Comparison of Nucleoside Triphosphate Levels in the Wild-type Strain with Those in Sporulation-deficient and Solvent-deficient Mutants of *Clostridium acetobutylicum* P262

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The role of GTP and other ribonucleoside phosphates in the differentiation of *Clostridium acetobutylicum* and two sporulation-deficient mutants was investigated. The clostridial stage (*cls-1*) mutant was unable to produce the clostridial stage, solvents, granulose, capsules or endospores, whereas the sporulation-deficient (*spo-1*) mutant produced the clostridial stage, solvents, granulose and capsules, but did not form a forespore septum. The nucleoside triphosphate profiles of the wild-type and *spo-1* mutant were similar and were characterized by a trough in nucleotide levels which occurred just prior to the pH break point, the onset of the stationary growth phase, clostridial stage formation and the transition from the acidogenic to the solventogenic phase. The nucleoside triphosphate concentrations during the exponential growth phase were much lower than those found during the stationary phase. The nucleotide levels in the *cls-1* mutant during the exponential phase were comparable to those observed in the wild-type and *spo-1* mutant. However, the nucleotide levels in the *cls-1* mutant did not increase during the stationary growth phase. The involvement of nucleotide levels, particularly that of GTP, in the differentiation of *C. acetobutylicum* was indicated by the effect of inhibitors, which have been shown to decrease ribonucleotide levels in other organisms and cause an increase in sporulation.

INTRODUCTION

The obligate anaerobe *Clostridium acetobutylicum* is used for the production of acetone, butanol and ethanol (ABE). In some strains of *C. acetobutylicum*, such as the industrial strain P262, the onset of solvent production and sporulation is characterized by a change in morphology from a highly motile, acid-producing, vegetative rod to a swollen, granulose-containing, phase-bright, cigar-shaped, solvent-producing, 'clostridial' cell (Long *et al.*, 1983; Jones & Woods, 1986). An understanding of the triggers which lead to the onset of solvent production and differentiation is important for developments aimed at improving the ABE fermentation.

In *Bacillus subtilis*, a decrease in GTP content is associated with the initiation of sporulation (Lopez *et al.*, 1979; Freese, 1981; Ochi & Freese, 1983). A similar decrease in GTP or S-adenosylmethionine content was shown to be associated with differentiation (meiosis and sporulation) in *Saccharomyces cerevisiae* (Freese *et al.*, 1984*b*). Recently, aerial mycelium formation in *Streptomyces* MA406-A-1 has been linked with a decrease in GTP content (Ochi, 1986). Since the role of GTP and other ribonucleoside phosphates in the differentiation of *C. acetobutylicum* has not been reported, we investigated changes in the ribonucleoside phosphate pools of the wild-type strain P262 and two sporulation-deficient mutants of this

Abbreviation: ABE fermentation, acetone/butanol/ethanol fermentation.

strain. The clostridial stage (*cls-1*) mutant is unable to form the clostridial stage and can not produce solvents, capsule, granulose or endospores (Jones *et al.*, 1982). The second mutant (*spo-1*) forms the clostridial stage and produces solvents, capsule and granulose but does not form a forespore septum (Jones *et al.*, 1982).

METHODS

Bacterial strains. The wild-type *C. acetobutylicum* strain P262 has been described previously (Jones *et al.*, 1982; Long *et al.*, 1983). The *cls-1* and *spo-1* mutants were isolated from this strain by mutagenesis with ethyl methanesulphonate as described by Jones *et al.* (1982).

Media and culture conditions. The *C. acetobutylicum* strains were grown under stringent anaerobic conditions in the *C. acetobutylicum* minimal medium (CAMM) of Long *et al.* (1983), with the glucose concentration reduced from 60 g l⁻¹ to 50 g l⁻¹ and the nitrogen source changed from ammonium phosphate to ammonium nitrate (7.2 g l⁻¹). CAMM was inoculated with exponential-phase cells (OD₆₀₀ 0.45 to 0.50) harvested from the clostridial basal medium (CBM) of O'Brien & Morris (1971). All cultures were incubated at 34 °C and shaken gently every 6 h.

Morphology and end-product determinations. Total cell counts, clostridial stage counts, forespore counts and spore counts were determined with a Thoma counting chamber (Weber Scientific International) and a Zeiss photomicroscope fitted with phase- and interference-contrast optics. Acetate, butyrate, acetone, butanol and ethanol were determined by gas chromatography on a Hewlett Packard 5880A gas chromatograph fitted with a flame ionization detector, using 2-propanol as an internal standard. The column and running conditions have been described previously (Long *et al.*, 1984).

Preparation of nucleotide extracts. Nucleotide extracts were prepared from cells grown in CAMM. The extraction methods of Olempska-Beer and Freese (1984) and Payne & Ames (1982) were combined and modified for use with *C. acetobutylicum*. Cells (100 ml) were collected rapidly on a 100 mm diameter nitrocellulose filter (Schleicher and Schüll BA 85 or AE 95) by vacuum filtration. The filter was placed cell side down in a glass Petri dish containing 5 ml ice-cold 3 M-formic acid saturated with 1-butanol. After a 30 min extraction period, the liquid was removed and the filter and Petri dish were washed twice with ice-cold sterile water. The three liquid fractions were combined and adjusted to pH 8.8 with 25% (w/v) ammonia solution. The salt concentration of the extract was increased to 1 M by the addition of 3 M-ammonium acetate, pH 8.8. Debris was removed by centrifugation at 40000 g for 20 min. The supernatant was applied to a column (1.5 cm long × 1 cm diameter) packed with Affi-Gel 601 (Bio-Rad) which had been equilibrated with 1 M-ammonium acetate, pH 8.8. The absorbance of the effluent was monitored at 254 nm and unwanted and interfering components were washed through the column with a mobile phase of 1 M-ammonium acetate, pH 8.8. When the *A*₂₅₄ of the effluent reached baseline, the mobile phase was changed to 100 mM-acetic acid to initiate elution of the nucleotides. The desired sample which contained the nucleotides (about 10 ml) was collected and freeze-dried. The freeze-dried sample was resuspended in water, centrifuged and assayed by high-performance liquid chromatography (HPLC). The reliability of this extraction method was routinely tested by performing duplicate extractions where a known concentration of a standard nucleotide mixture was added to one sample just prior to the formic acid treatment (Freese *et al.*, 1984a).

The nucleotide extraction method of Olempska-Beer & Freese (1984) utilized 1 M-formic acid saturated with 1-butanol. In our system, the concentration of formic acid had to be increased to 3 M to overcome the high buffering capacity of CAMM, which contained calcium carbonate. This was consistent with the results of Bagnara & Finch (1972), who reported that formic acid was unsatisfactory for the extraction of nucleotides if the buffering capacity of the culture medium caused the pH of the extractant to rise above approximately 3.0. The formic acid extract had to be purified prior to the HPLC analysis. Similar purification procedures were also reported as an essential step for the analysis of nucleotides from plant tissues (Meyer & Wagner, 1985; Payne & Ames, 1982).

Analysis of nucleotide extracts. The nucleotides in the cell extracts were separated using strong anion-exchange HPLC. The computer-HPLC data collection and analysis system used to analyse the nucleotide composition of the extracts has been described previously (Santangelo, 1988). All experiments were done at least three times, with similar results; representative data are presented.

Nucleotide synthesis inhibitors. Decoyinine and psicofuranine were generously donated by Dr R. L. Keen, Upjohn Company, Kalamazoo, Michigan, USA. Caffeine and 3-methylxanthine were purchased from Fluka. Theobromine, theophylline, 6-mercaptopurine, 2-amino-6-mercaptopurine and 6-azathymine were purchased from Sigma. Since CAMM supports a near-maximum spore titre for *C. acetobutylicum* P262, it was an unsuitable medium for inhibitor studies where an increase in sporulation was to be determined. The tryptone/yeast extract/glucose medium (TYG) of Hongo *et al.* (1968), which resulted in a lower sporulation level, was therefore used for these inhibitor studies. The inhibitors were added during the mid-exponential growth phase, when the OD₆₀₀ of the culture was 0.40 to 0.45. Percentage sporulation was determined after 48 h.

RESULTS

Growth, physiology and morphology

The ABE fermentation profile typical of *C. acetobutylicum* P262 in CAMM is shown in Fig. 1(a, b). Cells transferred to this medium show a characteristic 15 h lag phase after which cell numbers begin to increase (Long *et al.*, 1983). The exponential growth phase, from 15 to 35 h, was characterized by motile, rod-shaped cells. During this stage, glucose consumption resulted in acid production. The pH break point occurred when the acid concentration reached a maximum of approximately 4 g l⁻¹ (between 30 and 35 h). The break point was associated with the onset of the stationary growth phase and clostridial stage formation. Just prior to the pH break point, the cell morphology began to change from motile rods to phase-bright, granulose-filled clostridial cells, and by 40 h 90 to 100% of the cells had converted to the clostridial stage. The physiological changes associated with the pH break point included a decrease in acid concentration, initiation of solvent production, and an increase in the rate of glucose consumption. After 45 h, the acid concentration stabilized at approximately 2.7 g l⁻¹. Glucose exhaustion occurred at approximately 50 h and shortly thereafter solvent levels reached a peak of approximately 10 g l⁻¹. At this time a maximum number of cells containing forespore septa (60%) was observed, as well as a small percentage of mature spores (15%). The highest proportion of phase-bright spores (approximately 50%) was observed at the end of the fermentation (67 h).

A similar fermentation profile for the *spo-1* mutant is shown in Fig. 1(c, d). The exponential phase was characterized by motile rods, glucose consumption and acid production. The pH break point (pH 5.3) was reached between 35 and 40 h and was associated with a peak in acid levels. Stationary phase occurred after approximately 40 h and by 55 h 90 to 100% of the cells were phase-bright clostridial forms. Solvents were first detected immediately after the pH break point and reached a maximum of 9.6 g l⁻¹ at the end of the fermentation (67 h). The major difference between this and the wild-type fermentation was that no forespore septa or mature spores were observed, indicating that the *spo-1* mutant was an early sporulation mutant (*spo0*). Minor differences involving for example maximum cell number, time of peak in motility, and residual glucose levels were observed between the fermentation profiles of the P262 wild-type and *spo-1* strains.

The *cls-1* mutant showed a normal motile, acid-producing, glucose-consuming exponential phase, but the switch to solvent production did not occur and no clostridial-stage cells were observed (Fig. 1e, f). After approximately 35 h the rate of glucose consumption slowed and acid levels continued to increase. By 55 h the fermentation ceased and virtually no solvents were produced (<0.25 g l⁻¹). Only 30% of the available glucose was utilized and approximately 7 g acids l⁻¹ were produced.

Nucleotide profiles

The nucleoside triphosphate profile for the wild-type *C. acetobutylicum* P262 strain demonstrated that throughout the fermentation ATP was the most abundant nucleoside triphosphate followed by UTP, GTP and CTP (Fig. 2a). The nucleoside triphosphate concentrations during the exponential growth phase (15 to 35 h) were much lower (by as much as 40-fold in the case of ATP) than the values obtained during most of the stationary growth phase (40 to 50 h). From 17 to 23 h there was a gradual decrease in the concentration of all the nucleoside triphosphates, and just prior to the pH break point (25 to 35 h) the concentration of all the nucleoside triphosphates decreased to almost zero (27 h). The nucleotide levels then increased rapidly as the morphology of the culture changed from phase-dark rods to phase-bright clostridial cells. The highest pool values for all the nucleoside triphosphates coincided with the peak in the number of clostridial-stage cells and the onset of forespore septum development, indicating that the nucleoside triphosphate pool values were higher in the stationary-phase clostridial stage cells than in the exponential-phase rods. The decrease in the number of clostridial cells after 47 h correlated with a decrease in the nucleoside triphosphate concentrations, maximum solvent levels and the initiation of spore maturation.

Fig. 1. Morphological and physiological profiles for *C. acetobutylicum*. (a, b) Profiles for the wild-type strain P262; (c, d) profiles for the *spo-1* mutant; (e, f) profiles for the *cls-1* mutant. Symbols for graphs (a), (c) and (e) correspond to the following: $\blacktriangle$, glucose concentration (% w/v); $\blacksquare$, total acids (g l^{-1}); $\bullet$, total solvents (g l^{-1}); $\square$, log cell number; $\circ$, pH. The plots in (b), (d) and (f) (on facing page) are

Fig. 1 (continued)

expressed as percentages of the total cell count, and the symbols correspond to the following: ▲, motility; ■, phase-bright clostridial cells; ●, phase-dark rods; ○, forespore-containing cells; □, mature spores. In (f), only motility is shown; the *cls-1* mutant remained as 100% phase-dark rods throughout the fermentation.

Fig. 2. Nucleoside triphosphate profiles for *C. acetobutylicum*: (a) wild-type strain P262, (b) the *spo-1* mutant, and (c) the *cls-1* mutant. ■, ATP; ●, GTP; ▲, CTP; ▼, UTP.

The nucleoside triphosphate profile for the *spo-1* mutant (Fig. 2b) was similar to that for the wild-type P262 strain. ATP was the most abundant nucleoside triphosphate in exponential-phase cells, followed by GTP, UTP and CTP. The *spo-1* mutant did not produce forespore septa, but the cells did produce a clostridial stage. The concentrations of all nucleoside triphosphates increased as the percentage of clostridial cells increased, and the peak concentrations coincided with the peak in the percentage of clostridial-stage cells. In contrast to the wild-type strain, the decrease in the nucleoside triphosphate levels after 50 h was not associated with a decrease in the percentage of clostridial-stage cells. However, the metabolism of the *spo-1* mutant culture slowed down as solvent levels reached their maximum and the glucose concentration decreased.

The *cls-1* mutant was unable to produce solvents or to sporulate and did not differentiate into clostridial-stage cells. The nucleotide levels in exponential-phase cells of this mutant (Fig. 2c) were comparable to those observed in the wild-type strain and the *spo-1* mutant. ATP was the most abundant nucleoside triphosphate, followed by GTP, UTP and CTP. The nucleoside triphosphate levels decreased as the cell number increased and acids were produced, reaching

Table 1. *Percentage sporulation of wild-type C. acetobutylicum* P262 with and without the addition of compounds known to alter nucleotide levels

The optimum concentration of these compounds is the concentration that gave the greatest increase in percentage sporulation. The results are representative of two experiments that give similar results.

Compound	Optimum concn ($\mu\text{g ml}^{-1}$)	Percentage sporulation	
		Without addition	With addition
Cordycepin	300	33	42
Decoyinine	100	31	44
Psicofuranine	400	35	53
2-Aminomercaptopurine	500	39	60
6-Mercaptopurine	400	31	51
Caffeine	500	31	60
3-Methylxanthine	500	35	60
Theobromine	200	32	63
Theophylline	500	36	57
6-Azathymine	100	36	56

almost zero by 35 h. At this time, the cells had stopped dividing and the rate of acid production and glucose consumption had decreased. In contrast to the nucleoside triphosphate profiles obtained with the wild-type strain and the *spo-1* mutant, the nucleoside triphosphate levels did not increase during the stationary phase, and after 40 h UTP and CTP were not detected.

Nucleotide synthesis inhibitors

Ten compounds which increased the percentage of sporulating cells are shown in Table 1. Cordycepin, decoyinine and psicofuranine are adenosine analogues altered in the ribose moiety (Zain-ul-Abedin *et al.*, 1983). Psicofuranine and decoyinine, which both inhibit GMP synthetase (Freese *et al.*, 1979), were more effective than cordycepin at increasing the percentage of sporulating cells. The mercaptopurines (6-mercaptopurine and 2-aminomercaptopurine) were the next most effective compounds. The methylxanthines caffeine and theobromine provoked the largest increase in percentage sporulation, whereas the other two methylxanthines tested, theophylline and 3-methylxanthine, increased sporulation to a lesser extent. The pyrimidine analogue 6-azathymine also increased the percentage of sporulating cells.

DISCUSSION

Nutrient shift-down experiments, in which rapidly dividing cells from a rich medium are transferred to a nutrient-depleted medium, have been used to induce sporulation in *Bacillus* species (Sterlini & Mandelstam, 1969). However, nutrient depletion does not induce sporulation in *C. acetobutylicum* P262, which sporulates only under conditions where growth is limited in the presence of excess glucose and ammonia (Long *et al.*, 1984). These studies were therefore carried out in the minimal medium, CAMM, of Long *et al.* (1983), which provides a sufficiently synchronous developmental cycle to allow for these correlative morphological, physiological and biochemical studies (Woods & Jones, 1986).

ATP was the most abundant nucleoside triphosphate produced by the wild-type strain and the *spo-1* and *cls-1* mutants. An interesting observation was the extremely high concentrations of nucleotides in non-growing clostridial-stage cells of the wild-type strain and the *spo-1* mutant, since the fermentation of glucose to solvents yields 2 mol ATP per mol glucose, as compared to approximately 3.3 mol ATP per mol glucose when acids are produced (Jones & Woods, 1986). However, the clostridial cells do not appear to elongate or divide and the nucleotides may accumulate as a result of the absence of growth. The high pool values of these energy-rich

compounds may explain the association of energy-consuming processes such as granulose formation and capsule production with the clostridial stage (Reysenbach *et al.*, 1986).

The units that have been used for reporting nucleoside triphosphate levels in *B. subtilis* are pmol per OD₆₀₀ unit (Ochi & Freese, 1983; Zain-ul-Abedin *et al.*, 1983) or pmol per AM unit (Freese *et al.*, 1981), where one AM unit is the amount of cells that produces an OD₆₀₀ of 1 if suspended in 1 ml. Unfortunately, these authors make no reference to cell number, nor do they mention the path length used in their optical density measurements. Therefore, the results presented here, which are based on cell number, are not directly comparable to those above which are based on optical density. With our strain of *C. acetobutylicum*, 10⁸ cells ml⁻¹ gives an OD₆₀₀ of approximately 1 if a 1 cm path length is used. Therefore if the maximum nucleoside triphosphate concentrations reported by Zain-ul-Abedin *et al.* (1983) are converted from pmol per OD₆₀₀ unit to ng per OD₆₀₀ unit, the values obtained are approximately 375, 115, 91 and 41 for ATP, GTP, UTP, and CTP respectively. Our results indicate that the nucleoside triphosphate levels for 1 × 10⁸ *C. acetobutylicum* cells fall within the same range as reported for 1 OD₆₀₀ unit of *B. subtilis* cells.

The isolation of various classes and combinations of sporulation, clostridial cell, solvent, granulose and capsule mutants demonstrates that although the individual pathways function independently of each other, the induction of these events appears to be linked by some common regulatory mechanism (Woods & Jones, 1986). The concentrations of nucleoside triphosphates in the wild-type strain and in the *cls-1* and *spo-1* mutants decreased during the late-exponential growth phase and reached their lowest levels just prior to the pH break point. This trough also coincided with a decrease in motility and rate of cell division. In the wild-type strain and the *spo-1* mutant the conversion of cells to the non-dividing clostridial stage and the onset of solvent production coincided with a rapid and very large increase in nucleotide pools. In the *cls-1* mutant, which failed to produce solvents or the clostridial stage, no increase in the level of nucleoside triphosphates was detected. The accumulation of high concentrations of nucleoside triphosphates in the *spo-1* mutant suggests that the increase in these compounds was associated with the conversion of cells to the solvent-producing clostridial stage and was not dependent on spore formation.

A decrease in GTP has been shown to be associated with the initiation of differentiation and sporulation in *B. subtilis* (Lopez *et al.*, 1979; Freese, 1981; Ochi & Freese, 1983), *Saccharomyces cerevisiae* (Freese *et al.*, 1984b), and a streptomycete (Ochi, 1986). A variety of inhibitors which are known to decrease ribonucleotide levels in *B. subtilis* bring about an increase in sporulation (Freese *et al.*, 1979; Ochi & Freese, 1983; Zain-ul-Abedin *et al.*, 1983). Although there is no direct evidence implicating the low levels of GTP which occur during the late-exponential growth phase in *C. acetobutylicum* P262 it is possible that a decrease in this or other nucleoside triphosphates plays a role in differentiation of *C. acetobutylicum*. The involvement of these compounds is supported by the increase in sporulation which was obtained in cultures treated with the various inhibitors shown in Table 1. When these inhibitors were added to stationary-phase cultures just after the pH break point, little or no increase in sporulation occurred (not shown). The methylxanthines were the most effective compounds in stimulating sporulation, which is consistent with the results of Sacks & Thompson (1977). One inhibitor which would not be expected to affect purine ribonucleotide levels is the pyrimidine analogue 6-azathymine. This compound also caused an increase in sporulation in *C. acetobutylicum*. Similar results were obtained by Craven & Blankenship (1982) with *Clostridium perfringens*.

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