Fermentation acids inhibit amino acid deamination by *Clostridium sporogenes* MD1 via a mechanism involving a decline in intracellular glutamate rather than protonmotive force

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Fermentation acids inhibited the growth and ammonia production of the amino-acid-fermenting bacterium *Clostridium sporogenes* MD1, but only when the pH was acidic. Such inhibition was traditionally explained by the ability of fermentation acids to act as uncouplers and decrease protonmotive force (Δp), but *C. sporogenes* MD1 grows even if the Δp is very low. Cell suspensions incubated with additional sodium chloride produced ammonia as rapidly at pH 5.0 as at pH 7.0, but cells incubated with additional sodium lactate were sensitive to even small decreases in extracellular pH. Similar results were obtained if the sodium lactate was replaced by sodium acetate or propionate. When extracellular pH declined, ΔpH increased even if sodium lactate was present. The cells accumulated intracellular lactate anion when the pH was acidic, and intracellular glutamate declined. Because amino acid deamination is linked to a transamination reaction involving glutamate dehydrogenase, the decrease in ammonia production could be explained by the decrease in intracellular glutamate. This latter hypothesis was consistent with the observation that extracellular glutamate addition restored amino acid deamination even though glutamate alone did not allow for the generation of ammonia.

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INTRODUCTION

Mankind has used fermentation to preserve animal feed for thousands of years, but spoilage can still be a problem if acid accumulation does not cause a sustained decrease in pH (Ohmomo *et al.*, 2002). This problem is exacerbated by secondary fermentations that consume acids or produce ammonia. If pH increases, the growth of moulds and other harmful micro-organisms is favoured (Wilkinson, 1999). Silage fermentation is promoted by lactic-acid-producing bacteria that consume sugar, but some silages (e.g. alfalfa haylage) have little sugar and a very high amino acid content. If amino acids are deaminated by clostridia, silage pH can increase (Ohmomo *et al.*, 2002). Amino acid degradation also decreases the nutritive value of silage as animal feed.

Clostridium sporogenes can utilize amino acids as the sole energy source (Nisman, 1954), and it can readily be isolated from silages (Flythe & Russell, 2004). *C. sporogenes* also ferments carbohydrates, but only if amino acids are present (Lovitt *et al.*, 1987). Protonmotive force (Δp) has a variety of cellular functions, and bacteria typically have a Δp of at least -100 mV (Harold, 1986). However, recent work with *C. sporogenes* MD1 indicated that growing cells had a Δp smaller than -15 mV, but Δp values as large as -120 mV could be detected if growth was inhibited by acidic pH, deprivation of an essential amino acid, or inhibitors of protein synthesis (Flythe & Russell, 2005).

The inhibition of bacterial growth by fermentation acids was traditionally explained by the ability of undissociated acids to move across the cell membrane, act as uncouplers, and decrease Δp (Jay, 1986). The question then arose of how fermentation acids would inhibit a bacterium that has virtually no Δp when it is growing. Fermentation acid toxicity has also been correlated with intracellular pH regulation (Russell & Diez-Gonzalez, 1998). If a bacterium lets its intracellular pH decrease, the pH gradient across the cell membrane (ΔpH) is lowered, and the accumulation of fermentation acid anions is not as great. This latter mechanism helps to explain why some bacteria are more sensitive to fermentation acids than others.

Abbreviations: Δp , protonmotive force; ΔpH , transmembrane pH gradient; $\Delta \Psi$, transmembrane electrical potential; TCS, tetrachlorosalicylanilide; TPP⁺, tetraphenylphosphonium ion.

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The experiments described here sought to determine the effect of fermentation acids on the membrane bioenergetics of *C. sporogenes* MD1. We hypothesized that fermentation acids would prevent Δp generation at acidic pH. Because preliminary experiments indicated that this simple hypothesis was incorrect, we decided to examine glutamate pools and amino acid transamination.

METHODS

Growth conditions. *C. sporogenes* MD1 was isolated as described by Flythe & Russell (2004) and grown anaerobically (39 °C) in basal medium that contained (per litre) 292 mg KH_2PO_4 , 480 mg Na_2SO_4 , 480 mg NaCl , 100 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 64 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 600 mg cysteine hydrochloride and 6.9 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$. The basal medium was boiled to remove O_2 and allowed to cool under O_2 -free N_2 . Enzymic casein hydrolysate (15 mg ml^{-1} , Gibco Laboratories) was added as an energy source. Initial pH was adjusted by adding HCl or NaOH.

Cell suspensions. Overnight *C. sporogenes* MD1 cells (16–24 h, 160 μg protein ml^{-1} , pH 6.7) were harvested by centrifugation (2300 g, 5 min) in anaerobic culture tubes with rubber stoppers (Bellco Glass). The supernatants were removed by aspiration and the N_2 atmosphere was maintained by continuous gassing. Cell pellets were resuspended in basal medium. The suspensions were transferred to serum bottles and amended with casein hydrolysate and/or glutamic acid. The pH was adjusted with HCl and the cell suspensions incubated for 20 min unless otherwise indicated (39 °C).

Adjustment of culture pH. *C. sporogenes* MD1 did not decrease culture pH when casein hydrolysate was the energy source, but pH could be decreased by adding HCl. To reduce pH, *C. sporogenes* MD1 was grown in an anaerobic culture vessel (250 ml, 100 ml basal medium) equipped with a pH electrode. The vessel was continuously purged with N_2 and enough HCl (6 M) was added to achieve a 0.25 unit reduction in pH. HCl was added every 15 min.

Protonmotive force. The $\Delta p\text{H}$ and transmembrane electrical potential, $\Delta\Psi$, were determined by methods employing silicone oil centrifugation as previously described (Kashket & Barker, 1977). Briefly, $\Delta p\text{H}$ was estimated from the distribution of [^{14}C]benzoate (1 μM , 0.81 GBq mmol^{-1} ; Amersham Pharmacia Biotech) across the cell membrane using the Henderson–Hasselbalch equation. The $\Delta\Psi$ was calculated from the uptake of [^3H]tetraphenylphosphonium bromide ([^3H]TPP $^+$) (10 μM , 8.51 GBq mmol^{-1} ; Amersham Pharmacia Biotech) using the Nernst equation ($-2.3 [RT/F] \times \log[(\text{concentration in})/(\text{concentration out})]$). Intracellular volume (3.6 μl mg^{-1} protein) was estimated from the difference between the distribution of $^3\text{H}_2\text{O}$ (50 μl ml^{-1} , 185 kBq μl^{-1} ; Amersham Pharmacia Biotech) and the extracellular marker [^{14}C]taurine (0.5 μM , 4.26 GBq mmol^{-1} ; Amersham Pharmacia Biotech). [^3H]TPP $^+$ and [^{14}C]benzoate uptakes were corrected for extracellular contamination using [^{14}C]taurine, an osmolyte that is not taken up, and $^3\text{H}_2\text{O}$. Growing cultures or washed cell suspensions were transferred anaerobically to N_2 -filled tubes that contained [^3H]TPP $^+$ or [^{14}C]benzoate, and incubated at 39 °C for 5 min. Corrections for non-specific [^3H]TPP $^+$ binding were made by incubating the cells with a combination of nigericin (5 μM) and valinomycin (5 μM) for 10 min to eliminate the $\Delta\Psi$.

Intracellular lactate. Cell suspensions (160 μg protein; 1 ml) in basal medium were incubated for 25 min (39 °C) with [^{14}C]lactic acid (100 mM, 3.1 MBq mmol^{-1} ; Amersham Pharmacia Biotech) prior to silicone oil centrifugation (equal parts mixture of Dexter Hysol 550 and 560). The tubes were frozen and the pellets were clipped into scintillation vials with scintillation fluid. The cell pellets

were mixed by vortexing and permitted to dissolve overnight at 25 °C before scintillation (Beckman LS56500). Intracellular lactate concentration was calculated from the ratio of supernatant to pellet radioactivity and the intracellular volume (see above). Nigericin- and valinomycin-treated cells were used to correct [^{14}C]lactate uptakes for the extracellular space in the pellet (see above).

Intracellular glutamate. Growing cells or washed cell suspensions (1 ml; 0.16 mg protein) were placed in microcentrifuge tubes containing silicone oil (400 μl , 7 parts Dexter Hysol 550 to 3 parts Dexter Hysol 560) layered on top of 100 μl 14% perchloric acid plus 9 mM EDTA. After centrifugation (13 000 g, 1 min), the perchloric acid layer was removed, neutralized with 100 μl 0.3 M K_2CO_3 and 0.8 M KOH, mixed by vortexing, and frozen at –20 °C. After thawing and centrifugation to remove precipitate (13 000 g, 5 min), samples were analysed for glutamate by a method employing glutamate dehydrogenase (Chen & Russell, 1989). Briefly, glutamate dehydrogenase (EC 1.4.1.4) and NADP^+ were mixed in a triethanolamine buffer. The sample was added and the production of NADPH was observed at 340 nm in a spectrophotometer (model 260, Gilford Instruments).

Other analyses. Cell protein from NaOH-hydrolysed cells (0.2 M NaOH, 100 °C, 15 min) was determined by the Lowry method using bovine serum albumin as a standard. Ammonia was assayed by the colorimetric method of Chaney & Marbach (1962). Optical density (600 nm) was determined using a spectrophotometer (model 260, Gilford Instruments).

Statistics. All experiments were performed three or more times. In all cases, the coefficient of variation (standard deviation divided by the mean) was less than 10%.

RESULTS

Growing cultures

C. sporogenes MD1 grew rapidly (0.4 h^{-1}) in basal medium (pH 6.7) that contained additional sodium chloride (100 mM) or sodium lactate (100 mM). In each case, the final OD_{600} was 1.2, ammonia accumulation was approximately 30 mM, and pH did not decline (data not shown). *C. sporogenes* MD1 could not initiate growth without a long lag time if the pH was less than 6.0, but by adding HCl in stepwise fashion, it was possible to assess the effect of the extracellular pH on growth (Fig. 1a). Cultures with additional sodium chloride were less susceptible to a decrease in extracellular pH than those that had additional sodium lactate. When the pH was less than 6.25, cultures with sodium lactate had a lower OD_{600} than those with sodium chloride, and ammonia production was also inhibited (Fig. 1b).

Cell suspensions

When stationary-phase *C. sporogenes* MD1 cultures grown at pH 6.7 were harvested by centrifugation and resuspended in basal medium (39 °C, 30 min), the impact of pH on the initial rate of ammonia production could be estimated. Cells incubated with additional sodium chloride (100 mM) produced ammonia as rapidly at pH 5.0 as at pH 7.0 (Fig. 2). However, cells incubated with additional sodium lactate (100 mM) were sensitive to even small decreases in

Fig. 1. (a) Effect of stepwise HCl addition on the OD₆₀₀ (circles) and pH (triangles) of *C. sporogenes* MD1 cultures. (b) Ammonia accumulation (squares). In both panels, open symbols show cultures that were grown in basal medium that contained additional sodium chloride (100 mM), and filled symbols show cultures that were grown in basal medium that contained additional sodium lactate (100 mM).

extracellular pH, and little ammonia production was observed at pH 5.0. Similar results were obtained if the sodium lactate was replaced by sodium acetate or propionate. Cells treated with a protonophore (5 μ M tetrachlorosalicylanilide, TCS) or a combination of nigericin and valinomycin (5 μ M each) were also sensitive to a decrease in extracellular pH.

Intracellular ATP, protonmotive force and intracellular pH

C. sporogenes MD1 cultures that were growing exponentially at pH 6.7 had an intracellular ATP content of 10 nmol (mg protein)⁻¹. Late-stationary-phase cells had an ATP content of less than 1 nmol (mg protein)⁻¹ at pH 6.7, but cells exposed to acidic pH (with or without sodium lactate) had as much ATP as those growing exponentially at pH 6.7 (data not shown). The ability of the *C. sporogenes* MD1 cells

Fig. 2. Effect of pH on the initial rate of ammonia production by *C. sporogenes* MD1 cell suspensions. Cells were resuspended in basal medium that had additional sodium chloride ($\circ$), sodium lactate ($\bullet$), sodium acetate ($\blacktriangle$), sodium propionate ($\blacksquare$), TCS (5 μ M) ($\triangle$), or a combination of nigericin and valinomycin (5 μ M each) ($\square$). The concentration of all the sodium salts was 100 mM. In each case, cell suspensions were acidified with HCl, and the initial cell protein concentration was 160 μ g ml⁻¹.

to maintain an ATP pool at acidic pH was consistent with their ability to generate a Δ p. The Δ p was at least fivefold greater at pH 5.2 than at pH 6.5, but this potential could be eliminated by a combination of nigericin and valinomycin (Fig. 3a). The protonophore TCS also decreased Δ pH, but TCS bound TPP⁺ and confounded the measurement of Δ Ψ . The intracellular pH of *C. sporogenes* MD1 cells declined as a function of extracellular pH, but the Δ pH was approximately 0.8 pH unit at pH 5.0. Because similar results were observed with cells resuspended in basal medium containing additional sodium chloride or sodium lactate (100 mM each) (Fig. 3b), the fermentation-acid-dependent decline in ammonia production (Fig. 2) could not be explained by a decrease in intracellular pH or Δ p. If TCS or a combination of nigericin and valinomycin was present, Δ pH could not be detected and the intracellular pH was therefore lower (Fig. 3b).

Glutamate efflux

When *C. sporogenes* MD1 cells were resuspended in basal medium that had additional sodium lactate (100 mM), intracellular lactate increased at acidic pH, and this accumulation was similar to the amount predicted by Δ pH and the Henderson–Hasselbalch equation (Fig. 4a). Because less than 2 % of the lactic acid (pK_a 3.91) was in the undissociated form even if the intracellular pH was 5.75 (Fig. 4a), the lactic acid accumulation was primarily lactate anion. When intracellular lactate anion increased, the specific activity of amino acid deamination declined (Fig. 4b). *C. sporogenes* MD1 cells resuspended with either sodium lactate or sodium chloride (100 mM) had more

Fig. 3. Effect of extracellular pH on the Δp (triangles) of *C. sporogenes* MD1 cell suspensions with additional sodium chloride (open symbols) or sodium lactate (filled symbols). Circles represent $\Delta\Psi$ (in a) and the intracellular pH (in b), and the effects of nigericin and valinomycin are also shown (squares). The dashed line in (b) indicates equal extracellular and intracellular pH. In each case, the initial cell protein concentration was $160\ \mu\text{g ml}^{-1}$, and extracellular pH was adjusted by adding HCl. The concentrations of sodium salts and ionophores were 100 mM and 5 μM , respectively.

than 100 mM intracellular glutamate at pH 6.5 (Fig. 5). Acidic pH had little impact on intracellular glutamate if sodium chloride was present, but glutamate was virtually undetectable if sodium lactate was present and the pH was 5.0. Similar results were obtained if the cells were incubated with 100 mM sodium acetate (Fig. 5), TCS (5 μM) or nigericin and valinomycin (5 μM) and the pH was 5.0 (data not shown).

Extracellular glutamate and deamination

C. sporogenes MD1 cell suspensions did not deaminate glutamate in the absence of casein hydrolysate, and glutamate addition did not stimulate ammonia production from casein hydrolysate at pH values from 6.5 to 5.0 (Fig. 6). However, large amounts of extracellular glutamate counteracted the lactate-dependent inhibition of ammonia

Fig. 4. (a) Effect of extracellular pH on the intracellular lactate anion accumulation of *C. sporogenes* MD1 cells that were resuspended in basal medium containing sodium lactate (100 mM). The dashed line shows the amount of lactate anion predicted from ΔpH and the Henderson-Hasselbalch equation. (b) Relationship of intracellular lactate anion accumulation and the deamination rate (from Fig. 3). The open circle indicates the deamination rate in the absence of sodium lactate. In each case, the initial cell protein concentration was $160\ \mu\text{g ml}^{-1}$, and pH was adjusted by adding HCl.

production. When the pH was 5.7 and lactate was 100 mM, the deamination rate was $380\ \text{nmol (mg protein)}^{-1}\text{ min}^{-1}$, but the rate increased to $650\ \text{nmol (mg protein)}^{-1}\text{ min}^{-1}$ if 60 mM glutamate was added to the cell suspensions. At pH 5.2, lactate was even more inhibitory, and glutamate once again restored ammonia production.

DISCUSSION

Textbooks still indicate that fermentation acids can act as uncouplers, and this idea is consistent with the observation that many undissociated fermentation acids can pass across the cell membrane and release protons in the more alkaline interior (Jay, 1986). The idea that lactate might act as an uncoupler in *C. sporogenes* MD1 was supported by the observation that the protonophore TCS, or a combination of nigericin and valinomycin, inhibited ammonia

Fig. 5. Effect of 100 mM sodium chloride (open bars), sodium acetate (hatched bars) or sodium lactate (filled bars) addition on the intracellular glutamate concentration of *C. sporogenes* MD1 cell suspensions that were incubated at pH 6.5 (a) or 5.0 (b). Error bars indicate the standard deviation ($n=3$).

production. However, fermentation acids did not decrease ATP or Δp at acidic pH. Because Δp was not affected, it appeared that the ability of fermentation acids to inhibit the ammonia production of *C. sporogenes* was occurring via a mechanism that did not involve a decline in Δp .

Many bacteria maintain a relatively constant intracellular pH over a wide range of extracellular pH (Padan *et al.*, 1981), but fermentative bacteria often let their intracellular pH decline when the environment is acidic (Russell & Hino, 1985; Kashket, 1987). The utility of letting intracellular pH decline when fermentation acids are present can be explained by the potential effects of anions. If undissociated fermentation acids move across the cell membrane and ionize in the more alkaline interior, the anions accumulate. When *C. sporogenes* MD1 was exposed to acidic extracellular pH and lactate was present, ΔpH increased from 0.2 unit at pH 7.0 to 0.8 unit at pH 5.0 and intracellular lactate anion accumulated exponentially. The increase in intracellular lactate was in accordance with the pK_a value of lactate, intracellular pH, and the Henderson–Hasselbalch equation.

The strategy of letting intracellular pH decline is facilitated by schemes of energy derivation that remain active at acidic pH (Russell & Diez-Gonzalez, 1998). Because the protonophore (TCS) and the ionophores decreased intracellular pH, the inhibition of ammonia production could have been at least partially explained by a decrease in intracellular pH. However, the same argument could not be made for sodium chloride- and lactate-treated cells. Ammonia production was inhibited by sodium lactate but not sodium chloride when the extracellular pH was acidic, but the declines in intracellular pH values were similar. The ammonia production rate of *C. sporogenes* MD1 declined when intracellular lactate anion accumulated, and the question then

Fig. 6. Effect of extracellular glutamate addition on the ammonia production rate of *C. sporogenes* MD1 cell suspensions. Cells were resuspended (basal medium plus 100 mM sodium lactate and glutamic acid as indicated), and the pH was adjusted to either 5.7 (filled circles) or 5.2 (open circles). In each case, the initial cell protein concentration was $160 \mu\text{g ml}^{-1}$. Controls lacking casein hydrolysate are also shown (squares).

arose how lactate was affecting the deamination of amino acids.

When Roe *et al.* (1998) treated *Escherichia coli* cells with acetic acid, intracellular pH decreased transiently, but the cells accumulated acetate anion. Because the accumulation of acetate anion caused a decrease in intracellular glutamate, it appeared that the cells were balancing one anion with another. The restoration of intracellular pH after the removal of acetate was dependent on glutamate biosynthesis, however, 'the question of how [the] growth inhibition was achieved...remained unresolved' (Roe *et al.*, 2002). Later work indicated that acetic acid also led to an inhibition of methionine biosynthesis and possibly homocysteine toxicity (Roe *et al.*, 2002).

In *C. sporogenes*, the first step in oxidative amino acid deamination typically involves a transamination reaction linked to glutamate dehydrogenase (Gottschalk, 1986). In this reaction, amino groups are transferred to 2-oxoglutarate, and glutamate is produced. Glutamate is then deaminated to release the ammonia. The glutamate dehydrogenase of *C. symbiosum* has a pH optimum of 8.0 (Coughlan *et al.* (2001). Furthermore, glutamate dehydrogenase has an equilibrium constant ($4.5 \times 10^{-14} \text{ M}$) that strongly favours glutamate formation rather than glutamate deamination (Frieden, 1959). Because relatively high concentrations of glutamate would be necessary for ammonia production, and glutamate is an intracellular osmolyte in a variety of bacteria (Neidhardt *et al.*, 1990), we decided to examine the effect of fermentation acids on the intracellular pool of glutamate. Our results indicated that lactate accumulation caused a marked decline in the

intracellular glutamate of MD1. Because this effect was not seen unless the pH was acidic and lactate was present, it appeared that lactate was inhibiting transamination via a decrease in intracellular glutamate. Based on the observation that acetate also caused glutamate efflux, it appeared that the effect was not lactate-specific.

The hypothesis that the decline in glutamate was responsible for the inhibition of ammonia production was consistent with the observation that the addition of extracellular glutamate restored the ammonia production of *C. sporogenes* MD1. Because glutamate alone was not deaminated and glutamate did not stimulate ammonia production from casein hydrolysate unless lactate was present and pH was acidic, the potential argument that glutamate itself was simply being deaminated could be dismissed. Further work will be needed to define the signal triggering glutamate efflux, but the mechanism is probably related to osmotic homeostasis.

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