Temperature-dependent Azide Sensitivity of Growth and ATPase Activity in the Facultative Thermophile, *Bacillus coagulans*

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Inhibition by sodium azide of the growth of *Bacillus coagulans* decreased but the cytochrome content, particularly cytochrome *d*, increased with increasing growth temperature. Higher cytochrome *d* content correlated with increased cyanide resistance of NADH oxidase, but azide resistant activity was found in mesophilic and thermophilic cultures. Anaerobic growth at 37 °C was totally inhibited by 1 mM-azide. At 55 °C growth occurred with 4 mM-azide, but the cell yield was reduced by 60%. ATPase activity was sensitive to azide but inhibition varied with both growth and assay temperatures. ATPase from cells grown at 55 °C was least sensitive when assayed at 55 °C. Possible changes in ATPase which could account for the temperature-dependent azide sensitivity are discussed.

INTRODUCTION

Facultative thermophiles are capable of growth in both the thermophilic and mesophilic temperature ranges (Farrell & Campbell, 1969). Several reports have suggested that unlike obligate thermophiles, in which the majority of proteins are intrinsically thermostable (Williams, 1975), facultative thermophiles may contain thermolabile enzymes which require modification or stabilization to operate at high growth temperatures. Recently, studies on several enzymes from *Bacillus flavothermus* and a facultatively thermophilic *Streptomyces* sp. resulted in the conclusion that 'a facultatively thermophilic organism can harbour two types of enzyme, namely those with increasing thermostability depending on growth temperature and those which remain thermolabile' (Heinen & Lauwers, 1983). Earlier, the glyceraldehyde phosphate dehydrogenase of *Bacillus coagulans* KU was shown to be very labile, even from cells grown at 55 °C, unless stabilized by high ionic strength (Crabb *et al.*, 1975). This led to a generalized theory of facultative thermophily which proposed that the enzymes of such organisms were not intrinsically thermostable (Crabb *et al.*, 1975; Amelunxen & Murdock, 1978). However, recently the ATPase of *B. coagulans* has been shown to be thermostable (Edwards & Jones, 1983). More work is therefore needed to establish if facultative thermophiles do possess particular and possibly unique mechanisms which allow growth at thermophilic temperatures. The increasing use of microbial enzymes (especially from *Bacillus* sp.) for biotechnological purposes makes studies of intrinsic and conferred thermostability of considerable interest.

Bacillus coagulans was chosen for the present study because of its wide temperature range (Gordon *et al.*, 1973) and because growth rates alter little between mesophilic (37 °C) and thermophilic (55 °C) conditions (Novitsky *et al.*, 1974; Amelunxen & Murdock, 1978). *Bacillus coagulans* can be readily distinguished from other bacilli growing at 55 °C or above by its tolerance to sodium azide and the production of lactic acid as the major fermentation product (Gordon *et al.*, 1973; Gibson & Gordon, 1974). The insensitivity to azide may reflect differences in terminal respiratory oxidases or a shift to fermentative growth. This report examines the effects of both temperature and sodium azide on the energy-yielding metabolism of *B. coagulans*.

METHODS

Organism and growth conditions. *Bacillus coagulans* (NCIB 8080) was grown aerobically in tryptone/yeast-extract/glucose (TYG) broth in conical flasks as previously described (Edwards & Jones, 1983) or in a 10 l batch fermenter (L. H. Engineering Co. Ltd, Stoke Poges, Bucks, UK) containing 7 l broth aerated at 7 l air min⁻¹ and stirred at 300 r.p.m. Sterile antifoam agent (Silicone Antifoam Emulsion A, Sigma) was added as required. The fermenter was inoculated with a 5% (v/v) overnight aerobic culture.

Anaerobic growth was in tubes containing 10 ml TYG broth which had been autoclaved and cooled to the growth temperature prior to inoculation with 0.05 ml overnight anaerobic culture. Tubes were incubated in an anaerobic jar (GasPak, BBL).

Growth was monitored at 550 nm and converted to dry weight using a calibration curve prepared using cells grown in TYG.

Preparation of cell extracts. Cell extracts for ATPase assays were prepared as described previously (Edwards & Jones, 1983). For other assays, samples (1 litre) of fermenter culture were harvested by centrifugation at 15000 g for 10 min at 4 °C, washed once in cold 50 mM-potassium phosphate buffer (pH 7.4) containing 5 mM-MgCl₂ and resuspended in the same buffer. Cells were disrupted by sonication for a total of 2–3 min using a MSE Ultrasonic Disintegrator (MSE Ltd, Crawley, UK) at an amplitude setting of 12 µm peak to peak. Cell breakage was assessed using phase-contrast microscopy. Whole cells were removed by centrifugation at 10000 g for 10 min and the supernatant, containing 4–10 mg protein ml⁻¹ used as the cell extract. Membrane fraction was prepared by lysozyme treatment as described by McArthur & Reynolds (1979).

ATPase assays. ATPase was assayed by a proton-release method essentially as described previously by West & Mitchell (1974) and Edwards *et al.* (1978). Cell extracts (0.5–1 mg protein) were added to ATPase buffer (total assay volume 3 ml) which contained 0.15 M-KCl, 28 mM-glycyl-glycine and 1 mM-MgCl₂ (pH 8). After temperature equilibration and addition of azide, the pH was adjusted to pH 8 with either 50 mM-KOH or 50 mM-HCl. The reaction was initiated by the addition of 50 µl 0.1 M-ATP, pH 8.0. The progressive decrease in pH resulting from ATP hydrolysis was monitored using a long-reach pH micro electrode (Russell pH Ltd, Fife, UK) linked via an EIL 7030 pH meter (EIL, Chertsey, Surrey, UK) to a Vitatron chart recorder (MSE, Crawley, Sussex, UK). No ATP hydrolysis occurred with buffer alone. ATPase activity was determined from the initial rate of hydrolysis (1–2 min). The mixture was calibrated by the addition of 5 µl 0.05 M-HCl on the basis that hydrolysis of one molecule of ATP releases 1.0 proton at pH 8.0 (Alberty, 1968).

Cytochrome content. Difference spectra of cell extracts or membrane fractions were plotted using a SP1800 spectrophotometer (Pye Unicam, Cambridge, UK). Samples were reduced by the addition of solid sodium dithionite and oxidized either by vigorously shaking in air or by the addition of solid potassium ferricyanide. CO-Reactive components were identified from reduced plus CO minus reduced difference spectra. CO gas was bubbled through dithionite reduced membrane fraction for between 1 and 10 min. Cytochrome concentrations were calculated using extinction coefficients given by Smith (1978).

Other assays. NADH oxidase was measured spectrophotometrically at 340 nm. The reaction mix contained (final volume, 1 ml): 50 µmol potassium phosphate buffer (pH 7.4), 5 µmol MgCl₂ and cell extract (50–200 µg protein). The reaction was started by the addition of NADH (2 µmol). Inhibitors (NaN₃ or KCN) were added before the substrate.

Protein was determined by the Lowry method using bovine serum albumin as the standard.

RESULTS

Aerobic growth in the presence of azide

Growth at 55 °C in the presence of sodium azide is used as a diagnostic test for *Bacillus coagulans* (Gordon *et al.*, 1973). This resistance was not found at lower incubation temperatures. At 55 °C aerobic growth in broth containing 1 mM-azide was only slightly slower (doubling time 58 min) than in the absence of the inhibitor (doubling time 42 min), but the final yield of cells was decreased (Fig. 1). With 2 mM-azide the cell yield was reduced by 75%. If the growth temperature was reduced, inhibition by azide became progressively more severe so that at 45 °C (doubling time 45 min in absence of azide) no growth was detected in broth containing 2 mM azide (Fig. 1). Preincubation of medium containing azide for 18 h at 55 °C before cooling and inoculation at lower temperatures indicated that the differences in growth inhibition were not due to instability of azide at 55 °C.

Fig. 1. Effect of sodium azide on the growth of *Bacillus coagulans*. Growth at (a) 55 °C or (b) 45 °C was measured in the absence (○) or presence of 1 mM- (■) and 2 mM- (□) sodium azide.

Fig. 2. Room temperature difference spectra of membrane fractions. Reduced (dithionite) minus oxidized (air) difference spectra were plotted of membrane fractions prepared from late-exponential phase cultures grown at (a) 55 °C or (b) 35 °C. Protein concentrations were (a) 19.8 mg ml⁻¹ and (b) 10.1 mg ml⁻¹.

Effect of temperature on respiratory chain composition

To investigate whether the azide resistance at high temperatures was attributable to changes in the respiratory chain, the cytochrome composition of *B. coagulans* grown at various temperatures was measured. Both quantitative and qualitative differences were apparent between organisms grown at 55 °C and 35 °C. The cytochromes in reduced minus oxidized spectra of membrane fractions prepared from 55 °C-grown cultures were cytochrome *b* ($\alpha_{\max}$ 565 nm), cytochrome *aa*₃ ($\alpha_{\max}$ 605 nm) and cytochrome *d* ($\alpha_{\max}$ 628 nm). No cytochrome *c* was detected (Fig. 2*a*). The presence of two terminal oxidases was also indicated in a reduced plus CO minus reduced spectrum. This did not show the presence of cytochrome *o* even after prolonged exposure to CO. In membrane fractions from a 35 °C-grown culture only cytochromes *aa*₃ and *b* were observed (Fig. 2*b*). The maximum of cytochrome *b* in 35 °C grown cells was 559 nm. The reduced plus CO minus reduced spectrum also indicated that only one oxidase (cytochrome *aa*₃) was present.

When the growth temperature was increased from 35 °C to 40 °C, slight traces of cytochrome *d* were sometimes observed in stationary phase but not in exponentially-growing cultures. A

Table 1. *Effects of growth temperature on cytochrome composition and NADH oxidase activity in Bacillus coagulans*

Growth temperature	Growth phase	Cytochrome concn [nmol (mg protein) ⁻¹]			NADH oxidase activity	
		<i>aa</i> ₃	<i>b</i>	<i>d</i>	Specific activity*	Inhibition 5 mM-KCN (%)
55 °C	Early-exponential	0.109	0.233	0.053	0.106	81
	Late-exponential	0.205	0.461	0.436	0.042	68
	Stationary	0.402	0.838	0.829	0.039	45
40 °C	Early-exponential	0.021	0.114	0	0.072	83
	Late-exponential	0.054	0.148	0	0.053	77
	Stationary	0.053	0.117	0.031	0.036	71

* $\mu\text{mol NADH oxidized min}^{-1} (\text{mg protein})^{-1}$.

more detailed examination of cytochrome composition during growth at 40 °C and 55 °C is presented in Table 1. The cytochrome content was always higher at the higher growth temperature and at 55 °C substantial increases in all three cytochromes (but particularly cytochrome *d*) occurred as stationary phase was reached. At 40 °C some increase in the amount of cytochrome *aa*₃ in stationary phase cells was measured, but the level was eight fold lower than at 55 °C.

Increased cytochrome *d* content was accompanied by increased resistance of respiration to cyanide. NADH oxidase activity was only slightly higher in organisms grown at 55 °C compared with 40 °C but up to 55% of the activity was resistant to 5 mM-KCN (Table 1).

It seemed possible that the increased azide resistance at 55 °C compared to 45 °C or below was due, in part, to the presence of an additional cytochrome oxidase, cytochrome *d*. However, NADH oxidase activity, measured either spectrophotometrically or with an oxygen electrode, was very resistant to azide, irrespective of the growth temperature. With 10 mM-azide the inhibition of NADH oxidase was only 16% and 21% in membrane fractions prepared from cultures grown at 55 °C and 35 °C, respectively. Furthermore, the addition of azide to cultures growing at a variety of temperatures did not induce additional synthesis of cytochrome *d* or change the cytochrome composition.

The presence of cytochrome *d* in cultures grown at 55 °C could also reflect a greater thermostability of this oxidase compared with cytochrome *aa*₃. Measurement of NADH oxidase activity remaining after heating cell-extracts for 5 min at temperatures up to 75 °C showed that there was little difference in cells grown at 55 °C or 35 °C. In both, the enzyme was stable up to 60 °C. Above this temperature it was rapidly inactivated so that after heating at 70 °C, no enzyme activity could be detected.

Anaerobic growth in the presence of azide

Growth under anaerobic conditions was examined to determine whether azide affected respiration and fermentative metabolism equally. At 55 °C anaerobic growth was possible even in the presence of 4 mM-azide although the cell-yield was reduced at all inhibitor concentrations (Table 2). The final pH of all the cultures was approximately 4.1. Increasing the medium glucose concentration did not change the final pH but instead resulted in unmetabolized glucose being present in the culture supernatant. Lactic acid was the main product detected in the culture supernatants and the amount formed was not altered by the presence of azide (data not shown). At lower temperatures, anaerobic growth like aerobic growth became more and more sensitive to azide. At 37 °C growth was completely inhibited by 2 mM-azide (Table 2). In the absence of azide, cell yields and acid production were similar at both growth temperatures (Table 2).

Effect of azide on ATPase

The fact that both aerobic and anaerobic growth in the presence of azide resulted in reduced cell yields prompted experiments to investigate the effect of azide on the ATPase of *B. coagulans*.

Table 2. Anaerobic growth in the presence of sodium azide

Bacillus coagulans was grown anaerobically at 55 °C or 37 °C in TYG broth with or without sodium azide. Culture pH and cell yield were determined after 18 h growth. Initial pH = 6.2.

Azide (mM)	55 °C		37 °C	
	Dry wt (mg ml ⁻¹)	pH	Dry wt (mg ml ⁻¹)	pH
0	0.26	4.10	0.27	4.20
0.5	0.20	4.05	0.13	4.45
1	0.18	4.03	0.06	4.90
2	0.14	4.03	0	5.95
4	0.10	4.10	0	6.20

Fig. 3

Fig. 3. Inhibition by azide of ATPase activity. Cell-free extracts prepared from cells grown at (a) 55 °C or (b) 37 °C were assayed at 55 °C (○) or 37 °C (●) in the presence of 0 to 1 mM-sodium azide and percentage inhibition was calculated.

Fig. 4

Fig. 4. Effect of assay temperature on azide inhibition of ATPase activity. Cell extracts from cells grown at 55 °C (○) or 37 °C (●) were assayed in the presence and absence of 0.3 mM-sodium azide and the percentage inhibition was calculated.

When assayed at mesophilic temperatures (e.g. 37 °C), the ATPase from cells grown at 37 °C was inhibited slightly less by azide (0.1–1 mM) than cells grown at 55 °C (Fig. 3). In contrast, when assayed at 55 °C ATPase activity in cells grown at 55 °C was much less sensitive, whereas ATPase from 37 °C-grown cells was slightly more sensitive (Fig. 3). The sensitivity to azide therefore depends both on the growth and assay temperature. To confirm this, the activity in the presence of a fixed concentration of azide (0.3 mM) was determined over the whole growth temperature range (27–58 °C) for both 55 °C- and 37 °C-grown culture. The results (Fig. 4) show that for 55 °C-grown cells the ATPase became progressively less sensitive to azide as the temperature of the assay was increased, while for 37 °C-grown cells the reverse trend was seen. However, the effect of temperature on the azide sensitivity of ATPase from 37 °C-grown cells was much less pronounced.

DISCUSSION

It was unexpected that growth of *Bacillus coagulans* should become more sensitive to azide as the incubation temperature was lowered. This suggested that some major change in metabolism was occurring during the transition from thermophilic to mesophilic conditions. In view of the

known effects of azide as the respiratory poison and the previous work on thermoadaptation of *Bacillus stearothermophilus* (Jung *et al.*, 1974), where fermentation and respiration are active at 55 °C but only respiration at 37 °C, it was anticipated that azide would be found to inhibit respiration in *B. coagulans* at least under mesophilic conditions.

A study of the cytochrome composition of *B. coagulans* grown at different temperatures revealed both quantitative and qualitative differences. The large amount of cytochrome *d* present in cells grown at 55 °C and especially in stationary-phase cultures is probably a reflection of lower oxygen solubility and therefore availability in dense cultures. Cytochrome *d* has been found in other bacteria under conditions of oxygen limitation (Rice & Hempfling, 1978) or in stationary phase cultures (Castor & Chance, 1959). However, attempts to induce synthesis of cytochrome *d* in cells grown at 35 °C by imposing severe oxygen limitation have failed (M. V. Jones, unpublished data). The synthesis of this cytochrome may therefore respond to both oxygen tension and temperature.

That cytochrome *d* functions as an alternative terminal oxidase in *B. coagulans* is indicated by the increased cyanide insensitive NADH oxidation. Cytochrome *d* has been shown to have low cyanide sensitivity in other bacteria (Pudek & Bragg, 1974; Knowles, 1976). It does not appear to have any particular role in the thermostability of the respiratory system as NADH oxidase from cells with or without detectable cytochrome *d* was equally thermostable. This result is again an exception to the hypothesis that facultative thermophiles do not synthesize inherently thermostable proteins at mesophilic growth temperatures (Crabb *et al.*, 1975; Heinen & Lauwers, 1983).

Despite changes in cytochrome composition at different growth temperatures there was little difference in the azide sensitivity of NADH oxidation. Concentrations of azide sufficient to completely inhibit growth had little or no effect on respiration. The finding that anaerobic growth at mesophilic temperatures was equally sensitive to azide as aerobic growth indicated that azide was probably affecting a central metabolic activity. There was no evidence for a change in metabolic capability as seen in *B. stearothermophilus* (Jung *et al.*, 1974) and anaerobic growth was equally efficient (as measured by cell yields) at both mesophilic and thermophilic temperatures.

Both mitochondrial and bacterial ATPases are known to be sensitive to azide (Linnett & Beechley, 1979) and the inhibitor will bind to the soluble (BF₁) portion of the enzyme (Munoz *et al.*, 1969; Yoshida *et al.*, 1977). Concentrations of 0.1 mM-azide gave over 50% inhibition of ATPase from *E. coli* (Evans, 1969) and *Micrococcus lysodeikticus* (Munoz *et al.*, 1969). We found the ATPase of *B. coagulans* to be slightly less sensitive to azide at 37 °C. However, the degree of inhibition depended on both the assay and the growth temperature. The ATPase in cell-free extracts prepared from cells grown and assayed at 55 °C was substantially more resistant to azide than ATPase from 37 °C-grown cultures. The progressive decrease in azide inhibition with increased temperature reflects the pattern of growth in azide-containing broth. We propose that a major factor in the temperature-dependent azide sensitivity of *B. coagulans* is a modification of the ATPase with growth temperature.

Temperature-dependent changes in ATPase activity could occur through modifications in membrane-lipids as has been shown for several membrane-bound enzymes (Welker, 1976; De Kruffy *et al.*, 1973). Alternatively, as azide can interact with the soluble portion of the ATPase molecule, a change in the subunit structure of the BF₁ might be possible. It has been shown that in the obligatory thermophilic bacterium PS3 the γ subunit was necessary for activity at high temperatures. In the absence of the γ subunit the reconstituted enzyme was thermolabile and insensitive to azide (Yoshida *et al.*, 1977). Studies with the ATPase of the facultative thermophile, *B. coagulans*, will provide an interesting comparison with this work and further information on the mechanisms of thermophily.

REFERENCES

ALBERTY, R. A. (1968). The effect of pH and metal ion concentration on the equilibrium hydrolysis of adenosine triphosphate to adenosine diphosphate. <i>Journal of Biological Chemistry</i> 243 , 253-260.	AMELUNXEN, R. E. & MURDOCK, A. L. (1978). Mechanisms of thermophily. <i>CRC Critical Reviews in Microbiology</i> 6 , 343-393.
	CASTOR, L. & CHANCE, B. (1959). Photochemical

- determination of the oxidases of bacteria. *Journal of Biological Chemistry* **234**, 1587–1592.
- CRABB, J. W., MURDOCK, A. L. & AMELUNXEN, R. E. (1975). A proposed mechanism of thermophily in facultative thermophiles. *Biochemical and Biophysical Research Communications* **62**, 627–633.
- DE KRUYFF, B., VAN DUICK, P. W. M., GOLDBACH, R. W., DEMEL, R. A. & VAN DEENEN, L. L. M. (1973). Influence of fatty acid and sterol composition on the lipid phase transition and activity of membrane bound enzymes in *Acholeplasma laidlawii*. *Biochimica et biophysica acta* **330**, 269–282.
- EDWARDS, C. & JONES, M. V. (1983). Production of a thermostable ATPase by the facultative thermophile, *Bacillus coagulans*. *Archives of Microbiology* **135**, 74–76.
- EDWARDS, C., SPODE, J. A. & JONES, C. W. (1978). The properties of adenosine triphosphatase from exponential and synchronous cultures of *Alcaligenes eutrophus* H16. *Biochemical Journal* **172**, 253–260.
- EVANS, D. J. (1969). Membrane adenosine triphosphatase of *Escherichia coli*: activation by calcium ions and inhibition by monovalent cations. *Journal of Bacteriology* **100**, 914–922.
- FARRELL, J. & CAMPBELL, L. L. (1969). Thermophilic bacteria and bacteriophages. *Advances in Microbial Physiology* **3**, 83–109.
- GIBSON, T. & GORDON, R. E. (1974). *Bacillus*. In *Bergey's Manual of Determinative Bacteriology*, pp. 529–550. Edited by R. E. Buchanan & N. E. Gibbons. Baltimore: Williams & Wilkins.
- GORDON, R. E., HAYNES, W. C. & PANG, N. C. (1973). The genus *Bacillus*. *United States Department of Agriculture Handbook* no. 427.
- HEINEN, W. & LAUWERS, A. M. (1983). Changes in enzyme stability and fatty acid composition of *Streptomyces* sp., a facultative thermophilic Actinomycete. *Archives of Microbiology* **134**, 247–250.
- JUNG, L., JOST, R., STOLL, E. & ZUBER, H. (1974). Metabolic differences in *Bacillus stearothermophilus* grown at 55 °C and 37 °C. *Archives of Microbiology* **95**, 125–138.
- KNOWLES, C. J. (1976). Microorganisms and cyanide. *Bacteriological Reviews* **40**, 652–680.
- LINNETT, P. E. & BEECHLEY, R. B. (1979). Inhibitors of the ATP synthetase system. *Methods in Enzymology* **LV**, 472–515.
- MCARTHUR, H. A. I. & REYNOLDS, P. E. (1979). Peptidoglycan carboxypeptidase and endopeptidase activities of *Bacillus coagulans* NCIB 9365. *Journal of General Microbiology* **111**, 327–355.
- MUNOZ, E., SALTON, M. R. J., NG, M. H. & SCHOR, M. T. (1969). Membrane ATPase of *Micrococcus lysodeikticus*: Purification and properties of the soluble enzyme and properties of the membrane-bound enzyme. *European Journal of Biochemistry* **7**, 490–501.
- NOVITSKY, T. J., CHAN, M., HIMES, R. H. & AKAGI, J. M. (1974). Effect of temperature on growth and cell wall chemistry of a facultative thermophilic *Bacillus*. *Journal of Bacteriology* **117**, 858–865.
- PUDEK, M. R. & BRAGG, P. D. (1974). Inhibition by cyanide of the respiratory chain oxidases of *Escherichia coli*. *Archives of Biochemistry and Biophysics* **164**, 682–693.
- RICE, C. W. & HEMPFLING, W. P. (1978). Oxygen-limited continuous culture and respiratory energy conservation in *Escherichia coli*. *Journal of Bacteriology* **134**, 115–124.
- SMITH, L. (1978). Bacterial cytochromes and their spectral characteristics. *Methods in Enzymology* **LIII**, 202–212.
- WELKER, N. E. (1976). Effect of temperature on membrane proteins. In *Extreme Environments: Mechanisms of Microbial Adaptation*, pp. 229–254. Edited by M. R. Heinrich. New York: Academic Press.
- WEST, I. C. & MITCHELL, P. (1974). The proton-translocating ATPase of *Escherichia coli*. *FEBS Letters* **40**, 1–4.
- WILLIAMS, R. A. D. (1975). Caldoactive and thermophilic bacteria and their thermostable proteins. *Science Progress* **62**, 373–393.
- YOSHIDA, M., SONE, N., HIRATA, H. & KAGAWA, Y. (1977). Reconstitution of adenosine triphosphatase of thermophilic bacterium from purified individual subunits. *Journal of Biological Chemistry* **252**, 3480–3485.