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Ammonium/Urea-Dependent Generation of a Proton Electrochemical Potential and Synthesis of ATP in *Bacillus pasteurii*

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The influence of ammonium and urea on the components of the proton electrochemical potential (Δp) and de novo synthesis of ATP was studied with *Bacillus pasteurii* ATCC 11859. In washed cells grown at high urea concentrations, a Δp of -56 ± 29 mV, consisting of a membrane potential ($\Delta\psi$) of -228 ± 19 mV and of a transmembranic pH gradient (ΔpH) equivalent to 172 ± 38 mV, was measured. These cells contained only low amounts of potassium, and the addition of ammonium caused an immediate net decrease of both $\Delta\psi$ and ΔpH, resulting in a net increase of Δp of about 49 mV and de novo synthesis of ATP. Addition of urea and its subsequent hydrolysis to ammonium by the cytosolic urease also caused an increase of Δp and ATP synthesis; a net initial increase of $\Delta\psi$, accompanied by a slower decrease of ΔpH in this case, was observed. Cells grown at low concentrations of urea contained high amounts of potassium and maintained a Δp of -113 ± 26 mV, with a $\Delta\psi$ of -228 ± 22 mV and a ΔpH equivalent to 115 ± 20 mV. Addition of ammonium to such cells resulted in the net decrease of $\Delta\psi$ and ΔpH without a net increase in Δp or synthesis of ATP, whereas urea caused an increase of Δp and de novo synthesis of ATP, mainly because of a net increase of $\Delta\psi$. The data reported in this work suggest that the ATP-generating system is coupled to urea hydrolysis via both an alkalinization of the cytoplasm by the ammonium generated in the urease reaction and a net increase of $\Delta\psi$ that is probably due to an efflux of ammonium ions. Furthermore, the findings of this study show that potassium ions are involved in the regulation of the intracellular pH and that ammonium ions may functionally replace potassium to a certain extent in reducing the membrane potential and alkalinizing the cytoplasm.

Bacillus pasteurii has been intensively studied since its early description (4) because of the interesting observation that optimal growth of this bacterium is observed in alkaline media containing high concentrations of ammonium salts; urea can substitute for ammonia because of the high urease activity of the bacterium (5, 8). The physiological basis for these requirements was studied by Wiley and Stokes (29, 30), who suggested that the alkaline pH was needed to convert NH_4^+ to free NH_3 and that the latter was necessary for the transport of substrates at low concentrations across the cell membrane. Other authors suggested that the dependence on high ammonium concentrations was due to the absence of an ammonium transport system and of a glutamine synthetase in this bacterium (19). Recently it has been shown that transport of glutamine is stimulated by ammonium in *B. pasteurii*; this stimulation was observed in cells grown at high ammonium or urea concentrations, whereas in cells grown at low concentrations of urea or ammonium, no stimulatory effect of ammonium was observed (10). The stimulation of amino acid transport by ammonium has also been observed in *Amphibacillus xylanus* (13); this stimulation has been assumed to be due to the direct activation of a sodium-translocating ATPase by ammonium, which generates an electrochemical sodium potential necessary for substrate transport (14).

In the present study, the influence of ammonium and urea on components of the proton motive force (Δp), the membrane potential ($\Delta\psi$) and the proton gradient (ΔpH), and that on the synthesis of ATP were examined in order to assess the role of ammonium and urea in the generation and the maintenance of these chemical/electrochemical parameters in *B. pasteurii*. Ammonium and urea were found to exert profound effects on both the membrane potential and the intracellular pH, thereby affecting the synthesis of ATP in this moderate alkaliphile.

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MATERIALS AND METHODS

Microorganism and culture conditions. *B. pasteurii* ATCC 11859 was grown aerobically at 28°C in a medium containing 20.0 g of yeast extract per liter and the amount of urea indicated in each experiment; the pH was adjusted to 9.0 with NaOH prior to autoclaving. The cells were harvested from early exponential growth and washed twice with a buffer containing 20 mM CAPSO (3-cyclohexylamino-2-hydroxy-1-propanesulfonic acid), 20 mM NaCl, and 100 μg of chloramphenicol ml^{-1} at pH 9.0 (buffer A).

Measurement of ΔpH and $\Delta\psi$. Washed cells were resuspended in buffer A to give a cell density of between 0.2 and 0.3 mg of protein ml^{-1}; 1 ml of the suspension was incubated at 25°C for 5 min with stirring, before the radiolabelled probe was added. $\Delta\psi$ was determined by addition of 2.5 μM [*phenyl*-^{14}C]tetraphenylphosphonium bromide ([*phenyl*-^{14}C]TPP) (1.85×10^5 Bq; 0.74×10^8 Bq $mmol^{-1}$) and centrifugation in Eppendorf tubes (3 min at $12{,}000 \times g$) of 250 μl of the cell suspension after 2, 8, and 15 min through a 200-μl silicon oil layer (a mixture of 6 volumes of silicon oil AR 200 [d = 1.04; Serva, Heidelberg, Germany] and 1 volume of silicon oil DC 200 [d = 0.96; Serva]). One hundred microliters of the cell-free supernatant was added to 0.4 ml of 0.4 N NaOH before 5.5 ml of the scintillation cocktail (Quicksafe A; Zinsser Analytic) was added. After the remainder of the supernatant and most of the oil were carefully aspirated, the tip of the Eppendorf tube containing the cell pellet was cut off and the cell pellet was added to 0.4 ml of 0.4 N NaOH; after 24 h of incubation at 30°C, 5.5 ml of scintillation cocktail was added and radioactivity was determined by liquid scintillation counting. Since the apparent $\Delta\psi$ determined by the measurement of the distribution of [*phenyl*-^{14}C]TPP may depend on the [*phenyl*-^{14}C]TPP concentration used (2, 9), the concentration of [*phenyl*-^{14}C]TPP was varied between 0.5 and 10.0 μM in one set of experiments; no significant differences in the values of $\Delta\psi$ determined after a 15-min incubation with various concentrations of [*phenyl*-^{14}C]TPP were observed (results not shown). ΔpH ($z\Delta$pH, with z = 59 mV) was determined from the distribution of 2.5 μM [^{14}C]methylamine (1.85×10^5 Bq; 2.07×10^8 Bq $mmol^{-1}$) after 2, 8, and 15 min of incubation with this compound, as described above. This technique could be used without interference from an ammonium transport system, since *B. pasteurii* lacks such a system (19). Controls for energy-independent probe binding were performed for each assay of both the determination of the membrane potential and the intracellular pH by preincubating cells with 20 μM gramicidin D for 15 min prior to the addition of the radiolabelled compounds; the values for $\Delta\psi$ were corrected for nonspecific binding by the exponential mean model according to

TABLE 1. Influence of monovalent inorganic cations and urea on *B. pasteurii* grown at different urea concentrations[a]

Compound added (concn)	Electrochemical parameter or ATP content[b] in cells grown at urea concn							
	15 mM				300 mM			
	$\Delta\psi$ (mV)	ΔpH (mV)	Δp (mV)	ATP (mM)	$\Delta\psi$ (mV)	$z\Delta$pH (mV)	Δp (mV)	ATP (mM)
None	−228 ± 22	115 ± 20	−113 ± 26	4.05 ± 0.50	−228 ± 19	172 ± 38	−56 ± 29	0.41 ± 0.21
None[c]	−215 ± 21	32 ± 24	−183 ± 28	5.22 ± 0.42	−178 ± 16	22 ± 17	−146 ± 22	5.10 ± 1.52
Urea (50 mM)	−245 ± 22	85 ± 18	−160 ± 35	6.62 ± 0.35	−248 ± 19	115 ± 15	−133 ± 30	0.84 ± 0.50
Urea (50 mM)[d]	−235 ± 18	75 ± 9	−160 ± 22	6.50 ± 1.20	−236 ± 12	96 ± 10	−140 ± 15	5.80 ± 0.50
NH_4Cl (50 mM)	−170 ± 20	50 ± 18	−120 ± 20	4.00 ± 1.16	−167 ± 28	62 ± 22	−105 ± 10	6.60 ± 1.20
KCl (50 mM)	−205 ± 15	90 ± 10	−115 ± 12	3.95 ± 0.09	−145 ± 18	105 ± 10	−30 ± 6	0.43 ± 0.10
NaCl (50 mM)	−228 ± 12	102 ± 20	−126 ± 22	4.05 ± 0.22	−232 ± 14	165 ± 14	−67 ± 12	0.45 ± 0.20

[a] Values for electrochemical parameters and ATP contents were determined 2 and 3 min, respectively, after the addition of the indicated compounds to cells harvested from exponential growth, washed twice, and resuspended in buffer A at 30°C, except as indicated.
[b] All values are means ± standard errors of the means for three independent experiments.
[c] Cells were washed twice and resuspended in the supernatant of the first centrifugation step (growth medium) at 30°C.
[d] Urea was added 15 min prior to the determination of the electrochemical parameters and ATP content.

the method of Zaritsky et al. (31). In some experiments, both the ΔpH across the membranes and the $\Delta\psi$ were monitored by the change of fluorescence of 9-aminoacridine or rhodamine 6G, respectively (16), with the modifications described previously (10). The total and intracellular water space were earlier determined to be 4.5 and 2.25 μl mg of protein^{-1}, respectively (10); all calculations of the electrochemical parameters are based on these values.

Determination of cellular ATP, potassium, and ammonium. Washed cells were suspended in buffer A to give a cell density of between 0.1 and 0.15 mg of protein ml^{-1}. ATP was extracted by rapidly mixing 100 μl of this cell suspension with 900 μl of boiling Tris-EDTA (20 mM Tris–1 mM EDTA, pH 7.7) for 10 s; after cooling on ice, light emission was recorded 10, 30, and 50 s after the addition of 100 μl of ATP-monitoring reagent (firefly lantern extract FLE 50; Sigma, Deisenhofen, Germany) to 100 μl of the extract in a luminometer (LUMIStox, Dr. Lange GmbH, Berlin, Germany); the ATP content was calculated after extrapolation to 0 s from a calibration curve with Na_2-ATP as a standard. Potassium was determined by flame photometry after separation of the cells from the external medium by silicon oil centrifugation (18). Internal and external ammonium concentrations were determined colorimetrically as described elsewhere (28).

Determination of ATPase activity. ATPase activity was determined in membrane vesicles prepared from exponentially growing cells. Spheroplasts prepared according to the method of Kaback (11) were suspended in 20 mM Tris-HCl, pH 8.5, containing 20 mM KCl and 3 mM $MgCl_2$ and submitted to ultrasonic treatment (5 s ml^{-1} at an output of 60 W; Branson Sonifier B 12); the membranes were washed twice in 20 mM Tris-HCl–20 mM KCl–3 mM $MgCl_2$, pH 8.5. ATPase activity was assayed in this buffer containing 10 mM Na_2-ATP at the pH and the ammonium concentrations indicated at 30°C by measuring the release of P_i as described elsewhere (26). One enzyme unit was defined as the release of 1 μmol of P_i per min.

Protein assay. Protein was determined as previously described (27), with the modification that only 10% of the indicated amount of potassium iodide was used; bovine serum albumin served as a standard.

Reproducibility of results. The data in Fig. 1 to 5 and in Table 4 are representative results from experiments that were repeated at least three times. The experiments presented in Tables 1 to 3 were repeated three times, and the ranges are indicated.

Chemicals. [^{14}C]methylammoniumchloride (2.07×10^9 Bq mmol^{-1}) was from Amersham Buchler and Co. KG, Braunschweig, Germany; [*phenyl*-^{14}C]TPP (0.74×10^9 Bq mmol^{-1}) was purchased from NEN DuPont de Nemours, Bad Homburg, Germany. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was obtained from Serva; *N*,*N*′-dicyclohexylcarbodiimide (DCCD) was supplied by Gibco BRL, Paisley, United Kingdom. Valinomycin, gramicidin, and the fluorescence dyes were from Sigma. All other chemicals were purchased from Merck, Darmstadt, Germany.

RESULTS

Electrochemical parameters of *B. pasteurii*. The values for $\Delta\psi$ and ΔpH were determined both in cells washed twice and suspended in buffer A and in cells washed twice with buffer A and resuspended in the supernatant of the first centrifugation step (growth medium). The data in Table 1 indicate that the intracellular pH of cells resuspended in buffer A was significantly more acidic than that of cells resuspended in growth medium; this effect was more pronounced in cells grown at a high urea concentration than in cells grown at a low concentration of this compound (Table 1). The values for $\Delta\psi$ were identical in cells grown at different urea concentrations and resuspended in buffer A; significantly lower values of $\Delta\psi$ were measured in cells resuspended in the growth medium, especially after growth at a high urea concentration (Table 1). The determination of intracellular pH with permeant amines at low values of ΔpH is known to be unreliable; however these data were confirmed in experiments in which the intracellular pH was estimated by measuring the fluorescence change of 9-aminoacridine (results not shown). The findings suggested that compounds of the growth medium were necessary for the maintenance of an alkaline pH in *B. pasteurii*, especially when grown at a high urea concentration. Since the only difference between the media was the urea concentration used for growth and since urea was completely hydrolyzed by the time the cells were harvested, the influences of both urea and ammonium on ΔpH and $\Delta\psi$ were investigated. The experiments were performed at pH 9.0, near the pK of ammonium (9.25), and no increase or decrease of the extracellular pH of more than 0.1 pH unit was measurable during the assay periods in these studies.

The addition of 50 mM ammonium to cells grown at a high concentration of urea resulted in immediate alkalinization of the cytoplasm accompanied by a net decrease of $\Delta\psi$ and resulted in a net increase of Δp. These changes were insignificant when ammonium was added at concentrations between 2 and 10 mM (results not shown); the addition of up to 300 mM ammonium had the same effects on $\Delta\psi$ and ΔpH as shown in Fig. 1. In cells grown at a low urea concentration, the addition of ammonium also resulted in a net decrease of $\Delta\psi$ and an alkalinization of the cytoplasm; however, no significant net increase of Δp was measured (Fig. 1).

The addition of urea at concentrations between 25 and 200 mM resulted in an increase of Δp in cells grown at both high and low urea concentrations (Fig. 2); at urea concentrations between 1 and 5 mM, almost no effect on Δp was observed (results not shown). Measurements of the change of fluorescence of rhodamine 6G indicated a rapid initial increase of $\Delta\psi$ (within less than 15 s after the addition of urea) under these conditions (results not shown). These findings were confirmed by measurements of the distribution of the lipophilic cation [*phenyl*-^{14}C]tetraphenylphosphonium over the cell membrane: an initial net increase of $\Delta\psi$, accompanied by a slow increase of intracellular pH, resulted in an overall increase of Δp (Fig. 2). In order to determine the specificities of ammonium and urea

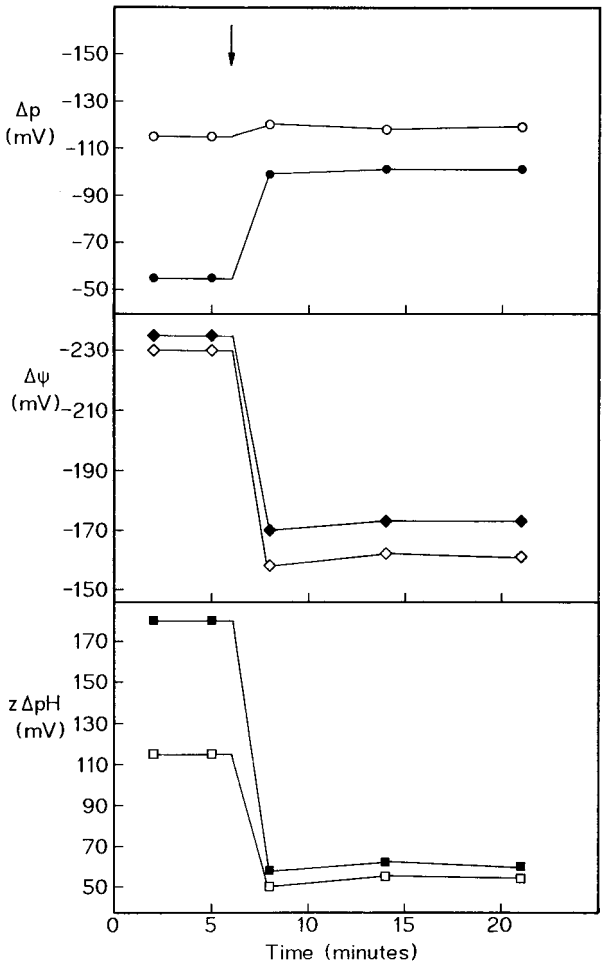

FIG. 1. Effect of ammonium on components of Δ*p* in *B. pasteurii*. Cells grown at urea concentrations of 15 mM (open symbols) or 300 mM (closed symbols) were harvested from exponential growth, washed twice, and incubated in buffer A at 30°C. The arrow indicates the addition of ammonium chloride at a final concentration of 50 mM.

in affecting the electrochemical parameters in *B. pasteurii*, the influences of both other monovalent cations and permeant amines on Δψ and ΔpH were studied. The addition of methylamine and ethanolamine resulted in an increase of the intracellular pH, a net decrease of Δψ, and a net increase of Δ*p* similar to those observed upon the addition of ammonia (results not shown); of the other monovalent cations tested, only potassium significantly affected the electrochemical parameters, decreasing Δψ and increasing the intracellular pH (Table 1).

Intracellular ATP concentration and ATPase activity. Both urea and ammonium profoundly affected Δ*p* in *B. pasteurii*, and the influence of these compounds on the ATP content was subsequently studied in cells grown at different concentrations of urea. The ATP content of *B. pasteurii* obviously correlated with the determined electrochemical parameters (Table 1); while low intracellular ATP concentrations were observed in resting cells grown at high urea concentrations, about a 10-fold increase in concentration was measured in cells grown at low concentrations of urea. ATP synthesis was strongly stimulated by urea and ammonium in cells grown at high urea concentrations but was only slightly stimulated in cells grown at low urea concentrations (Table 1); the addition of other permeant amines (methylamine and ethanolamine) had the same effects on ATP synthesis as those observed upon addition of ammonia (results not shown). This urea- and ammonium-mediated stimulation of ATP synthesis was observed in a range between pH 7.5 and 10.5 in 50 mM Tris-HCl, buffer A, and 50 mM glycine-NaOH (results not shown); incubation of the cells at a pH below 7.5 or above 11 resulted in a rapid loss of viability in both the presence and absence of potassium and/or sodium ions (results not shown). Acetohydroxamic acid, a potent inhibitor of bacterial ureases, almost completely abolished the urea-stimulated but not the ammonium-stimulated ATP synthesis (Table 2). The inhibition of ATPase by DCCD and the reduction of Δψ by the addition of KCl and valinomycin severely inhibited both the urea- (Table 2) and the ammonium-stimulated synthesis of ATP (results not shown); the protonophore CCCP had only a weak inhibitory effect (Table 2). The presence of the ionophore gramicidin completely inhibited de novo ATP synthesis; contrary to the results with CCCP, the ΔpH was rapidly abolished by gramicidin, as observed in experiments measuring the fluorescence quench of 9-aminoacridine (Fig. 3).

The time course of ammonium/urea-stimulated ATP synthesis was studied in cells grown at high concentrations of urea. Despite the immediate increase of Δ*p* upon the addition of

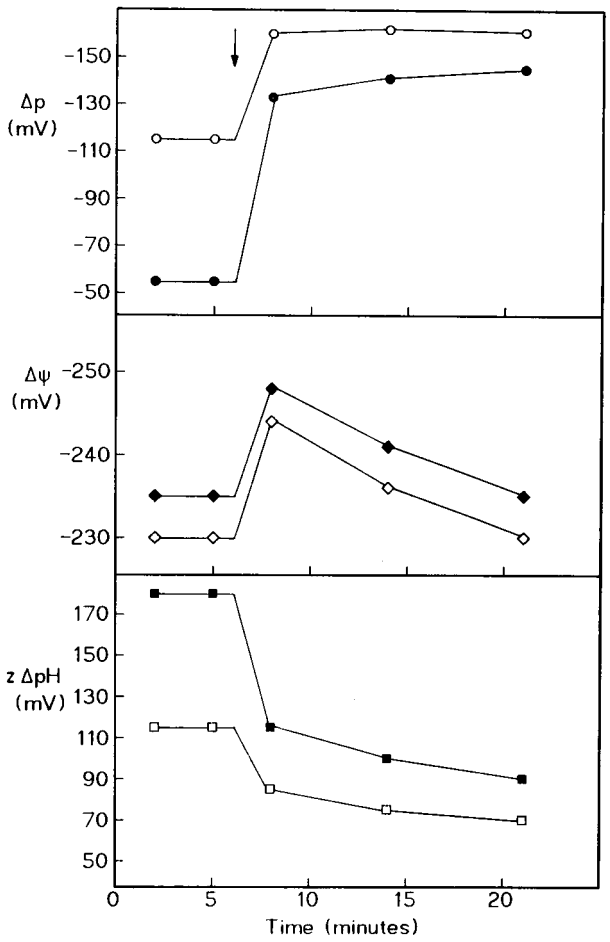

FIG. 2. Effect of urea on components of Δ*p* in *B. pasteurii*. Cells grown at urea concentrations of 15 mM (open symbols) or 300 mM (closed symbols) were harvested from exponential growth, washed twice, and incubated in buffer A at 30°C. The arrow indicates the addition of urea at a final concentration of 50 mM.

TABLE 2. Effect of metabolic inhibitors and acetohydroxamic acid on urea-stimulated ATP synthesis in *B. pasteurii*[a]

Compound(s) added (concn[s])	Intracellular ATP (mM)[b]
None	6.45 ± 0.45
Acetohydroxamic acid (1 mM)	0.92 ± 0.66
Acetohydroxamic acid (1 mM) + NH_4Cl (50 mM)	4.20 ± 1.08
CCCP (50 μM)	6.00 ± 0.55
CCCP (200 μM)	4.65 ± 1.13
DCCD (20 μM)	5.50 ± 1.00
DCCD (100 μM)	0.90 ± 0.44
Valinomycin (2 μM)	3.60 ± 0.75
Valinomycin (2 μM) + KCl (20 mM)	0.30 ± 0.10
Gramicidin (10 μM)	0.49 ± 0.08

[a] Cells grown with 300 mM urea and harvested from exponential growth were washed twice and resuspended in buffer A. The cells were incubated in the presence of the indicated compounds for 10 min at 30°C prior to the addition of urea at a final concentration of 50 mM; the ATP content was determined 10 min later. CCCP, DCCD, gramicidin, and valinomycin were added as solutions in dimethyl sulfoxide (0.5% [vol/vol]); dimethyl sulfoxide alone had no effect.

[b] Values are means ± standard errors of the means for three independent experiments.

urea (Table 1), the urea-mediated stimulation of ATP synthesis started with a lag after the addition of urea, when the internal pH had risen above 7.0 (Fig. 4). The addition of ammonium led both to an immediate rise of the cytoplasmic pH and to de novo synthesis of ATP (Fig. 4); the simultaneous addition of both ammonium and urea resulted in both the rapid alkalinization of the cytoplasm and the immediate onset of ATP synthesis (Fig. 4).

The influence of ammonium and urea on the ATPase activity was studied in more detail in order to differentiate between a direct stimulation of ATPase by ammonium, as it has been described for the enzyme of a facultatively anaerobic alkaliphile strain Ep01 (*A. xylanus*; 14), and an indirect stimulation via an alkalinization of the cytoplasm and formation of Δp. In *B. pasteurii*, ATPase activity exhibited a broad pH optimum between pH 7.5 and 9.0, with only low levels of activities

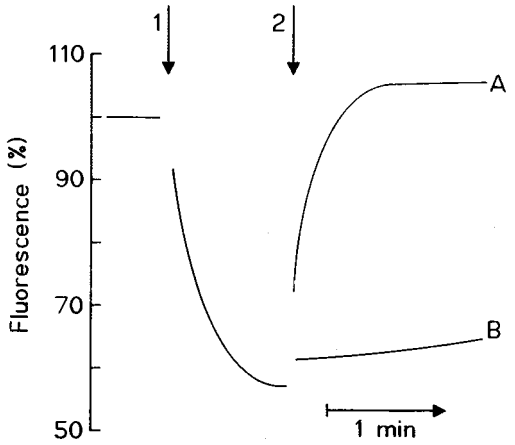

FIG. 3. Effects of CCCP and gramicidin on pH in *B. pasteurii*. Cells grown at a urea concentration of 300 mM were harvested from exponential growth, washed twice, and resuspended in buffer A at 30°C. At the times indicated (arrows), the cells (arrow 1; final cell density, 0.08 mg of protein ml^{-1}) and 10 μM gramicidin (arrow 2; trace A) or 100 μM CCCP (arrow 2; trace B) were added to buffer A containing 2 μM 9-aminoacridine. Downward deflection shows the decrease in fluorescence intensity and is indicative of an increase of ΔpH, with an acidic intracellular pH. The fluorescence monitor did not allow exact measurements 10 s prior to and after the addition of cells and ionophores.

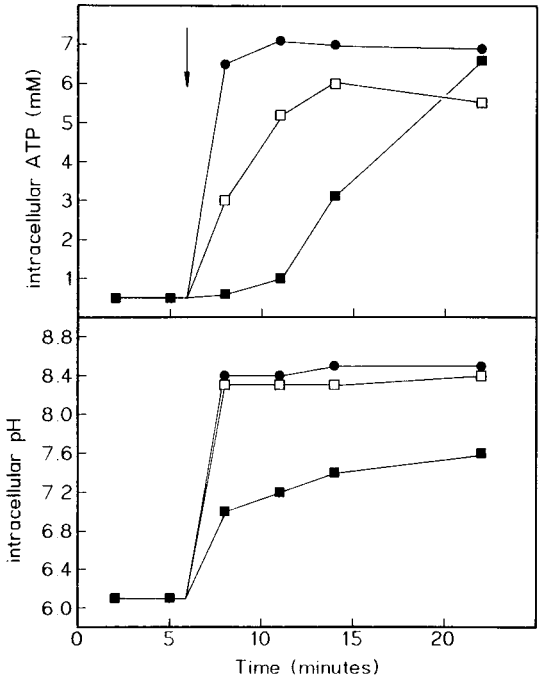

FIG. 4. Effects of ammonium and/or urea on the intracellular pH and ATP content in *B. pasteurii*. Cells grown at a urea concentration of 300 mM were harvested from exponential growth, washed twice, and incubated in buffer A at 30°C. The arrow indicates the addition of 50 mM ammonium chloride (□), 50 mM urea (■), or 50 mM both compounds (●).

observed at pH 7.0 (Fig. 5), explaining the observed lag of urea-stimulated ATP synthesis despite the immediate increase of Δp (Table 1; Fig. 4). Urea and ammonium at concentrations between 5 and 500 mM did not stimulate ATPase activity at any pH; ammonium became inhibitory at concentrations above 250 mM (Fig. 5), concentrations that are observed in *B. pasteurii* under growth conditions (see below).

Transmembrane gradient of potassium and ammonium. Potassium is known to exert profound effects on the intracellular

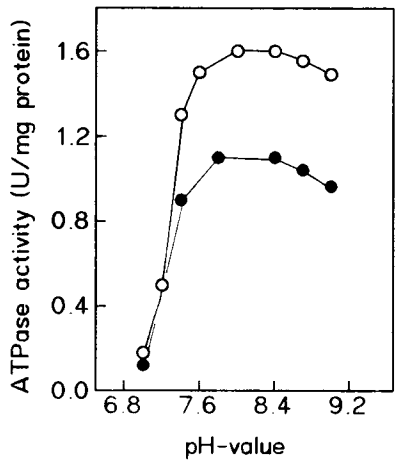

FIG. 5. Influence of ammonium chloride and pH on ATPase activity in membranes of *B. pasteurii*. Cells grown at a urea concentration of 300 mM were harvested from exponential growth and washed twice in 20 mM Tris-HCl, pH 8.5, containing 20 mM KCl and 3 mM $MgCl_2$. ATPase activity in membranes prepared in this buffer was determined in the presence (●) or the absence (○) of 250 mM ammonium chloride.

TABLE 3. Intracellular and extracellular concentrations of ammonium and potassium in *B. pasteurii* grown at different urea concentrations

Urea concn (mM) at which cells were grown	Concn (mM)			
	K^+_{in}	K^+_{out}	$NH_4^+{}_{in}$	$NH_4^+{}_{out}$
15[b]	705 ± 54	18 ± 2	320 ± 45	45 ± 5
15[c]	688 ± 41	ND[d]	15 ± 8	0.1 ± 0.1
300[b]	26 ± 14	30 ± 2	620 ± 82	595 ± 25
300[c]	22 ± 4	ND	152 ± 44	0.8 ± 0.2

[a] Values are means ± standard errors of the mean for three independent experiments. K^+_{in}, intracellular potassium; K^+_{out}, extracellular potassium; $NH_4^+{}_{in}$, intracellular ammonium; $NH_4^+{}_{out}$, extracellular ammonium.
[b] Cells centrifuged once and resuspended in the supernatant.
[c] Cells washed twice with and resuspended in buffer A.
[d] ND, not detectable (<0.1 mM).

pH in alkaliphilic bacteria (16, 17, 22), and ammonium functionally replaced potassium to a certain extent in a number of microorganisms studied so far (6, 7). Since the addition of ammonium to resting cells of *B. pasteurii* had the drastic effects on intracellular pH described above, the transmembrane gradients of ammonium and potassium were determined. As shown in Table 3, significant differences in intracellular potassium and ammonium concentrations were observed in cells grown at different urea concentrations. For *Ureaplasma urealyticum*, ATP synthesis has been described as being coupled to a chemical potential of ammonium generated upon the addition of urea (24, 25, 28). When the transmembranic gradient of ammonium in *B. pasteurii* was determined under similar conditions, it was obvious that even if the intracellular ammonium concentration increased upon the addition of urea to resting cells grown at high urea concentrations, the concentration gradient ($NH_4^+{}_{in}/NH_4^+{}_{out}$, where $NH_4^+{}_{in}$ and $NH_4^+{}_{out}$ are intracellular and extracellular ammonium concentrations, respectively) was highest prior to the addition of urea (Table 4).

DISCUSSION

The results presented in this work clearly show that both ammonium and urea exhibit profound effects on electrochemical parameters and ATP synthesis in *B. pasteurii*, especially when the cells were grown under optimal conditions, i.e., at a high concentration of urea. High ammonium concentrations in *B. pasteurii* were measured under these conditions, and washing of these cells resulted in a depletion of internal ammonium (Table 3). This resulted in unusually high values of Δψ, since cells resuspended in the growth medium exhibited significantly lower values of Δψ (Table 1), which are in the range of those observed in other alkaliphiles (9). Obviously intracellular ammonium ions reduce Δψ and allow the cells to pump out more protons to alkalinize their cytoplasm under these growth conditions, and the loss of ammonium results in an increase of Δψ and decrease of the cytoplasmic pH (Table 1). The addition of ammonium or other permeant amines to such cells resulted in a net increase of Δ*p*; this increase was due to the alkalinization of the cytoplasm, since a net decrease of Δψ was observed under these conditions (Fig. 1; Table 1). These effects were observed only upon the addition of ammonium at concentrations above 20 mM, which are in the physiological range for growing cells of *B. pasteurii*. Similar observations were made for *Enterococcus hirae*; 5 mM ammonium had almost no effect on the cytoplasmic pH of this organism, whereas rapid alkalinization occurred upon the addition of 50 mM of this permeant amine (12). For *B. pasteurii* growing at high urea concentrations, ammonium is obviously necessary for a sufficient alkalinization of the cytoplasm; the observed reduction of the membrane potential upon the addition of ammonium to washed cells may result from both an influx of NH_4^+, directly reducing Δψ, and a diffusion of NH_3 into the cell, resulting in the observed alkalinization of the cytoplasm and allowing an influx of protons via ATP synthetase.

The addition of urea to cells grown at a high concentration of this compound led to a net increase of Δψ and an alkalinization of the cytoplasm, resulting in an increase of Δ*p* (Fig. 2, Table 1); this increase depended on the intracellular urease activity, since it was prevented by the urease inhibitor acetohydroxamic acid (Table 2). It may be assumed that after the hydrolysis of urea, a protonation of ammonia generated by the hydrolysis of urea occurs and that this is followed by its efflux. A concentration difference of ammonium (inside the cells/outside the cells) of up to 30-fold was observed after the addition of 50 mM urea to resting cells of *B. pasteurii* (Table 4), a difference which corresponds to a Δ*p* of 85 mV for ammonium, calculated as described previously (20). The concentration gradient of ammonium may act as a driving force for such an efflux; when appearing in the extracellular space, a significant amount of the excreted ammonium is deprotonated at an extracellular pH of 9.0; therefore, the NH_4^+ concentration difference would even be higher. However, the highest ammonium concentration gradients, of approximately 150-fold, were observed in washed cells of *B. pasteurii* prior to the addition of extracellular urea (Table 4); under these conditions, the efflux of ammonium may be prevented at least in part by the Δψ of −228 mV, retaining the positively charged NH_4^+ in the cytoplasm. At an intracellular pH of 6.1 observed under these conditions (Fig. 5), a reduction of Δψ via an ATP synthetase-mediated proton flux and simultaneous synthesis of ATP is inhibited. Upon addition of urea, the intracellular pH rapidly rises about 0.9 units to pH 7.0 (Table 4), and the influx of protons via the ATP synthetase, generating ATP and reducing Δψ, may support an efflux of ammonium ions. Future studies will have to show if an efflux system for ammonium ions is involved in this process; the presence of such a system has been discussed for *U. urealyticum*, for which a net increase of Δψ of about 20 mV has been observed upon the addition of urea (28). This effect of urea was by far more pronounced in *B. pasteurii* than in *U. urealyticum*, both with respect to the increase of Δ*p* and the pH range at which stimulation of ATP synthesis was observed. While the stimulation of ATP synthesis occurred in a narrow pH range around 6.0 in *U. urealyticum*, ATP synthesis in *B. pasteurii* was enhanced within the pH range of 7.5 to 9.5. Furthermore, simultaneous addition of

TABLE 4. Effects of urea on the intracellular and extracellular ammonium concentrations, cytoplasmic pH, and ATP content in *B. pasteurii*[a]

Time (min)	Concn (mM)			pH_{in}
	$NH_4^+{}_{in}$	$NH_4^+{}_{out}$	ATP_{in}	
−5	142	0.8	0.58	6.1
−2	135	0.8	0.56	6.1
2	354	11.4	0.80	7.0
5	361	40.8	2.15	7.2
10	405	70.5	6.40	7.4
20	395	92.0	6.35	7.5

[a] Cells grown at a urea concentration of 300 mM were harvested from exponential growth, washed twice, and resuspended in buffer A at 30°C. At 0 min, 50 mM urea was added. $NH_4^+{}_{in}$, intracellular ammonium; $NH_4^+{}_{out}$, extracellular ammonium; ATP_{in}, intracellular ATP; pH_{in}, intracellular pH.

both ammonium and urea to resting cells of *B. pasteurii* resulted in the immediate synthesis of ATP, which was higher than after the addition of urea or ammonium alone (Fig. 4). Ammonium has been shown to drastically inhibit the urea-stimulated synthesis of ATP (25), in *U. urealyticum*, and this was ascribed to a reduction of the ammonium chemical potential by external ammonium.

As expected, ATP synthesis in *B. pasteurii* was severely inhibited by DCCD, an inhibitor of F_0F_1 ATPases (Table 2). Although very effective in inhibiting the uptake of glutamine in *B. pasteurii* (10), the protonophore CCCP had only minor effects on the urea-induced ATP synthesis in this organism (Table 2), and the addition of 100 μM CCCP led to almost no increase of the acidic intracellular pH (Fig. 3). The acidification of the cytoplasm in these cells may be caused by a Donnan potential; the involvement of such a Donnan potential in the acidification of the cytoplasm of the facultatively anaerobic alkaliphile strain Ep01 (15), now classified as *A. xylanus* (21), has also been discussed. In such a case, the formation of ΔpH would depend on the proton permeability of the membrane and would primarily not be dissipated in the presence of a protonophore. Assuming that CCCP abolishes Δp (by equating $\Delta\psi$ and ΔpH terms to be equal and opposite), a large flux of ammonia occurring upon the addition and hydrolysis of urea may swamp the effect of this protonophore in *B. pasteurii* and permit the synthesis of ATP; a similar suggestion has been made for the urea-induced synthesis of ATP in *U. urealyticum* (28). As expected, valinomycin and KCl, abolishing the membrane potential in *B. pasteurii* (results not shown), and gramicidin, inducing a flux of monovalent cations across the membrane and dissipating ΔpH (Fig. 3), completely inhibited the urea-stimulated de novo synthesis of ATP.

In cells grown at a low concentration of urea, both a higher Δp due to a more alkaline cytoplasm and higher intracellular ATP concentrations than in cells grown at high urea concentrations were measured. The addition of ammonium did not result in de novo ATP synthesis; the alkalinization of the cytoplasm due to the diffusion of ammonia into the cytoplasm and its subsequent protonation to NH_4^+ was accompanied by a decrease of the membrane potential, probably due to an influx of ammonium ions, and resulted in almost no net increase of Δp. Upon the addition of urea, net increases of Δp and de novo ATP synthesis were measured in these cells, observations similar to those made for cells grown at high urea concentrations. While a lag between the addition of urea and increase of intracellular ATP in cells grown at high urea concentrations was observed, an immediate rise of the cellular ATP concentration was measured upon the addition of urea in cells grown at low concentrations of this compound (Table 1), obviously due to the more alkaline cytoplasmic pH permitting an immediate onset of ATP synthesis. This more alkaline intracellular pH in *B. pasteurii* grown at a low urea concentration correlated with higher intracellular potassium concentrations compared with those of cells grown at a high urea concentration (Table 3), even after repeated washing of the cells. Potassium probably reduces the membrane potential, which allows the cell to pump out more protons, thus converting $\Delta\psi$ into a ΔpH. Such an interconversion of the components of Δp in the presence of potassium ions has been shown to occur in a number of microorganisms (1, 3), and K^+-depleted cells of *Streptococcus lactis* and *Rhodobacter sphaeroides* were unable to regulate their cytoplasmic pHs (1, 23). In *B. pasteurii*, the addition of potassium resulted in a net decrease of $\Delta\psi$, especially in cells grown at a high concentration of urea (Table 1); this was probably due to a rapid electrogenic K^+ uptake into these potassium-depleted cells (Table 3), and was accompanied by an alkalinization of the cytoplasm (Table 1). Because of their fast transmembranic diffusion and intracellular protonation, permeant amines may substitute for potassium in *B. pasteurii* in leading more directly to an alkalinization of the cytoplasm and an increase of Δp. This would allow the influx of protons via the ATP synthetase and lead to a reduction of the membrane potential and ATP synthesis (Fig. 1 and 4; Table 1). The direct stimulation of ATPase by ammonium, as described previously for *A. xylanus* (14), was not observed in *B. pasteurii.*

The results of the present study show that in *B. pasteurii*, ammonium is involved in the regulation of the internal pH and replaces potassium under certain growth conditions. Furthermore, several factors are involved in the stimulation of ATP synthesis. Upon the addition of urea and/or ammonium, an alkalinization of the cytoplasm occurs that results in both an increase of Δp and an activation of ATPase, which is almost inactive at pHs below 6.8 (Fig. 5). Efflux of NH_4^+ generated by the hydrolysis of urea further leads to an increase of the membrane potential and thereby to an increase of Δp.

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