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Metabolism of Adenylylated Nucleotides in *Clostridium acetobutylicum*

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In response to the stresses imposed by temperature upshift or addition of butanol, *Clostridium acetobutylicum* cultures accumulated diadenosine-5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) and adenosine 5′-P^1,P^4-tetraphospho-5′-guanosine (Ap_4G) to high levels. The two adenylylated nucleotides were also accumulated in batch culture in the absence of imposed stresses when the clostridia switched from the acidogenic phase of growth to the solventogenic phase. Most of the adenylylated nucleotides were extracellular. The intracellular concentrations of these compounds were low throughout batch growth and in cells stressed by added butanol. In contrast to other procaryotes, these clostridia did not possess enzymes to degrade the dinucleotides, as shown with both intact cells and cell-free preparations. Our findings are consistent with the hypothesis that endogenously produced solvents are stressful to the cells, stimulating the synthesis of adenylylated nucleotides. The nucleotides accumulate extracellularly because they cannot be degraded and because the cell membranes are permeabilized by the solvents produced.

Growing *Clostridium acetobutylicum* cells ferment sugars to acetic and butyric acids and, at the end of exponential growth, switch to the production of butanol, acetone, and ethanol and initiate sporulation (reviewed in references, 9, 10, and 21). The mechanisms by which solventogenesis and sporulation are initiated and regulated are not known. Unlike bacilli, clostridia do not sporulate in response to nutrient limitation (15).

The present study was initiated to explore the possibility that solventogenesis and sporulation occur as the result of a stress response. We investigated the metabolism of adenylylated nucleotides, such as diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A), because these compounds have been postulated to act as regulatory molecules in both procaryotic and eucaryotic cells (25). In procaryotes the evidence implicating adenylylated nucleotides in responses to stress includes the increases in cellular Ap_4A levels seen in enteric bacteria stressed by ethanol and temperature upshifts, as well as oxidative stress and a number of toxic compounds (3, 4, 13). From these data, Ap_4A was termed an alarmone (12, 13), that is, a regulatory molecule that signals the onset of environmental stress and triggers a stress response, the response being associated with the induction of a small number of proteins, including some heat shock proteins (reviewed in references 14, 16, and 17). However, it is now clear that adenylylated nucleotides are synthesized in conjunction with only some stress regulons and not others (24). *C. acetobutylicum*, like other organisms, responds to stresses, including temperature upshift, butanol addition, and exposure to oxygen, by inducing the synthesis of stress proteins (J. S. Terracciano, E. Rapaport, and E. R. Kashket, manuscript in preparation). The synthesis of some of these proteins is dependent on the growth phase, suggesting a developmental role for the clostridial stress proteins.

Here we report that temperature upshift and butanol addition effect the accumulation of high levels of Ap_4A and adenosine 5′-P^1,P^4-tetraphospho-5′-guanosine (Ap_4G) in *C. acetobutylicum* cultures. In the absence of an added stress, as the cells proceed into the solventogenic phase of growth, adenylylated nucleotides accumulate extracellularly, because the cells lack enzymes to degrade them and because the cell membranes are permeabilized by the solvents produced.

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MATERIALS AND METHODS

Growth of cells. *C. acetobutylicum* ATCC 4259 cells were grown at 28°C as described before (Terracciano et al., in preparation). The growth phases were identified from the fermentation end products, as measured by gas chromatography (22).

Heat and butanol shock. Temperature upshifts from 28 to 45°C and butanol stress were imposed on growing cultures as described before (Terracciano et al., in preparation).

Nucleotide determinations. Nucleotides were extracted as described previously (11) from 2.5-ml samples of cultures or filtrates of cultures filtered through membrane filters (0.45-μm pore size). Ice-cold trichloroacetic acid (TCA) (10% final concentration) was added, and the suspensions were chilled on ice for 45 min and then centrifuged at 7,700 × *g* for 25 min. The TCA was removed with tri-*n*-octylamine in Freon 113, and the neutral aqueous extracts were stored at −20°C for later Ap_4A determinations or at −80°C for ATP assays.

ATP was measured with the luciferin-luciferase reaction with a Turner photometer (Turner Designs, Mountain View, Calif.), using enzyme from Turner. Ap_4A assays were carried out by the coupled phosphodiesterase-luciferase method of Ogilvie (18), which consists of incubating the extracts with calf intestine alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) overnight at 37°C to degrade ATP. The samples were then frozen for 1 h to inactivate the alkaline phosphatase. Ap_4A was converted to ATP with snake venom phosphodiesterase (Cooper-Worthington Diagnostics, Freehold, N.J.), and the released ATP was then measured with the luciferin-luciferase reaction, using enzyme from Sigma Chemical Co., St. Louis, Mo. A known amount of Ap_4A (Sigma) was added to the extracts before the alkaline phosphatase to quantitate the reaction.

The differences in nucleotide content of the cultures and

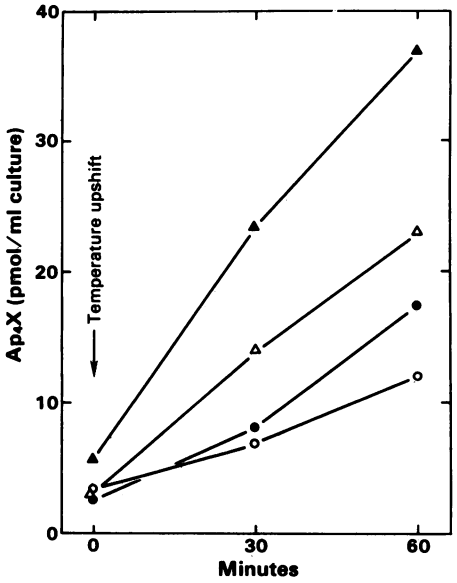

FIG. 1. Adenylylated nucleotide content of heat-stressed *C. acetobutylicum* cultures. The growth temperature was shifted from 28 to 45°C during the acidogenic phase (○), during the switch of solventogenesis (●), or early (△) and late (▲) during solventogenic phase. The nucleotides were extracted and assayed as described in the text. Ap_4X denotes Ap_4A plus Ap_4G.

the culture filtrates were used to calculate the intracellular nucleotide concentrations, using the intracellular aqueous volumes determined previously (22).

Degradation of Ap_4A by intact cells. [^{3}H]Ap_4A (0.1 mM, final concentration; 18 mCi/mmol) or [^{3}H]ATP (0.2 mM; 9 mCi/mmol) was added to cultures of *C. acetobutylicum* during the acid phase. The [^{3}H]Ap_4A (Du Pont-New England Nuclear Corp., Boston, Mass.) was purified before use by chromatography on a DEAE-cellulose column and elution with a linear gradient of 0 to 0.6 M ammonium bicarbonate, pH 7.6. TCA-soluble nucleotides were extracted from the cultures at various times as described above, and the adenylylated nucleotides were separated by one-dimensional thin-layer chromatography on polyethyleneimine-cellulose plates (Brinkmann Instruments, Inc., Westbury, N.Y.) (2, 3). The plates were developed with water, dried, and redeveloped with 1 M LiCl in the same dimension. Marker nucleotides (ATP, ADP, AMP, and Ap_4A, 20 nmol each; Sigma) were included in the samples, and spots were detected under UV light. The spots were cut out and extracted with 300 μl of 4 M ammonium hydroxide for 1 h at room temperature, and the radioactivity was counted.

The acid-insoluble radioactive material was separated by filtering the TCA-precipitated incubation mixtures through glass microfiber filters (Whatman, Inc., Clifton, N.J.). The filters were washed four times with 5% TCA and once with acetone, dried at 60°C for 10 min, and digested with Protosol (Du Pont-New England Nuclear) for 45 min at 60°C, and the radioactivity was counted.

Ap_4A hydrolase assay. *C. acetobutylicum* cells were grown to acid or solvent phase and *Escherichia coli* J53 cells (*met pro*) RP4 (Ap Km Tc IncP), from G. Jacoby, grown to exponential phase. The cultures were diluted to a concentration of 3 mg (dry weight) of cells per ml and sonicated as described before (Terracciano et al., in preparation). The sonically treated material was centrifuged at 7,700 × *g* for 30 min at 4°C, and the pellets were suspended in 1 ml of incubation buffer, which consisted of 50 mM Tris buffer (pH 7.8), 100 μM $CoCl_2$, and 10 μM EDTA. These preparations were incubated at 37°C with 20 μM [^{3}H]Ap_4A at 550 Ci/mol for various time periods. The nucleotides were extracted with TCA and resolved by one-dimensional chromatography on polyethyleneimine-cellulose plates, as described above.

In vivo $^{32}P_i$ labeling. $^{32}P_i$ (0.4 mmol at approximately 25 Ci/mol) was added to the cultures during the acidogenic phase of growth. At various times, 10-ml samples were extracted for nucleotides, as described above. Marker nonradioactive nucleotides (Ap_4A, Ap_3A, ATP, ADP, and AMP, 1.2 μmol each) were added to the extracts. After removal of TCA with tri-*n*-octylamine–Freon 113, the aqueous fractions were percolated through DEAE-cellulose columns (1.7 by 25 cm; Bio-Rad Laboratories, Richmond, Calif.) and eluted with linear gradients of 0 to 0.6 M ammonium bicarbonate, pH 7.8. Fractions of 4.0 ml were collected and counted for radioactivity, and the migration of the nucleotides was determined from the A_{260} of the added nucleotides. The fractions containing UV-absorbing material corresponding to adenylylated nucleotides were pooled, concentrated by lyophilization, suspended in 150 μl of water, and chromatographed in two dimensions on polyethyleneimine-cellulose plates (2, 3). The solvent for the first dimension was 1.5 M morpholine–1.2 M HCl–0.1 M boric acid, pH 8.7, and 3 M $(NH_4)_2SO_4$–2% (vol/vol) disodium EDTA, pH 5.5, was used for the second dimension (2). The plates were exposed to preflashed X-ray film (XAR5; Eastman Kodak Co., Rochester, N.Y.). The spots corresponding to radioactive areas were cut out, eluted with 0.5 ml of 4 M ammonium hydroxide, and counted for radioactivity.

RESULTS

Synthesis of adenylylated nucleotides in response to stress. *C. acetobutylicum* cells responded to a temperature upshift during growth or to the addition of butanol by synthesizing adenylylated nucleotides (Fig. 1 and 2). As the clostridia progressed through the various phases of growth, the response to temperature upshift became more pronounced, with Ap_4X (Ap_4A and Ap_4G; see below) of late solventphase cultures increasing from approximately 5 to 37 pmol/ml.

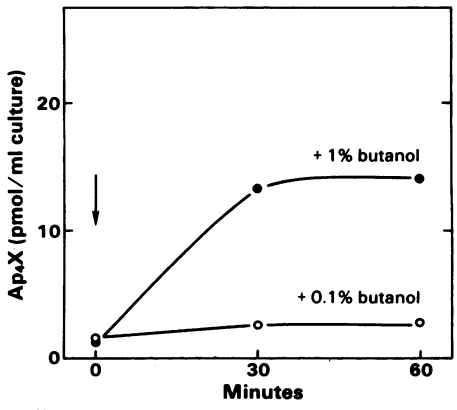

FIG. 2. Effect of butanol on adenylylated nucleotide content of *C. acetobutylicum* cultures. Butanol, 1.0% (●) or 0.1% (○) (vol/vol), was added to the cultures during the switch phase, as indicated by the arrow. The assays were performed as described in the legend to Fig. 1. Standard deviations were <1.2 and 0.3 pmol of Ap_4X per ml for the cultures challenged with 1% and 0.1% butanol, respectively.

TABLE 1. Intracellular concentrations of adenylylated nucleotides and ATP in stressed *C. acetobutylicum*

Growth phase	Stress	Duration (min)	Ap_4X		ATP (mM)
			μM	% of total in culture	
Acid	Temp upshift	0	0.3	40	0.9
		30	2.4	34	4.3
		60	5.4	37	3.5
Switch	Temp upshift	0	0.2	43	1.1
		30	2.3	48	4.2
		60	5.7	50	3.5
Early solvent	Temp upshift	0	0.1	17	0.7
		30	1.9	30	2.4
		60	2.1	28	0.6
Solvent	Temp upshift	0	0.3	60	0.6
		30	0.9	13	0.7
		60	0.06	9	0.6
Switch	0.1% butanol	0	0.2	16	1.1
		30	0.4	22	2.0
		60	0.1	6	2.6
Switch	1.0% butanol	0	0.4	38	1.3
		30	0.5	5	0.2
		60	0.3	2	<0.1

Butanol addition elicited a response similar to that of heat shock. Thus, addition of 1% (vol/vol) *n*-butanol to cells just initiating solvent synthesis (switch phase) increased the Ap_4X content to approximately 14 pmol/ml of culture (Fig. 2). Addition of 0.1 % butanol had a barely perceptible effect on the Ap_4X content of the cultures.

Clostridia produce equal amounts of Ap_4A and Ap_4G. The bioluminescence assay used does not differentiate between various adenylylated nucleotides (hence the designation Ap_4X). To identify which of the known adenylylated nucleotides were synthesized by the cells under various conditions, we added $^{32}P_i$ to the cultures during acid phase. The cultures were extracted when they had reached the switch phase (both with and without temperature upshift for 1 h), as well as early and late in stationary phase. The acid-soluble nucleotides were extracted, and the adenylylated nucleotides were isolated by DEAE-cellulose chromatography and characterized by two-dimensional thin-layer chromatography. There were no radioactive areas that could be assigned to Ap_4C or Ap_4U. The radioactive counts in the spots corresponding to Ap_4A and Ap_4G indicated that the same amount of Ap_4X was recovered as measured enzymatically and that approximately equal amounts of these dinucleotides were synthesized under all conditions tested (results not shown).

Distribution of adenylylated nucleotides between the cytoplasm and the medium. The chaotropic effect of alcohols and solvents on cell membranes has been established. For example, the butanol produced by *C. acetobutylicum* permeabilizes the cells and results in the loss of cellular metabolic intermediates (8) and transmembrane ion gradients (23). It was therefore of interest to determine the intracellular concentrations and the distribution of adenylylated nucleotides after stress and during the various growth phases.

The intracellular concentration of Ap_4X in acid-phase and switch-phase cells rose during 60 min of heat treatment and 40 to 50% of the pools of the dinucleotides were within the cells (Table 1). Later in the growth cycle the intracellular Ap_4X concentrations reached were lower than in the acid or switch phase, and larger fractions of the total were extracellular. In contrast to Ap_4X, no ATP was found extracellularly under any condition tested. Heat stress in acid- or switch-phase cells increased the cellular ATP from approximately 1 to 4 mM, but early in solvent phase the ATP concentrations increased less during heat stress and in solvent phase the intracellular ATP concentration remained low. This suggests that endogenously produced solvents increased the degree to which Ap_4X synthesis was stimulated by heat stress and reduced the retention of the nucleotides in the cells.

This effect of solvents was confirmed by the results obtained with butanol addition. In switch-phase cells, 0.1% butanol did not increase significantly the total Ap_4X in the culture (Fig. 2), but did decrease the fraction found within the cells. The ATP content of these cells increased upon 0.1% butanol addition, but less than after heat stress. With 1% butanol added, the ATP concentration did not increase. Presumably, the cells were permeabilized by the solvent and any ATP synthesized was hydrolyzed extracellularly.

Addition of 1% butanol resulted in a large decrease in the intracellular concentrations of both ATP and Ap_4X. The Ap_4X levels attained upon addition of 1% butanol reached a maximum value (15 pmol/ml of culture) probably because of the effect of butanol on the cell membrane.

Adenylylated dinucleotides of *C. acetobutylicum* cultures during batch growth. Since added butanol increased the Ap_4X content of clostridial cultures, we expected endogenously produced solvents to have the same effect, i.e., to exert a stress on these cells. Cultures inoculated from washed, germinated spores so that no solvents or extracellular Ap_4X was transferred with the inoculum were sampled during the growth cycle and assayed for Ap_4X and ATP (Fig. 3). The ATP content of the culture reached a maximum of approximately 5.4 nmol/ml of culture at the beginning of the solventogenic phase. As no ATP was present extracellularly, the internal ATP concentration was 0.72 mM at that time. As solvent phase progressed, the ATP in the culture decreased, reaching very low levels (0.19 nmol/ml or 57 μM intracellularly) after 8 days. In contrast, Ap_4X in the culture started to increase by day 3 and reached a maximum level after 8 days,

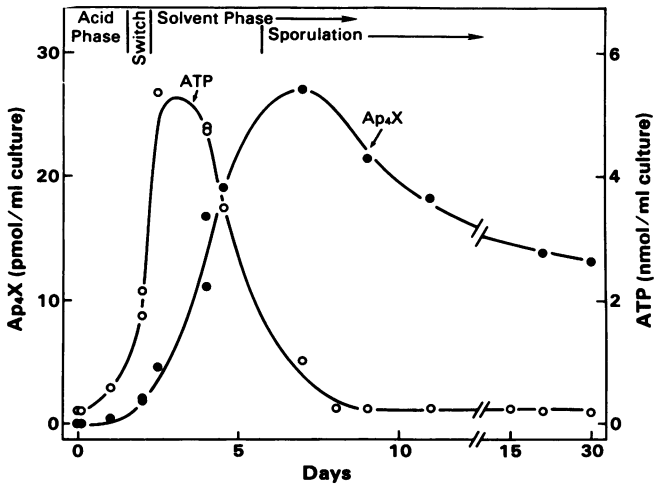

FIG. 3. Ap_4X and ATP content of *C. acetobutylicum* cultures during the batch growth cycle. The cells were grown at 28°C and assayed for nucleotides by methods described in the text.

when sporulation had started, as demonstrated by the appearance of phase-bright spores (not shown). The Ap_4X level then remained high, although it decreased somewhat to a plateau of approximately 13 pmol/ml at 30 days. In the absence of an exogenous stress, the intracellular concentration of Ap_4X was approximately 0.3 μM until at least 30 days (Table 1) and presumably throughout the growth cycle.

Degradation of Ap_4A by *C. acetobutylicum* and *E. coli* cells. The high Ap_4X levels during the later phases of batch cell culture could be the result of stress-induced stimulation of dinucleotide synthesis or of decreased degradation. To distinguish between these two possibilities, the ability of the clostridial cells to metabolize Ap_4X was tested. [^{3}H]Ap_4A was added to cultures during the acidogenic phase, the cells were extracted with TCA during subsequent growth phases, and the radioactivity of the TCA-soluble and -insoluble fractions was measured. No radioactivity was found in the acid-insoluble fraction of the TCA extracts, and all added radioactivity was recovered in the acid-soluble fraction as Ap_4A (Fig. 4A). This suggested that no degradation of [^{3}H]Ap_4A or incorporation of the adenine moiety into acid-insoluble material, such as nucleic acid, had taken place. As a control, [^{3}H]ATP was added in similar experiments. The radioactivity in the acid-soluble fraction decreased from a high level to approximately 30% of the initial value within 5 min and to almost undetectable levels within 24 h after addition of the nucleotide to intact cells. The radioactivity was subsequently identified as ATP and Ap_4A in the acid-soluble fraction (Fig. 4B).

It was quite possible that intact *C. acetobutylicum* cells are impermeant to [^{3}H]Ap_4A (unless permeabilized by alcohols or solvents), and thus the assay would not reflect any degradative ability of the cells toward endogenous Ap_4A. We therefore tested cell-free extracts for ability to degrade the dinucleotide. Sonic extracts of both acid-phase and solvent-phase *C. acetobutylicum* did not degrade Ap_4A, as the added [^{3}H]Ap_4A was recovered unmodified after incubation with these preparations (Fig. 5). In contrast, sonic extracts prepared from exponential-phase *E. coli* metabolized added [^{3}H]Ap_4A, as only 3 nmol of the added 16 nmol was recovered as the dinucleotide after 1 h. This indicates that *C. acetobutylicum* did not contain an Ap_4A-degradative enzyme.

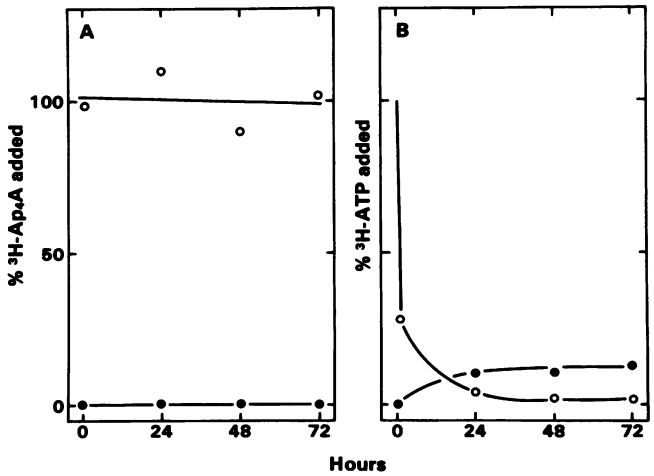

FIG. 4. Metabolism of Ap_4A and ATP by intact *C. acetobutylicum* cells. [^{3}H]Ap_4A or [^{3}H]ATP was added to cultures during acid phase. After further incubation for 24 (switch phase), 48 (early in solvent phase), or 72 (mid-solvent phase) h, the cultures were extracted for TCA-soluble (○) and TCA-insoluble (●) radioactive counts, as described in the text. Ap_4A and ATP were isolated from the acid-soluble material by thin-layer chromatography, as described in the text, and the radioactivity was counted.

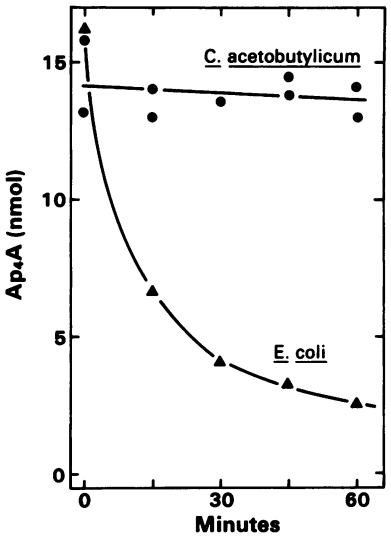

FIG. 5. Degradation of Ap_4A by cell-free extracts. Cells of *E. coli* and *C. acetobutylicum* were sonicated and assayed for degradation of [^{3}H]Ap_4A, as described in the text.

DISCUSSION

C. acetobutylicum cells synthesize adenylylated nucleotides in response to heat stress and to the addition of alcohol, like other bacteria (3, 4, 12, 13). Unlike *E. coli* and *Salmonella typhimurium*, however, the clostridial response to heat varies with the growth phase of the organism in batch culture. Temperature upshift after the cells have begun to synthesize solvents is probably a combined stress effect of heat and endogenous solvent. This would explain why, as the cells progress from acid (13 mM butanol present in the culture, contributed by the inoculum) to the switch (13 to 20 mM butanol) phase, then to early solvent (35 mM butanol), and finally to the solvent phase (70 mM butanol), they respond to heat by synthesizing increasing amounts of adenylylated nucleotides.

We found that [^{3}H]Ap_4A was not degraded by intact *C. acetobutylicum* or by cell-free extracts from either acid-or solvent-phase cells. This is an unusual feature of these clostridia, as the eucaryotic and other bacterial cells tested so far contain enzymes that catalyze the hydrolysis or phosphorolysis of Ap_4A (1, 5–7, 20). Therefore, the high levels of Ap_4A following stress or exposure to solvents are both a consequence of *C. acetobutylicum* cells lacking enzymes that catalyze the degradation of Ap_4A and due to increased synthesis of the dinucleotide. Finally, adenylylated nucleotides, such as Ap_4C or Ap_4U, or nonadenylylated nucleotides, such as Up_4U, Cp_4U, or Gp_4U, have been reported in stressed *Saccharomyces cerevisiae* and *E. coli* cells (4). In the former organisms, these nucleotides were postulated to be derived from the action of diadenosine-5′,5‴-P^1,P^4-tetraphosphate α,β-phosphohydrolase, while their origin in *E. coli* is not known. The absence of Ap_4A degradation could account for the failure to detect these compounds in *C. acetobutylicum*.

The function of Ap_4X in procaryotic organisms is not known. Because adenylylated nucleotides accumulate during the heat shock and stress responses, Ames and coworkers postulated that these compounds may in some way be associated with the stress response (13). However, in *E. coli* harboring a multicopy plasmid that overproduces diadenosine tetraphosphate hydrolase, the heat shock and hydrogen peroxide responses are unaffected, as detected by synthesis of stress proteins (19). In these cells the Ap_4A levels were three to eight times lower than in control cells before and 30 min after heat shock, respectively. Finally, adenylylated nucleotide accumulation in *E. coli* did not occur in response to all stresses imposed on the cells, inconsistent with a general alarmone role for these compounds (24).

The presence of Ap_4A-degrading enzymes in other bacteria (5, 6, 20) suggests that these enzymes are required by the cells. The lack of such enzymatic activity in *C. acetobutylicum* would be inconsistent with such a notion if it were not for the fact that the intracellular concentrations of these nucleotides are, in fact, low under all conditions tested, although the total levels in the cultures may be high. During the batch growth cycle the intracellular Ap_4X concentration did not exceed 0.4 μM and only rose to 5.7 μM 60 min after temperature upshift. The cellular Ap_4X concentrations reported for stressed *E. coli* (e.g., references 4 and 24) are higher than the *C. acetobutylicum* values and were measured by extracting whole cultures and calculated by assuming that all of the nucleotides were within the cells.

The low intracellular nucleotide concentrations in *C. acetobutylicum* are a result of the cell membranes becoming permeable to small molecules (8, 23) because of the chaotropic effect of alcohols and solvents on the membrane. Thus, the cells could support increased intracellular ATP concentrations after temperature upshift in acid or switch phase, but not when the solvent concentrations became too high in the later growth phases. When butanol was added to the cultures, the cellular ATP levels also could not be maintained. Thus, the Ap_4X response of the cells to butanol stress appears to be limited, in part, by the intracellular ATP concentration, and butanol has two opposite effects: as a stress, it causes increased Ap_4A synthesis, but it also lowers the intracellular ATP and Ap_4X.

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