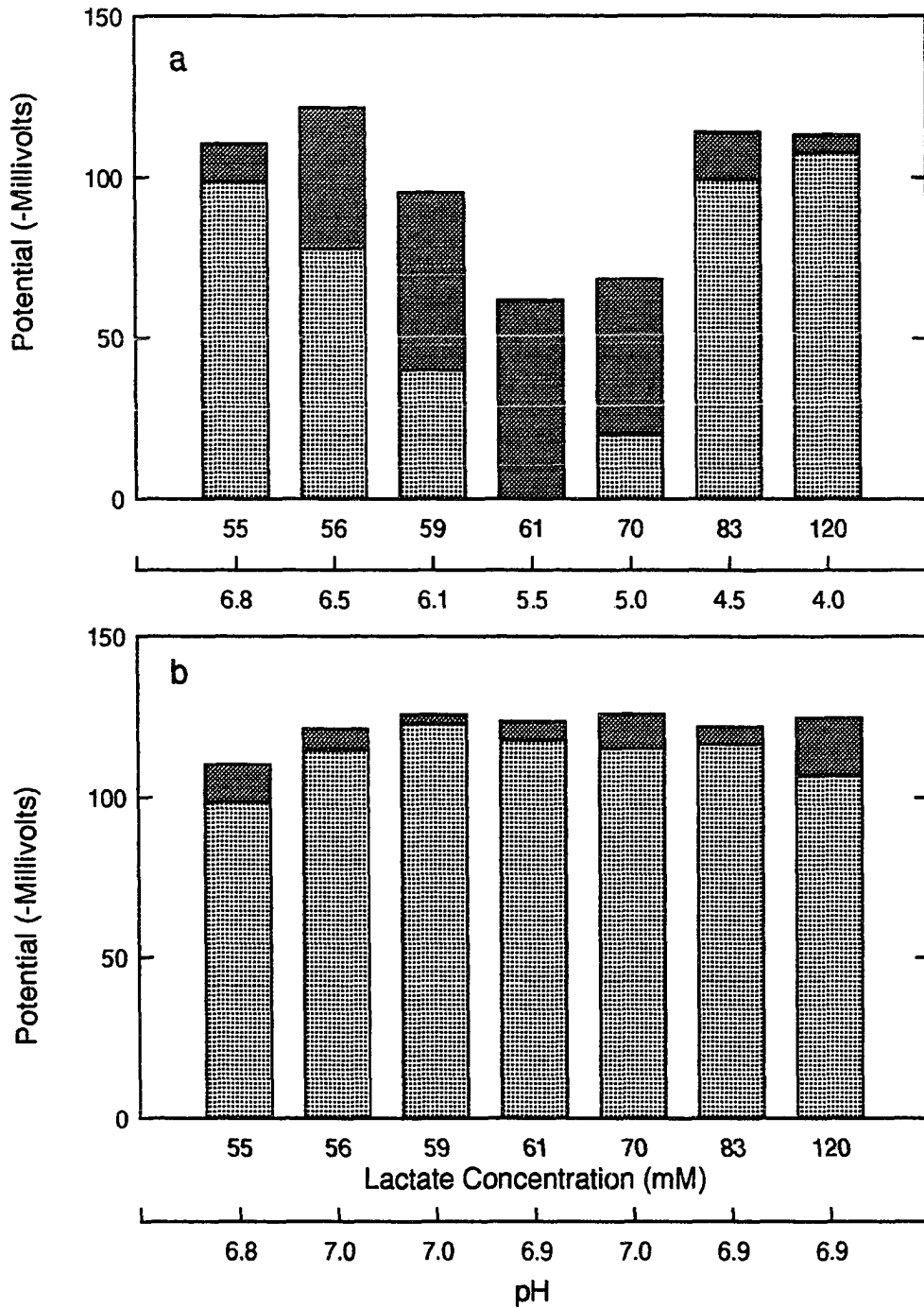
Figure 2: The ΔΨ and ZΔpH of P127 after exposure to increased lactate at acidic pH (a) and neutral pH (b). Values are the averages of duplicate exposures with 2 replicates at each exposure time.
Symbols: ▦ ΔΨ ▨ ZΔpH.

127

GENERAL CONCLUSIONS

It has been recognized that the optimum pH for growth of propionibacteria is between pH 6.0 and 7.1, and that growth rates decline at acidic pH (41). However, studies have not been conducted to explain these effects. Experiments reported in this research define the parameters of growth at low pH and help to explain the mechanism of acid- and low-pH-tolerance and inhibition.

Six strains of *Propionibacterium* (three strains of *P. acidipropionici*, one strain of *P. jensenii*, and two strains of *P. thoenii*) were identified as potential acid- and low-pH-tolerant strains. These strains were selected based on their ability to produce large amounts of propionic and acetic acids and obtain a final pH <4.4 at the end of culture growth with glucose, fructose, and maltose as carbon sources. The *P. acidipropionici* strains produced lower final pH values and on average higher acid concentrations than did the other strains.

The lowest pH values at which the strains could initiate growth and the lowest pH values at which they could remain viable were determined with lactic, propionic, and hydrochloric acids as acidulants. Generally, the strains could not grow below pH 5 in the presence of organic acids; however, they could remain viable at significantly lower pH values (down to pH 4.1) for 48 h. All strains were more pH-tolerant when the inorganic acid was used. The *P. thoenii* and *P. jensenii* strains were able to initiate growth and survive at lower pH values than could the *P. acidipropionici* strains. However, the *P. thoenii* and *P. jensenii* strains were more sensitive to increased propionate concentrations at neutral pH

than were the *P. acidipropionici* strains. Thus, the ability to produce and tolerate large amounts of acids was not directly related to a strain's ability to initiate growth or survive in low-pH conditions. It appeared that the response to acid anions and to protons was governed by two different mechanisms.

To determine how acid production and low pH affected *P. thoenii* P127, the proton motive force (Δp) of P127 was measured during batch growth at pH 7 and pH 5. The results demonstrated that the contribution of the individual components, pH gradient (ΔpH) and electrical potential ($\Delta \Psi$), to the overall Δp depended on the pH of the medium. At pH 7, the Δp was comprised of only the $\Delta \Psi$; the internal pH remained constant at pH 7. During growth at pH 5, the Δp consisted of both $\Delta \Psi$ and ΔpH and the internal pH decreased slowly throughout the fermentation with acid production.

The acid tolerance of fermentative organisms had been attributed to their ability to lower their internal pH as the external pH decreased in the presence of weak organic acids (15, 79, 80, 81, 82). Weak organic acids diffuse across the membrane in accordance with the pH gradient and dissociate at the more alkaline internal pH. The impermeable anion accumulates internally, at concentrations predicted by the Henderson-Hasselbalch equation; this accumulation is believed to contribute to inhibitory effects of the acid. Strain P127 was able to prevent large amounts of propionic and acetic acid from accumulating internally by lowering its ΔpH. However, a lower ΔpH resulted in a lower internal pH; this can also contribute to organic acid inhibition (7, 46, 69, 82).

When the internal pH of P127 fell below pH 5.8, the rate of consumption of substrate lactate declined and the cells entered death phase, even though the

lactate had not yet been depleted. These results suggest that pH 5.8 may be the critical internal pH threshold for strain P127. Cellular functions are inhibited at internal pH values below the critical threshold. Most internal enzymes have a narrow range in which they are active (7, 46, 69); pH 5.8 may be the lower limit for enzymes in this strain.

The ATP concentrations of P127 grown in batch culture controlled at pH 7 and pH 5 were measured. Throughout the entire fermentation, the ATP concentrations for the pH 5 culture were significantly lower than for the pH 7 culture. This may be due to the reverse action of the H^+-ATPase which consumes ATP to expel protons in order to maintain an alkaline internal pH with respect to the medium (4, 7, 67). At pH 5, strain P127 decreased its intracellular pH, lactate consumption rate, and intracellular ATP concentration. These results suggest that energy availability may contribute to the pH sensitivity of P127.

When strain P127 was exposed to increased lactate concentrations at acidic pH, the cells manipulated the components of the Δp to maintain a large transmembrane potential. The cells maintained a near neutral internal pH when the external pH ranged from 6.8 to 6.1. However, the internal pH then decreased with further decrease in the external pH. A large decline in internal pH occurred at external pH values below 5.0; this may suggest that growth inhibition was due to the inability of the cell membranes to remain impermeable to protons.

Strain P127 remained viable but could not grow at pH 4.5; viability was lost at pH 4.1. At an external pH of 4.5, the corresponding internal pH was measured as 4.7. This internal pH did not allow the cells to grow, but was not lethal. At pH

4.0, the ΔpH for cells was very small, and the internal pH was 4.1. These results

suggest that the cells need to maintain an internal pH above 4.1 to remain viable.

Examination of transmission electron micrographs of P127 cells grown at

pH 7 and pH 5 showed differences in cell wall and membrane structure at the two

conditions. At pH 5, cell walls appeared diffuse and seemed to lack a definitive

structure. Since the cells were fixed in buffers adjusted to the pH at which they

were grown, it is possible that the difference in appearance may be due to pH-

induced differences in the ability of the cells to take up stain. Examination of cells

grown at pH 5 and fixed at pH 7 would test this possibility and help to answer the

question of whether cell structure was altered by growth at low pH. In addition, a

comparison of the capsules of cells grown at pH 7 and pH 5 may reveal other

morphological changes at low pH.

The initial goal of this study was to isolate and/or develop an acid- and low-

pH-tolerant variant that could be used as a silage inoculant, as a probiotic feed

additive, or as a more productive strain for industrial fermentations. The

development of a strain tolerant to both acid and low pH seems unlikely, however;

tolerance to acid and to acid anions appeared to be two separate complex

mechanisms. Indeed, the propionate-tolerant variant of P9, strain P200910, had

been isolated after repeated exposure to increasing propionate concentrations at

neutral pH (91), but showed the least tolerance to low pH.

The development of a fermentative low-pH-tolerant variant would require

changes in several characteristics of the strain. A strain could increase its pH-

tolerance by maintaining a more neutral internal pH. In this way, the internal

enzymes could remain active. This would involve the overexpression of proton

pumps such as the H^+-ATPase. However, this would require that the cell have a large ΔpH; this would allow more of the product acids of the fermentation to accumulate inside the cell.

A strain could also increase its pH-tolerance by lowering the pH optima of its internal enzymes. Such a strain could maintain a lower ΔpH and would not accumulate as high an internal concentration of acid anions. However, the pH optimum of an enzyme depends on several factors, and changing the pH range of activity would not be easily accomplished. One approach could be through genetic manipulation of the enzyme's primary structure, to contain more basic amino acids. This would involve locating the active site(s), determining the amino acid residues involved in catalysis, and using site-directed mutagenesis to alter these residues. A strain could also be altered by random mutagenesis using a chemical mutagen followed by selection at low pH.

In addition, either approach to obtaining a low-pH-tolerant variant would require changes in the cell membranes to make them more impermeable to protons. Considering the complex mechanisms involved, it seems unlikely that a strain of *Propionibacterium* is a good candidate for low-pH-tolerance.

An alternative approach would be to gradually adapt the cells to low pH by exposing them to mildly acidic conditions. When logarithmically growing cells of P127 were exposed to a mild acid stress (pH 5.5) for one doubling, they were more resistant to a subsequent lethal pH exposure (pH 3.5) than were cells that were shifted directly from neutral pH to pH 3.5. We proposed that protein synthesis and the ability to maintain a ΔpH were necessary for adaptation. The

unadapted cells had an internal pH of 3.9 upon exposure to pH 3.5, while the adapted cells maintained an internal pH of 4.2.

Researchers have shown that the synthesis of outer membrane proteins and the activity of the H^+-ATPase in *S. typhimurium* were involved in acid adaptation (27, 28, 53). The membrane proteins may have increased the cell membrane's impermeability to protons, which allowed the cells to survive better at lethal acidic pH conditions. Investigation of the proteins that were synthesized upon acid exposure may further explain the adaptation process in P127. In addition, isolation of H^+-ATPase mutants of P127 would help determine the role of this enzyme in adaptation.

Other areas for future research include:

1) Measurement of the Δp of strain P127 during fed-batch fermentation. This would determine better how acid production affects this strain because in the presence of excess carbon source the culture would produce more acid than did the batch culture. This experiment would allow us to determine the propionic acid concentration at which the cell ceases growth and fermentation.

2) Isolation and characterization of the H^+-ATPase from unadapted and adapted cells of P127. The pH optima and basal activities of these enzymes would help explain the low-pH adaptation response.

3) Comparison of the components of the Δp of strain P9 and its propionate-tolerant variant, P200910, when these strains were grown and exposed to increased propionate concentrations. This would help explain the mechanism of propionate anion tolerance.

4) Isolation and characterization of membranes of cells grown at neutral and low pH. The composition of fatty acids, proteins, and lipids can then be determined for cells grown at neutral and low pH.

In addition, the development of the protocol for the measurement of the Δp will allow our laboratory to determine sugar transport systems in propionibacteria and the mode of action of the bacteriocins produced by strains of propionibacteria. Determination of sugar transport systems is accomplished by incubating cells in the presence of radiolabeled sugars and measuring the internal concentration of the sugar over time. A comparison of the Δp, internal concentrations, and rates of transport of the sugar for cells incubated in the presence and absence of ATPase inhibitors, protonophores, ionophores, and metabolic inhibitors would determine if transport was active or passive. The mode of action of the bacteriocins can be determined by measuring the components of the Δp of an indicator organism in the presence and absence of the bacteriocin.

APPENDIX A: PROTON MOTIVE FORCE CALCULATIONS

Internal Volume Calculations

The internal volume of cells was determined from the difference between the total and extracellular volumes of the cell pellets. The total volume was measured with ^3H_2O (1.25 μCi), and the extracellular volume was measured with [^{14}C] PEG (3.9 μM, 0.25 μCi). A stock solution of ^3H_2O (0.25 μCi/μl) was made from 0.25 ml of ^3H_2O (5 μCi/μl) and 0.475 ml of "cold" H_2O; PEG was used as purchased. The internal volume was measured at different times throughout culture growth; each sample time consisted of triplicate measurements. The internal volume was calculated as follows.

Total volume of the cell pellet (V_t) ^3H_2O counts:

$$V_t = \frac{DPM\ [^3H]pellet - DPM\ Bkgd}{DPM\ [^3H]supernatant - DPM\ Bkgd}$$

Where DPM Bkgd is the counts of a nonradioactive control culture.

Extracellular volume of the cell pellet (V_e) [^{14}C] PEG counts:

$$V_e = \frac{DPM\ [^{14}C]pellet - DPM\ Bkgd}{DPM\ [^{14}C]supernatant - DPM\ Bkgd}$$

Internal volume (V_{in}) (μl mg^{-1} dry weight):

$$V_{in} = \frac{(\text{avg } V_t - \text{avg } V_e)(\text{volume of supernatant } (\mu l))}{\text{pellet weight (mg)}}$$

In the initial internal volume measurements [^{14}C] inulin (0.5 μCi) was used to measure the extracellular volume. However, there were large inconsistencies among the calculated internal volumes at each sampling time, including some negative values. After consulting with Dr. Thomas Montville (Rutgers, The State University of New Jersey) and Dr. Herbert Strobel (University of Kentucky), we tried PEG instead of inulin. Both consultants suggested that inulin was such a large molecule that sometimes it may adhere to cells. Also, bacteria behave differently and thus not all probes worked for every organism. Dr. Strobel's lab uses PEG or nontransported amino acids to avoid the binding of the large molecules. After we began using PEG, we no longer measured negative values for internal volumes.

The internal volume was measured at various stages of culture growth for cultures grown at pH 7 and pH 5 (Table A1). The average internal volume at pH 7 was 0.96 +/- 0.13 μl mg^{-1} dry weight and at pH 5 it was 0.51 +/- 0.068 μl mg^{-1} dry weight. At pH 5.5 for the acid tolerance response experiment, internal volume was 0.65 +/- 0.039 μl mg^{-1} dry weight. Although the volume appeared to decrease with culture age, all values were used to calculate the average. Small changes in internal volume do not have a significant effect on the final $\Delta\Psi$ and ΔpH calculations, which are calculated as the logarithm of the concentration ratios. The $\Delta\Psi$ and zΔpH values for the pH 7 and pH 5 cultures differed by only 14

mV when they were calculated with the average internal volume +/- two standard

deviations (1.22 and 0.70 μl mg^{-1} dry weight for pH 7 and 0.65 and 0.27 μl mg^{-1}

dry weight for pH 5 cultures).

Table A1: Calculated internal volumes of P127 at various stages of growth at pH 7 and pH 5.

pH	Time (h)	Internal Volume (μl mg^{-1} dry weight)
7	24	1.16
7	24	1.14
7	72	0.95
7	96	0.82
7	96	0.91
7	96	0.90
7	C.C.[a]	0.86
7	C.C.[a]	0.97
5	48	0.47
5	72	0.59
5	96	0.52
5	168	0.41
5	168	0.57
5	666	0.47
5.5	ATR[b]	0.60
5.5	ATR[b]	0.68
5.5	ATR[b]	0.65
5.5	ATR[b]	0.67

[a] C.C.= continuous culture experiment
[b] ATR = acid tolerance response experiment, cells were grown at pH 7 until mid-logarithmic phase and then for one doubling at pH 5.5.

Nonspecific Binding Calculations

The probes that are used to estimate the $\Delta\Psi$ (tetraphenylphosphonium,

TPP+) and ΔpH (salicylic acid) dissociate in solution and are charged. Charged

compounds will bind to cell membranes and thus corrections need to be made to

the concentration ratios of these probes. The correction factor was measured

from the amount of probe that accumulated in cells that had been incubated for 60

min with nigericin (5 μM), valinomycin (20 μM), and 40 mM K+ (added as

K_2HPO_4). Nigericin carries out the symport of H+ and K+; this dissipates the ΔpH.

Valinomycin catalyzes the electrical uniport of cations (especially K+) and thus

dissipates the $\Delta\Psi$. The nonspecific binding (NSB) correction factors were

calculated from:

$$\text{Probe}_{bound} \ (\text{DPM}/\mu l) = \frac{\text{DPM pellet -DPM Bkgd}}{[\text{internal volume } (\mu l/mg)][\text{pellet weight (mg)}]}$$

$$\text{Probe}_{free} \ (\text{DPM}/\mu l) = \frac{\text{DPM supernanant- DPM Bkgd}}{\text{Volume of supernatant } (\mu l)}$$

$$\text{NSB correction factor} = \frac{\text{Probe}_{bound}}{\text{Probe}_{free}}$$

The NSB correction factors were calculated for both salicylic acid and

TPP+ at each sample time and subtracted from the concentration ratios of the

respective probe. Initially, these correction factors were calculated from the plot of the concentration of the bound probe versus free probe for cells incubated with different concentrations of the probe. The NSB factor for each culture condition was then calculated from the slope of the line (Figures A1 and A2). We began calculating correction factors at each sample time after consultation with Dr. Thomas Montville. Culture age and medium composition affect the binding of the probes and thus it was necessary to calculate them at each sample time.
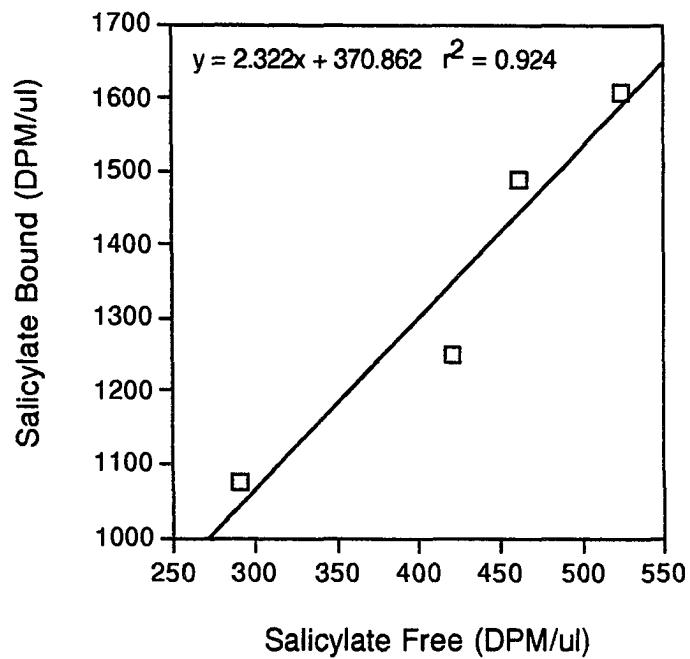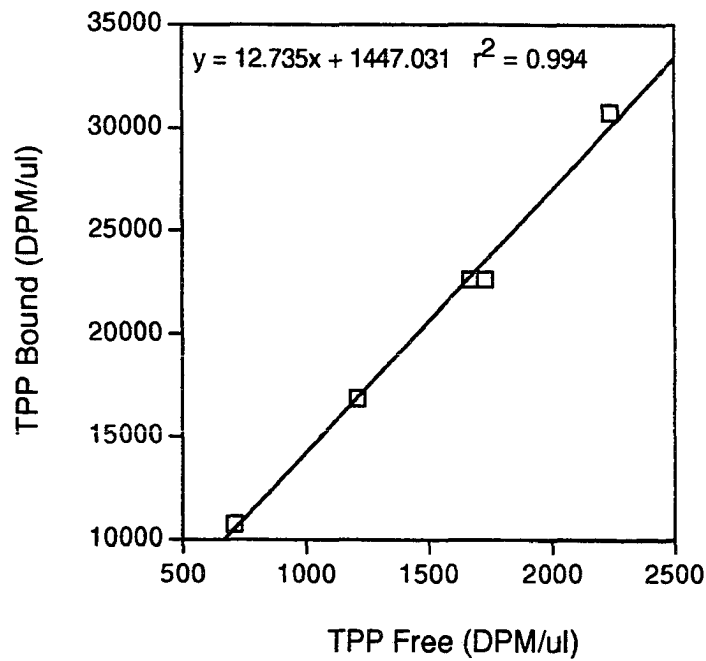
Figure A1: Bound versus free probe concentrations for a) TPP+ and b) salicylate for pH 7 cultures. NSB correction factors are given as the slope of the line.
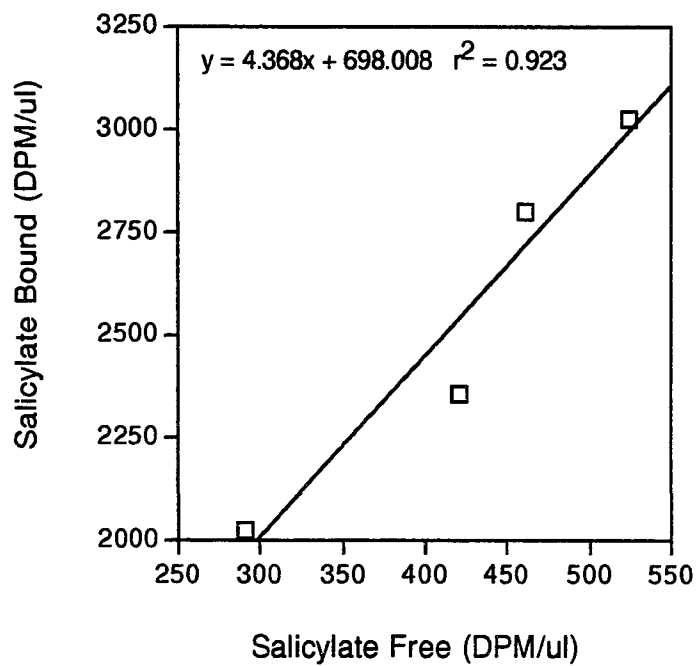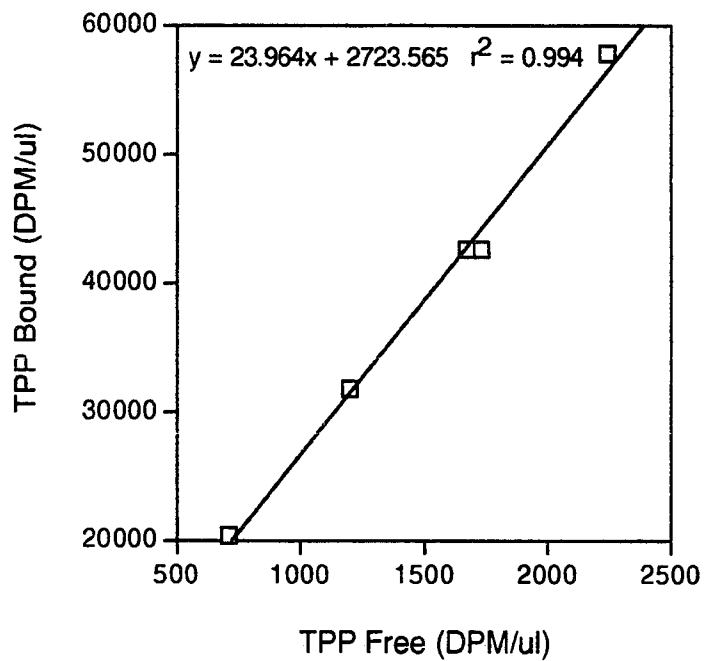
Figure A2: Bound versus free probe concentrations for a) TPP+ and b) salicylate for pH 5 cultures. NSB correction factors are given as the slope of the line.

ΔΨ Calculations

The ΔΨ was calculated based on the distribution of [^{3}H] TPP$^+$ (2.25 μM, 0.5 μCi). A stock solution (450 μM, 0.1 μCi/μl) was prepared from 0.1 ml of [^{3}H] TPP$^+$ (1 μCi/μl) and 0.9 ml of 500 μM "cold" TPP$^+$. The ΔΨ was calculated from:

$$TPP^+_{out} \ (DPM/\mu l) = \frac{DPM \ supernanant - DPM \ Bkgd}{Volume \ of \ supernatant \ (\mu l)}$$

$$TPP^+_{in} \ (DPM/\mu l) = \frac{DPM \ pellet - DPM \ Bkgd}{[internal \ volume \ (\mu l/mg)][pellet \ weight \ (mg)]}$$

$$TPP^+ \ corrected = \frac{TPP^+_{in}}{TPP^+_{out}} - NSB \ correction \ factor$$

$$\Delta\Psi = -Z \ log(TPP^+ \ corrected)$$

Where $z = 2.303 \ RT/nF$; R = gas constant (8.31 J/mol K); T = absolute temperature (K); n = charge of transported species = 1; F= Faraday's constant (96.519 J/mol mV). z is 59 mV at 25°C

ΔpH Calculations

The ΔpH was calculated from the distribution of $[^{14}C]$ salicylic acid (2.5 μM, 0.125 μCi). The stock solution was prepared with 0.24 ml of $[^{14}C]$ salicylic acid (1800 μM, 100 μCi) and 0.76 ml of 100 μM "cold" salicylic acid to give a final concentration of 500 μM and 0.025 μCi/μl. Salicylic acid was chosen because of its low pK_a (2.97), which allowed 99% dissociation of the probe in the extracellular and intracellular space even at pH 5. The ΔpH was calculated from:

$$\text{Salicylic acid}_{out} \ (\text{DPM}/\mu l) = \frac{\text{DPM supernatant} - \text{DPM Bkgd}}{\text{Volume of supernatant} \ (\mu l)}$$

$$\text{Salicylic acid}_{in} \ (\text{DPM}/\mu l) = \frac{\text{DPM pellet} - \text{DPM Bkgd}}{[\text{internal volume} \ (\mu l/mg)][\text{pellet weight} \ (mg)]}$$

$$\text{Salicylic acid corrected} = \frac{\text{salicylic acid}_{in}}{\text{salicylic acid}_{out}} - \text{NSB correction factor}$$

$$\Delta pH = \log(\text{salicylic acid corrected})$$

When the pH of the medium was < pKa + 1 (i.e. 3.97) the following formula was used:

$$\Delta pH = \log\left\{(\text{salicylic acid corrected})\left(10^{pK_a - pH_{out}} + 1\right) - 10^{pK_a - pH_{out}}\right\}$$

$$pH_{in} = pH_{out} + \Delta pH$$

The total Δp was calculated as:

$$\Delta p = \Delta \Psi - Z\Delta pH$$

The $\Delta \Psi$ and ΔpH were considered to be collapsed when the concentration ratio minus the NSB correction factor was < 1, which would result in a negative value after taking the logarithm. In these cases the $\Delta \Psi$ was not reported as positive; TPP^+ would not accumulate in cells with a positive interior and thus the values were reported as zero. Similarly, the ΔpH was reported as zero in these cases because salicylic acid would not accumulate in cells that were more acidic than the external medium.

APPENDIX B: EXTENDED FERMENTATION OF *P. THOENII* P127 AT PH 7

During extended incubation (480 to 1500 h) of *P. thoenii* P127 in batch culture controlled at pH 7, the culture experienced an increase in the proton motive force, viable cell counts, and acetic acid concentrations. The purity of the culture was verified by microscopic examination and plating onto sodium lactate agar and Brain Heart Infusion agar (Difco), which would allow a variety of fastidious organisms to grow. The plates were incubated aerobically and anaerobically at 32°C for five to seven days. The culture was also plated onto Sulfite Polymixin Sulfadiazine agar (Difco) and incubated anaerobically at 37°C for 48 h to check for the presence of clostridia.

These results may suggest that the culture had undergone some metabolic change which may have induced the synthesis of new enzymes. To get an overall view of cellular proteins, the intracellular and membrane-bound proteins of the culture at 1516 h and a control culture at 48 h were separated by SDS-PAGE.

The cells were harvested by centrifugation (9,000 x g, 15 min), washed in 10 mM MES (Fisher), and resuspended in a small amount of 10 mM MES to form a thick cell paste. The cell suspension was disrupted by passing through a chilled French pressure cell maintained at 18,000 lbs/in^2. Three passes were necessary to obtain adequate cell disruption. Cell debris and remaining intact cells were removed by centrifugation at 9,000 x g for 15 min. Aliquots (3-5 μl) of supernatant were subjected to SDS-PAGE. Electrophoresis was performed using 7.5% Tris-

HCl polyacrylamide gel (Mini-PROTEAN II Ready Gels; BioRad Laboratories,
Hercules, CA) and 10-20% gradient Tris-HCl polyacrylamide gel (Jule Inc.
Biotechnologies, New Haven, CT) with Tris-Glycine-SDS running buffer at
constant voltage (200 V) for 30 min. Gels were stained with coomassie blue stain
according to the manufacturer's instructions.

There were no significant differences in banding patterns of the larger
molecular weight proteins on the 7.5% polyacrylamide gel (data not shown).
However, there appeared to be an additional protein band on the 10-20%
gradient polyacrylamide gel at approximately 25 kDa in the 1516 h culture (Figure
B1). A better approach to detecting differences in protein content would be to
conduct a two-dimensional-PAGE, the first dimension using isoelectric focusing
and the second dimension using SDS-PAGE.

The cells may have synthesized enzymes for the metabolism of the
remaining amino acids in the medium. Propionibacteria have been found to
utilize several amino acids, especially aspartate, alanine, serine, and glycine (3).
The metabolism of aspartate yields succinate, acetate, carbon dioxide, and
ammonia. In addition, strains of propionibacteria have been found to produce
polysaccharides (1). The formation of polysaccharides also increased the
formation of acetic acid and carbon dioxide without any associated propionic acid
production.

This additional band might also correspond to a protein synthesized during
stationary phase. A variety of proteins have been found to be synthesized in *E.
coli* during entry into stationary phase (2). These proteins were involved in
maintaining viability during prolonged starvation.

References

1. **Crow, V.L.** 1988. Polysaccharide production by propionibacteria during lactose fermenation. Appl. Environ. Microbiol. **54**:1892-1895.

2. **Kolter,R., D.A. Siegele, and A. Tormo.** 1993. The stationary phase of the bacterial life cycle. Annu. Rev. Microbiol. **47**:855-874.

3. **Langsrud, T., T. Sorhaug, and G.E. Vegarud.** 1995. Protein degradation and amino acid metabolism by propionibacteria. Lait **75**:325-330.

Figure B1: SDS-PAGE analysis of P127 after 1516 h and 36 h (control) of incubation at pH 7 on a 10-20% gradient polyacrylamide gel. Lanes 1, 2, and 10: empty: Lanes 3 and 7: 1516 h cell lysate; Lanes 4 and 8: 36 h cell lysate; Lane 5: low molecular weight standard (Sigma); Lane 6: broad molecular weight standard (BioRad); Lane 9: colored low molecular weight standard (Sigma).

kDa

1 2 3 4 5 6 7 8 9 10

200.0
116.0
97.0
66.0
45.0
31.0
21.5
14.5
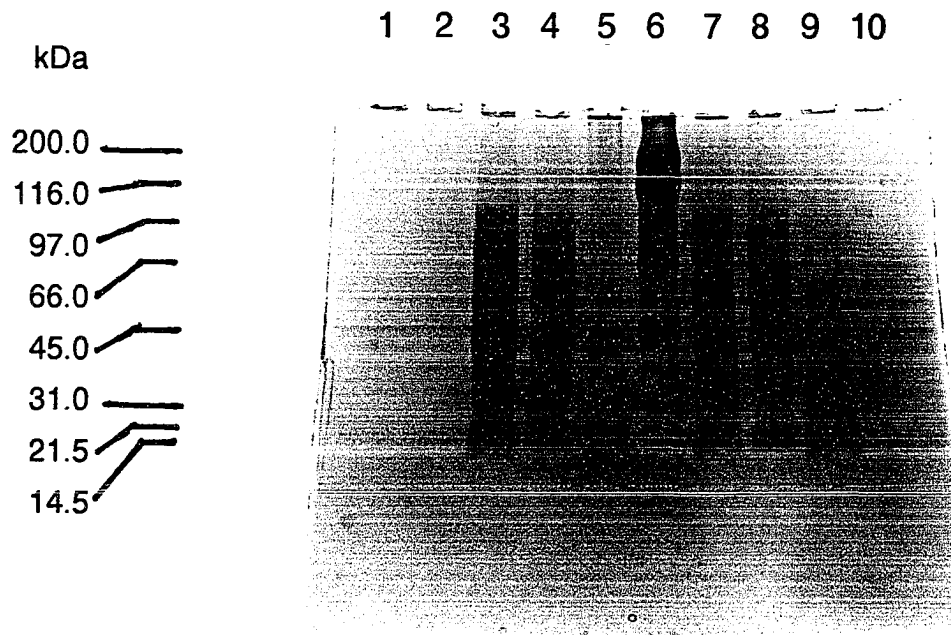
APPENDIX C: PIGMENT LOSS

During the acid adaptation experiment, we noted that upon exposure to pH 3.5 and 3.0 a significant percent of colonies lost their typical red-orange pigment when plated onto nonselective medium (Figure C1). The percentage of unpigmented colonies increased with time of exposure to low pH. However, pigment loss was not permanent; when unpigmented cultures were subcultured in neutral laboratory medium for 36 to 48 h and plated onto nonselective medium, approximately half of the resulting colonies were pigmented.

Strain P127 also lost pigmentation upon exposure to streptomycin, valinomycin, and chloramphemicol, as well as during heat shock and when grown on extremely overcrowded plates. The percentage of pigment loss was greater for cultures that had experienced a significant cidal effect from the stress (Figure C2). The combination of low pH and exposure to chloramphenicol presented two "hurdles" for the culture to overcome. Survivors of this combination showed the highest percentage loss of pigment. This suggests that pigment loss is associated with stress, e.g. low pH, presence of antibiotics, heat shock, or competition for nutrients.

Figure C1: Photographs of plates of P127 typically seen upon exposure to stress. A) typical healthy colonies, B) unadapted (pH 7) culture after exposure to pH 3.5 for 60 min and C) adapted (pH 5.5) culture after exposure to pH 3.5 and chloramphenicol for 60 min.
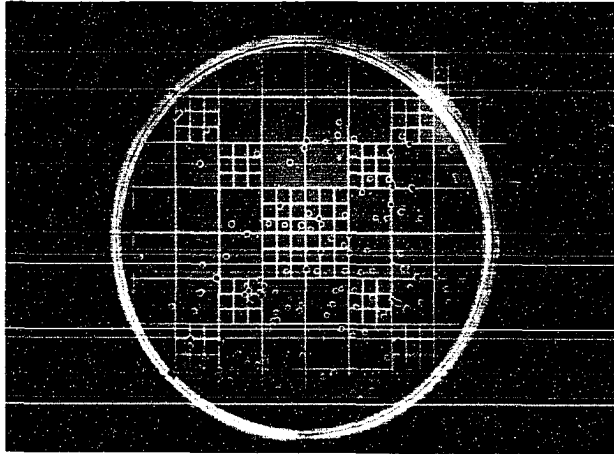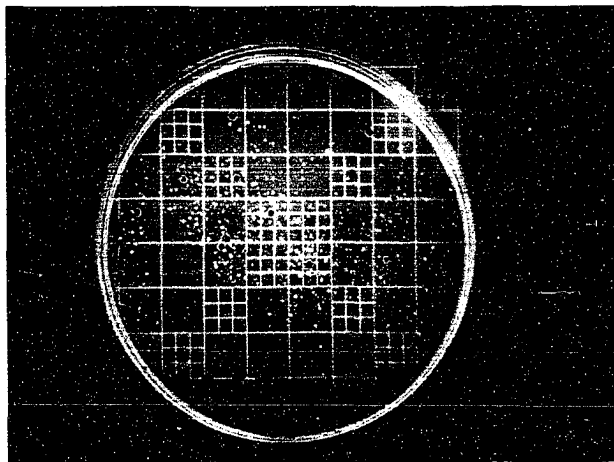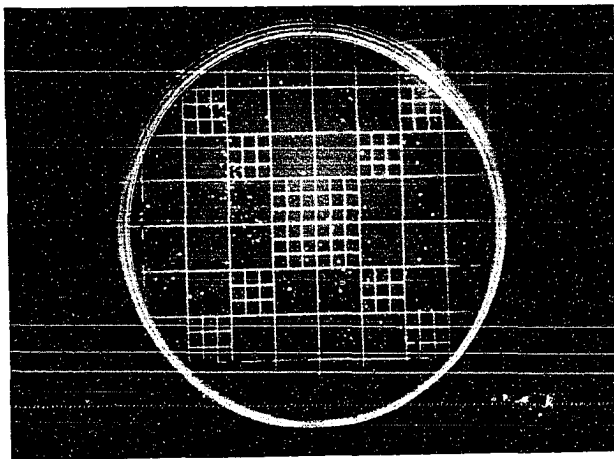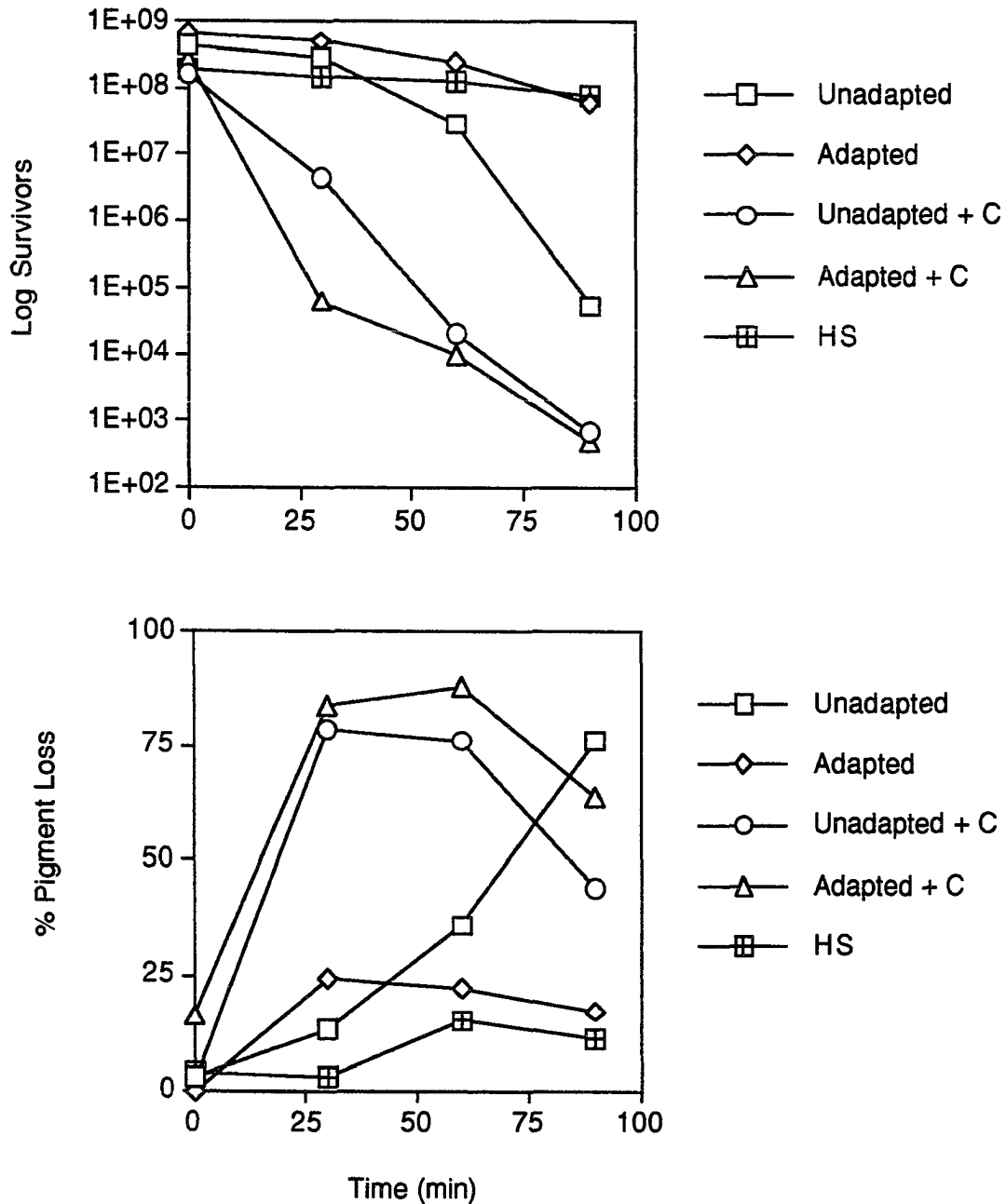
Figure C2: Comparison of total survivors and percent of pigment loss in cultures of P127 exposed to various stresses. Unadapted = cultures grown at pH 7 and then shifted to pH 3.5, Adapted = cultures exposed to pH 5.5 for one doubling prior to shifting to pH 3.5, C = chloramphenicol (100 μg/ml), HS = heat shock at 55°C.

REFERENCES

1. **Alexander, B., S. Leach, and W.J. ingledew.** 1987. The relationship between chemiosmotic parameters and sensitivity to anion and organic acids in the acidophile *Thiobacillus ferrooxidans*. J. Gen. Microbiol. **133**:1171-1179.

2. **Antipov, V.A., and V.M. Subbotin.** 1980. Use of probiotics- efficacy and prospects. Dairy Sci. Abstr. **44**:8567.

3. **Baer, A.** 1995. Influence of casein proteolysis by starter bacteria, rennet and plasmin on the growth of propionibacteria. Lait **75**:391-400.

4. **Baronofsky, J.J., W.J.A. Schreurs, and E.R. Kashket.** 1984. Uncoupling by acetic acid limits growth of and acetogenesis by *Clostridium thermoaceticum*. Appl. Environ. Microbiol. **48**:1134-1138.

5. **Belli, W.A., and R.E. Marquis.** 1991. Adaptation of *Streptococcus mutans* and *Enterococcus hirae* to acid stress in continuous culture. Appl. Environ. Microbiol. **45**:966-973.

6. **Bender, G.R., and R.E. Marquis.** 1987. Membrane ATPases and acid tolerance of *Actinomyces viscosus* and *Lactobacillus casei*. Appl. Environ. Microbiol. **53**:2124-2128.

7. **Booth, I.R.** 1985. Regulation of cytoplasmic pH in bacteria. Microbiol. Rev. **49**:359-378.

8. **Brendehaug, J., and T. Langrud.** 1985. Amino acid metabolism in propionibacteria: resting cell experiments with four strains. J. Dairy Sci. **68**:281-289.

9. **Brink, B.T., and W.N. Konings.** 1982. Electrochemical proton gradient and lactate concentration gradient in *Streptococcus cremoris* cells grown in batch culture. J. Bacteriol. **152**:682-686.

10. **Britz, T.J., and K.H.J. Riedel.** 1995. Numerical evaluation of the species groupings among the classical propionibacteria. Lait **75**:309-314.

11. **Buazzi, M.M., and E.H. Marth.** 1992. Sites of action by propionate on *Listeria monocytogenes*. Int. J. Food Microbiol. **15**:109-119.

12. **Cerna, B., M. Cerny, H. Betkova, P. Patricny, M. Soch, and I. Opatrna.** 1991. Effect of the Proma probiotics on calves. Dairy Sci. Abstr. **55**:1735.

13. **Cherrington, C.C., V. Allen, and M. Hinton.** 1992. The influence of temperature and organic matter on the bactericidal activity of short-chain organic acids on salmonellas. J. Appl. Bacteriol. **72**:500-503.

14. **Cobley, J.G., and J.C. Cox.** 1983. Energy conservation in acidophilic bacteria. Microbiol. Rev. **47**:579-595.

15. **Cook, G.M., and J.B. Russell.** 1994. The effect of extracellular pH and lactic acid on pH homeostasis in *Lactococcus lactis* and *Streptococcus bovis.* Curr. Microbiol. **28**:165-168.

16. **Crow, V.L.** 1986. Utilization of lactate isomers by *Propionibacterium freudenreichii* subsp. *shermanii*: regulatory role of intracellular pyruvate. Appl. Environ. Microbiol. **52**:352-358.

17. **Crow, V.L.** 1986. Metabolism of aspartate by *Propionibacterium freudenreichii* subsp. *shermanii*: effect on lactate fermentation. Appl. Environ. Microbiol. **52**:359-365.

18. **Crow, V.L.** 1987. Citrate cycle intermediates in the metabolism of aspartate and lactate by *Propionibacterium freudenreichii* subsp. *shermanii.* Appl. Environ. Microbiol. **53**:2600-2602.

19. **Crow, V.L.** 1987. Properties of alanine dehydrogenase and aspartase from *Propionibacterium freudenreichii* subsp. *shermanii.* Appl. Environ. Microbiol. **53**:1885-1892.

20. **Crow, V.L.** 1988. Polysaccharide production by propionibacteria during lactose fermentation. Appl. Environ. Microbiol. **54**:1892-1895.

21. **Cummins, C.S., and J.L. Johnson.** 1986. *Propionibacterium.*, p.1346-1353 *In* Sneath, P.H.A., N.S. Mair and J.G. Holt (ed), Bergey's manual of systemic bacteriology, vol 2. Williams and Wilkens, Baltimore.

22. **Dean, J.A. (ed.)** 1979. Lange's handbook of chemistry, 12th ed. McGraw-Hill Co., New York.

23. **Dupuis, C., C. Corre, and P. Boyaval.** 1993. Lipase and esterase activities of *Propionibacterium freudenreichii* subsp. *freudenreichii.* Appl. Environ. Microbiol. **59**:4004-4009.

24. **Dupuis, C., C. Corre, and P. Boyaval.** 1995. Proteinase activity of dairy *Propionibacterium.* Appl. Microbiol. Biotechnol. **42**:750-755.

25. **Dziezak, J.D.** 1986. Preservatives: antimicrobial agents. Food Technol. **35(9)**:104-111.

26. **Flores-Galarza, R.A., B.A. Glatz, C.J. Bern, and L.D. Van Fossen.** 1985. Preservation of high-moisture corn by microbial fermentation. J. Food Prot. **48**:407-411.

27. **Foster, J.W., and H.K. Hall.** 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. J. Bacteriol. **172**:771-778.

28. **Foster, J.W., and H.K. Hall.** 1991. Inducible pH homeostasis and the acid tolerance response of *Salmonella typhimurium*. J. Bacteriol. **173**:5129-5135.

29. **Foster, J.W.** 1991. Salmonella acid shock proteins are required for the adaptive acid tolerance response. J. Bacteriol. **173**:6896-6902.

30. **Gatje, G., and G. Gottschalk.** 1991. Limitation of growth and lactic acid production in batch and continuous cultures of *Lactobacillus helveticus*. Appl. Microbiol. Biotechnol. **34**:446-449.

31. **Goodwin, S., and J.G. Zeikus.** 1987. Physiological adaptation of anaerobic bacteria to low pH: metabolic control of proton motive force in *Sarcina ventriculi*. J. Bacteriol. **169**:2150-2157.

32. **Gottschalk, G.** 1986. Bacterial metabolism. Springer-Verlag, New York.

33. **Goulbourne, Jr., E., M. Matin, E. Zychlinsky, and A. Matin.** 1986. Mechanism of ΔpH maintenance in active and inactive cells of an obligately acidophilic bacterium. J. Bacteriol. **166**:59-65.

34. **Grinstead, D.A., and S.F. Barefoot.** 1992. Jenseniin G, a heat-stable bacteriocin produced by *Propionibacterium jensenii* P126. Appl. Environ. Microbiol. **58**:215-220.

35. **Harold, F.M.** 1986. The vital force: a study of bioenergetics. W.H. Freeman and Co., New York.

36. **Harold, F.M., and J. Van Brunt.** 1977. Circulation of H^+ and K^+ is not obligatory for bacterial growth. Science **197**:372-373.

37. **Herrero, A.A.** 1983. End-product inhibition in anaerobic fermentations. Trends Biotechnol. **1**:49-53.

38. **Herting, D.C., and E.E. Drury.** 1974. Antifungal activity of volatile fatty acids on grains. Cereal Chem. **51**:74-83.

39. **Hettinga, D.H., and G.W. Reinbold.** 1972. The propionic acid bacteria. A review. II Metabolism. J. Milk Food Technol. **35**:358-372.

40. **Hettinga, D.H., and G.W. Reinbold.** 1972. The propionic acid bacteria. A review. III Miscellaneous metabolic activities. J. Milk Food Technol. **35**:436-447.

41. **Hsu, S.T., and S.T. Yang.** 1991. Propionic acid fermentation of lactose by *Propionibacterium acidipropionici*. effects of pH. Biotechnol. Bioeng. **38**:571-578.

42. **Huang, L., L.N. Gibbins, and C.W. Forsberg.** 1985. Transmembrane pH gradient and membrane potential in *Clostridium acetobutylicum* during growth under acetogenic and solventogenic conditions. Appl. Environ. Microbiol. **50**:1043-1047.

43. **Huitson, J.J.** 1968. Cereals preservation with propionic acid. Proc. Biochem. **3**:31-32.

44. **Jimenez, J., and T. Benitez.** 1988. Selection of ethanol-tolerant yeast hybrids in pH-regulated continuous culture. Appl. Environ. Microbiol. **54**:917-922.

45. **Kashket, E.R.** 1985. The proton motive force in bacteria: a critical assessment of methods. Ann. Rev. Microbiol. **39**:219-242.

46. **Kashket, E.R.** 1987. Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. FEMS Microbiol. Rev. **46**:233-244.

47. **Kroll, R.G., and R.A. Patchett.** 1992. Induced acid tolerance in *Listeria monocytogenes*. Lett. Appl. Microbiol. **14**:224-227.

48. **Krulwich, T.A., and A.A. Guffanti.** 1986. Regulation of internal pH in acidophilic and alkalophilic bacteria. Meth. Enzymol. **125**:352-365.

49. **Langsrud, T., and G.W. Reinbold.** 1973. Flavor development and microbiology of Swiss cheese. A review. II Starters, manufacturing processes and procedures. J. Milk Food Technol. **36**:531-542.

50. **Langsrud, T., and G.W. Reinbold.** 1973. Flavor development and microbiology of Swiss cheese. A review. III Ripening and flavor production. J. Milk Food Technol. **36**:593-609.

51. **Langsrud, T., T. Sorhaug, and G.E. Vegarud.** 1995. Protein degradation and amino acid metabolism by propionibacteria. Lait **75**:325-330.

52. **Lee, I.S., J.L. Slonczewski, and J.W. Foster.** 1994. A low-pH-inducible stationary-phase acid tolerance response in *Salmonella typhimurium*. J. Bacteriol. **176**:1422-1426.

53. **Leyer, G.J., and E.A. Johnson.** 1993. Acid adaptation induces cross-protection against environmental stresses in *Salmonella typhimurium*. Appl. Environ. Microbiol. **59**:1842-1847.

54. **Lin, Y.L., and H.P. Blaschek.** 1983. Butanol production by a butanol-tolerant strain of *Clostridium acetobutylicum* in extruded corn broth. Appl. Environ. Microbiol. **45**:966-973.

55. **Luli, G.W., and W.R. Strohl.** 1990. Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. Appl. Environ. Microbiol. **56**:1004-1011.

56. **Lyon, W.J., and B.A. Glatz. 1991.** Partial purification and characterization of a bacteriocin produced by *Propionibacterium thoenii*. Appl. Environ. Microbiol. **57**:701-706.

57. **Maloney, P.C., E.R. Kashket., and T.H. Wilson.** 1974. A proton motive force drives ATP synthesis in bacteria. Proc. Nat. Acad. Sci. **71**:3896-3900.

58. **Mantere-Alhonen, S.** 1982. Die Propionibakterien der Molkereiindustrie als Darmkanalmikroben. Meijeritiet Aikak. **40**:19-23.

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

60. **McDonald, L.C., H.P. Fleming, and M.H. Hassan.** 1990. Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*. Appl. Environ. Microbiol. **56**:2120-2124.

61. **Mitchell, P., and J. Moyle.** 1969. Estimation of membrane potential and pH difference across the cristae membrane of rat liver mitochondria. Eur. J. Biochem. **7**:471-484.

62. **Mitchell, P.** 1979. Compartmentation and communication in living systems. Ligand conduction: a general catalytic principle in chemical, osmotic and chemiosmotic reaction systems. Eur. J. Biochem. **95**:1-20.

63. **Moon, N.J.** 1983. Inhibition of the growth of acid-tolerant yeasts by acetate, lactate and propionate and their synergistic mixtures. J. Appl. Bacteriol. **55**:453-460.

64. **Nanba, A., R. Nukada, and S. Nagai.** 1983. Inhibition by acetic and propionic acids of the growth of *Propionibacterium shermanii.* J. Ferment. Technol. **61**:551-556.

65. **Nannen, N.L., and R.W. Hutkins.** 1991. Intracellular pH effects in lactic acid bacteria. J. Dairy Sci. **74**:741-746.

66. **Nannen, N.L., and R.H. Hutkins.** 1991. Proton-translocating adenosine triphosphate activity in lactic acid bacteria. J. Dairy Sci. **74**:747-751.

67. **Nicholls, D.G., and S.J. Ferguson.** 1992. Bioenergetics 2. Academic Press Inc., San Diego.

68. **Ostlie, H., V. Floberhagen, G. Reinbold, E.G. Hammond, G. Vergarud, and T. Langsrud.** 1995. Autolysis of dairy propionibacteria: growth studies, peptidase activities, and proline production. J. Dairy Sci. **78**:1224-1237.

69. **Padan, E., and S. Schuldiner.** 1986. Intracellular pH regulation in bacterial cells. Meth. Enzymol. **125**:337-352.

70. **Perez-Chaia, A., A.M.S. de Saad, A.A.P. de Ruiz Holgado, and G. Oliver.** 1994. Proton-ATPase activity in cells of lactobacilli growth in the presence of propionate. J. Appl. Bacteriol. **77**:37-41.

71. **Perez-Chaia, A., A.M.S. de Saad, A.A.P. de Ruiz Holgado, and G. Oliver.** 1994. Influence of propionate on growth and fermentative activity of lactobacilli. J. Appl. Bacteriol. **77**:134-139.

72. **Perez-Chaia, A., M.E. Nader de Macias, and G. Oliver.** 1995. Propionibacteria in the gut: effect on some metabolic activities of the host. Lait **75**:435-445.

73. **Perez-Chaia, A., A.M.S. de Saad, A.A.P. de Ruiz Holgado, and G. Oliver.** 1995. Short-chain fatty acids modulate growth of lactobacilli in mixed fermentations with propionibacteria. Int. J. Microbiol. **26**:365-374.

74. **Piveteau, P.G., S. Condon, and T.M. Cogan.** 1995. Interactions between lactic and propionic acid bacteria. Lait **75**:331-343.

75. **Playne, M.J.** 1985. Propionic and butyric acids, p.731-759. *In* M. Moo-Young (ed.), Comprehensive biotechnology, vol 3. Pergamon Press, Oxford.

76. **Raeker, M.O., C.J. Bern, L.A. Johnson, and B.A. Glatz.** 1992. Preservation of high-moisture maize by various propionate treatments. Cereal Chem. **69**:66-69.

77. **Rosner, B., and B. Schink.** 1990. Propionate acts as carboxylic group acceptor in aspartate fermentation in *Propionibacterium freudenreichii.* Arch. Microbiol. **155**:46-51.

78. **Rottenberg, H.** 1979. Measurement of membrane potential and ΔpH in cells, organelles, and vesicles. Meth. Enzymol. **55**:547-569.

79. **Russell, J.B.** 1987. Effect of extracellular pH on growth and proton motive force of *Bacteroides succinogenes*, a cellulolytic ruminal bacterium. Appl. Environ. Microbiol. **53**:2379-2383.

80. **Russell J.B.** 1991. Resistance of *Streptococcus bovis* to acetic acid at low pH: relationship between intracellular pH and anion accumulation. Appl. Environ. Microbiol. **57**:255-259.

81. **Russell, J.B.** 1991. Intracellular pH of acid-tolerant ruminal bacteria. Appl. Environ. Microbiol. **57**:3383-3384.

82. **Russell, J.B.** 1992. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. J. Appl. Bacteriol. **73**:363-370.

83. **Salmond, C.V., R.G. Kroll, and I.R. Booth.** 1984. The effect of food preservatives on pH homeostasis in *Escherichia coli.* J. Gen. Microbiol. **130**:2845-2850.

84. **Sturr, M.G., and R.E. Marquis.** 1992. Comparative acid tolerances and inhibitor sensitivities of isolated F-ATPases of oral lactic acid bacteria. Appl. Environ. Microbiol. **58**:2287-2291.

85. **Swartzlander, J.H., and T.G. Rehberger.** 1993. Selection of a denitrifying strain of *Propionibacterium* and establishment in the rumen of beef cattle. abstr. Q-109, p.366. Abstr. 93rd Annu. Meet. Am. Soc. Microbiol. 1993.

86. **Tsau, J.L., A.A. Guffanti, and T.J. Montville.** 1992. Conversion of pyruvate to acetoin helps to maintain pH homeostasis in *Lactobacillus plantarum.* Appl. Environ. Microbiol. **58**:891-894.

87. **Tseng, C.P., J.L. Tsau, and T.J. Montville.** 1991. Bioenergetic consequences of catabolic shifts by *Lactobacillus plantarum* in response to shifts in environmental oxygen and pH in chemostat cultures. J. Bacteriol. **173**:4411-4416.

88. **Tuikov, R., M. Kristova, and I. Vladimirov.** 1980. Early weaning calves using milk additive and a preparation of freeze-dried bacteria. Dairy Sci. Abstr. **44**:1689.

90. **Wood, H.** 1981. Metabolic cycles in the fermentation by propionic acid bacteria, p.255-287 *In* R.W. Eastabrook and P. Srere (ed.), Current topics in cellular regulation, Vol. 18. Academic Press, New York.

91. **Woskow, S.A., and B.A. Glatz.** 1991. Propionic acid production by a propionic acid-tolerant strain of *Propionibacterium acidipropionici* in batch and semicontinuous fermentation. Appl. Environ. Microbiol. **57**:2821-2828.

92. **Woskow, S.A.** 1991. Improved production of propionic acid by strains of *Propionibacterium*. Ph.D. dissertation, Iowa State University. Ames, IA.

ACKNOWLEDGMENTS