Effect of tryptophan catabolites on the development of heat resistance in *Bacillus cereus* spores

Chandan Prasad and V. R. Srinivasan

Department of Microbiology, Louisiana State University, Baton Rouge, Louisiana 70803, U.S.A.

Abstract

Ethyloxamate and nicotinamide inhibited the development of heat resistance and the biosynthesis of pyridine-2,6-dicarboxylic acid (dipicolinic acid) in *Bacillus cereus* spores. Addition of quinaldic acid or hydroxyanthranilic acid to an ethyloxamate-grown culture resulted in an increase in the number of heat resistant spores. Nicotinamide induced heat sensitivity could be reversed to different degrees by the addition of kynurenine or xanthurenic acid. Explanations which may account for these observations are presented.

Introduction

Bacterial spores are generally far more resistant than their vegetative counterpart to certain processes such as sterilization or disinfection. This fact is of major concern in the sterilization of pharmaceuticals and also in the prevention of food spoilage. Most previous studies have been concerned with either the chemical changes during heat destruction or the effect of conditions of sporulation, heat treatment and recovery on the degree of heat resistance in bacterial spores (Schmidt, 1955; Tang and Grecz, 1965; Murrell and Scott, 1966).

Recently studies on the development of heat resistance in spores were undertaken by Murrell and Warth (1964); they made an extensive study on spore composition and heat resistance in a series of different bacilli. Warren (1968) has demonstrated a decrease in the heat precipitable protein with the advancement of sporulation and the increase in the heat resistant cells in *B. subtilis* cultures. However, the basis of heat resistance still remains obscure. This problem may be approached by studying the biochemical changes occurring during spore formation and the development of heat resistance, or by studying mutants producing heat sensitive spores, or more directly by the use of chemicals which induce the formation of heat sensitive spores. Several methods are now available for producing heat sensitive spores. These include sporulation in low calcium medium (Slepecky and Foster, 1959; Black, Hashimoto and Gerhardt, 1960), in the presence of phenylalanine (Church and Halvorson, 1959), ethyl malonate (Gollakota and Halvorson, 1960), ethyl oxamate (Gollakota and Halvorson, 1963), picolinamide (Upreti, Singh, Verma, Bhatia and Gollakota, 1969) or nicotinamide (Kumar and Gollakota, personal communication).

The present studies were designed to show the extent to which several intermediates of tryptophan catabolism contribute to the increase of heat resistance of spores of *Bacillus cereus* grown in the presence of ethyl oxamate or nicotinamide.

Materials and methods

Organism

Bacillus cereus TR-2, a phage resistant strain derived from *B. cereus* T, was used throughout these studies and was maintained on nutrient agar (Difco) slants.

Cultural conditions

The bacteria were grown in G-medium (Stewart and Halvorson, 1953) according to the active culture technique of Nakata and Halvorson (1960). The cultures were incubated on a rotary shaker maintained at 30°C.

Measurement of pH, growth and sporulation

Growth was determined by measuring optical density at 600 nm using Spec-20 (Bausch and Lomb). The viability of the culture was evaluated by plating on nutrient agar.

The degree of sporulation was determined by the number of cells that survived heating at 80°C for 30 min or the number of cells which remained viable after 15 min of exposure to octyl alcohol (1 drop of alcohol per 10 ml suspension).

The pH of the samples was measured on a Corning pH meter model 7. The time at which pH of the culture reached a minimum was referred to as t_0 ($t_0 = 4$ hr). Under our experimental conditions t_0 was also the time at which exponential growth ceased.

Effect of inhibitors

The effects of inhibitors on growth, sporulation and heat resistance of spores were studied by adding the compound to an active culture immediately after the final transfer was made (referred to as 'o' time).

Reversing agents

The ability of a compound to reverse the effect of an inhibitor was usually tested by the addition of inhibitor and the test compound to an active culture at o time. To study the effect of time of addition, the inhibitor was added at o time and the test compound at the indicated time.

Assay of dipicolinic acid (DPA)

DPA was assayed according to the method of Janssen, Lund and Anderson (1958). In some cases a reliable estimation of DPA could not be made due to the chromogenic nature of the reversing agents used.

Chemicals

DPA, DL-kynurenine, ethyloxamate and kynurenic acid were purchased from Aldrich Chemical Company, Wisconsin, U.S.A. L-tryptophan and 3-OH-anthranilic acid were obtained from Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A. Quinaldic acid and nicotinamide were the products of J. T. Baker Chemical Company, Phillipsburg, New Jersey, U.S.A. Anthranilic acid was bought from W. H. Curtin and Company, New Orleans, Louisiana, U.S.A.

Results

Experiment 1 Effect of nicotinamide and ethyloxamate on heat resistance

Nicotinamide (NA) or ethyloxamate (EO) was added at zero hour to an active culture of *B. cereus* growing at 30°C on a rotary shaker. Cell counts and DPA estimations were made after 24 hr of incubation. Table 1 presents a typical experiment showing the effect of NA and EO on sporulation and heat resistance.

Table 1 Effect of nicotinamide and ethyloxamate on sporulation of *Bacillus cereus* TR-2

Additions at zero hr	After 24 hours of incubation at 30 °C: Viable cells/ml	Heat stable cells/ml	% Heat stable cells
None	$5{\cdot}1 \times 10^8$	$5{\cdot}0 \times 10^8$	98
Nicotinamide (2 mg/ml)	$5{\cdot}0 \times 10^8$	$1{\cdot}2 \times 10^7$	2
Ethyloxamate (1·2 mg/ml)	$4{\cdot}0 \times 10^8$	$1{\cdot}7 \times 10^6$	1

Inhibitors were added in an active culture at zero hour and the cell counts were made after incubating the culture for 24 hr at 30 °C on a rotary shaker.

Neither of the two inhibitors affected the growth and sporulation. However, the proportion of heat resistant spores was very low in the cultures treated with NA (1 to 4% of control) or EO (1 to 10% of control) with a simultaneous decrease in DPA content.

Experiment 2 Reversion of nicotinamide and ethyloxamate induced heat sensitivity

Before testing the reversion of NA or EO induced heat sensitivity by tryptophan catabolites, it had to be established that these compounds would allow normal growth and sporulation of *B. cereus* under the conditions of our experimentation. This was tested and found to be true when the supplements were added at zero or 4 hr, except quinaldic acid and xanthurenic acid which were added only at 4 hr. Each of the following substances was added at the rate of 1 mg per 10 ml of the culture: L-tryptophan, DL-kynurenine, anthranilic acid, 3-hydroxyanthranilic acid, kynurenic acid, quinaldic acid and xanthurenic acid.

NA or EO were added at zero hour to actively growing cultures of *B. cereus*. At 4 hr, a number of common tryptophan catabolites (100 μg/ml, neutralized to pH 7·0 with 1 M NaOH) were added separately to inhibited cultures and incubated at 30°C on a rotary shaker. After 24 hr heat stable spore counts were taken. A typical experiment is presented in Table 2. There was a 300–500 per

Table 2 Effect of some tryptophan catabolites on the inhibition of development of heat resistance by ethyloxamate and nicotinamide

Compound added (100 μg/ml)	Ethyloxamate (1·2 mg/ml) Heat stable cells/ml	% of control	Nicotinamide (2 mg/ml) Heat stable cells/ml	% of control
None (control)	$7{\cdot}6 \times 10^7$	100	$2{\cdot}0 \times 10^6$	100
L-tryptophan	$3{\cdot}5 \times 10^7$	46	—	—
DL-kynurenine	$2{\cdot}6 \times 10^7$	35	$4{\cdot}1 \times 10^6$	205
Anthranilic acid	$2{\cdot}3 \times 10^7$	30	$2{\cdot}9 \times 10^6$	146
3-Hydroxy-anthranilic acid	—	—	$1{\cdot}5 \times 10^6$	75
Kynurenic acid	$1{\cdot}4 \times 10^7$	19	$2{\cdot}0 \times 10^7$	100
Xanthurenic acid	$5{\cdot}0 \times 10^7$	65	$1{\cdot}0 \times 10^7$	500
Quinaldic acid	$3{\cdot}3 \times 10^8$	435	$2{\cdot}0 \times 10^6$	100

All the cultures were grown in G-medium at 30 °C on a rotary shaker. Inhibitors and reversing compounds were added at zero and 4 hr respectively in an active culture. Cell counts were made after 24 hr.

cent increase in the number of heat resistant spores in a EO grown culture in the presence of quinaldic acid. In a nicotinamide inhibited culture, the increase in the heat resistant spores by addition of anthranilic acid, kynurenine or xanthurenic acid was 150, 200 and 200–500 per cent respectively.

Experiment 3 Effect of time of addition of quinaldic acid and hydroxyanthranilic acid in ethyloxamate treated cultures

The EO treated culture was the most sensitive to reversion by quinaldic acid immediately after the growth stopped (4·5 hr). As the sporulation progressed

Table 3 Effect of time of addition of quinaldic acid in an ethyloxamate treated culture on the development of heat resistance

Time of addition in hours (100 μg/ml)	After 24 hr of incubation at 30 °C: Viable cells/ml	Heat stable cells/ml	% of control
None (control)	$4{\cdot}0 \times 10^8$	$6{\cdot}7 \times 10^6$*	100*
4·5 ($t_{0\text{·}5}$)	$1{\cdot}2 \times 10^8$	$3{\cdot}5 \times 10^7$	522
5·0 (t_1)	$3{\cdot}4 \times 10^8$	$2{\cdot}1 \times 10^7$	313
5·5 ($t_{1\text{·}5}$)	$3{\cdot}2 \times 10^8$	$1{\cdot}5 \times 10^7$	224
6·1 (t_2)	$3{\cdot}3 \times 10^8$	$1{\cdot}2 \times 10^7$	179
10·0 (t_6)	$3{\cdot}5 \times 10^8$	$6{\cdot}4 \times 10^6$	96

Ethyloxamate (1·2 mg/ml) was added at zero hour to an active culture of *B. cereus* grown at 30 °C on a rotary shaker.

the ability of quinaldic acid to reverse EO effect decreased and it completely disappeared at 10 hr (t_6). The addition of quinaldic acid during growth phase resulted in cell lysis and inhibition of sporulation. On the contrary, hydroxyanthranilic could reverse the effect of EO only if it was added during growth phase (Tables 3 and 4).

Table 4 Effect of time of addition of 3-hydroxyanthranilic acid in an ethyloxamate treated culture on the development of heat resistance

Time of addition in hours (100 μg/ml)	After 24 hr of incubation at 30 °C: Viable cells/ml	Heat stable cells/ml	% of control
None (control)	$2{\cdot}1 \times 10^8$	$9{\cdot}5 \times 10^6$*	100*
2·5	$2{\cdot}2 \times 10^8$	$1{\cdot}2 \times 10^8$	1200
4·5	$2{\cdot}2 \times 10^8$	$1{\cdot}0 \times 10^7$	105
5·5	$2{\cdot}6 \times 10^8$	$4{\cdot}0 \times 10^6$	42
11·5	$2{\cdot}3 \times 10^8$	$3{\cdot}5 \times 10^6$	37

Ethyloxamate (1·2 mg/ml) was added at zero hour to an active culture of *B. cereus* grown at 30 °C on a rotary shaker.

Experiment 4 Effect of time of addition of DL-kynurenine or xanthurenic acid in nicotinamide grown cultures

Kynurenine or xanthurenic acid, when added to a nicotinamide treated culture at 4 hr, resulted in an increase in the number of heat resistant spores. The

Table 5 Effect of time of addition of DL-kynurenine in a nicotinamide treated culture on the development of heat resistance

Time of addition in hours (100 μg/ml)	After 24 hr of incubation at 30 °C: Viable cells/ml	Heat stable cells/ml	% of control
None (control)	$5{\cdot}0 \times 10^8$	$2{\cdot}6 \times 10^6$*	100*
2 (t_{-2})	$4{\cdot}8 \times 10^8$	$6{\cdot}0 \times 10^5$	23
4 (t_0)	$5{\cdot}1 \times 10^8$	$5{\cdot}2 \times 10^6$	200
6 (t_2)	$4{\cdot}6 \times 10^8$	$8{\cdot}0 \times 10^5$	31
10 (t_6)	$4{\cdot}9 \times 10^8$	$9{\cdot}0 \times 10^5$	35

Nicotinamide (2 mg/ml) was added at zero hour to an active culture of *B. cereus* grown at 30 °C on a rotary shaker.

present experiment was designed to find out if time of addition of these reversing agents had any effect by itself. Both of these supplements were effective only if they were added to the culture before pH started rising (t_0 or 4 hr). A typical experiment is presented in Tables 5 and 6.

Table 6 Effect of time of addition of xanthurenic acid in a nicotinamide treated culture on the development of heat resistance

Time of addition in hours (100 μg/ml)	After 24 hr of incubation at 30 °C: Viable cells/ml	Heat stable cells/ml	% of control
None (control)	$5{\cdot}2 \times 10^8$	$2{\cdot}6 \times 10^6$	100
2 (t_{-2})	$5{\cdot}0 \times 10^8$	$1{\cdot}1 \times 10^6$	42
4 (t_0)	$4{\cdot}9 \times 10^8$	$5{\cdot}6 \times 10^6$	215
6 (t_2)	$4{\cdot}8 \times 10^8$	$1{\cdot}4 \times 10^6$	54
10 (t_6)	$4{\cdot}8 \times 10^8$	$8{\cdot}0 \times 10^5$	31

Nicotinamide (2 mg/ml) was added at zero hour to an active culture of *B. cereus* grown at 30 °C on a rotary shaker.

Discussion

Our studies on the inhibition of development of heat resistance and the biosynthesis of DPA by ethyloxamate and nicotinamide is in good agreement with the previous observations (Gollakota and Halvorson, 1963; Kumar and Gollakota, personal communication). Figure 1 illustrates the time course of bacterial sporulation, development of heat resistance and its inhibition by nicotinamide and ethyloxamate. In an actively growing culture nicotinamide must be added before t_6 to obtain a fair degree of inhibition. Incidentally, t_6 is also the time when active synthesis of DPA starts. Kynurenine and xanthurenic acid were able to reverse the NA induced heat sensitivity to some extent if added at the time when the culture switched from growth phase to sporulation phase. It may be that kynurenine and xanthurenic acid or their metabolic product(s) are affecting some step(s) involved in commitment of heat resistance.

'In the differentiating systems, it is common that inductors are effective only during competent periods of cellular differentiation' (Needham, 1942). It is quite likely that similar phenomena are operative during sporulation of bacilli. This is reflected upon by our observation that EO was effective only if added

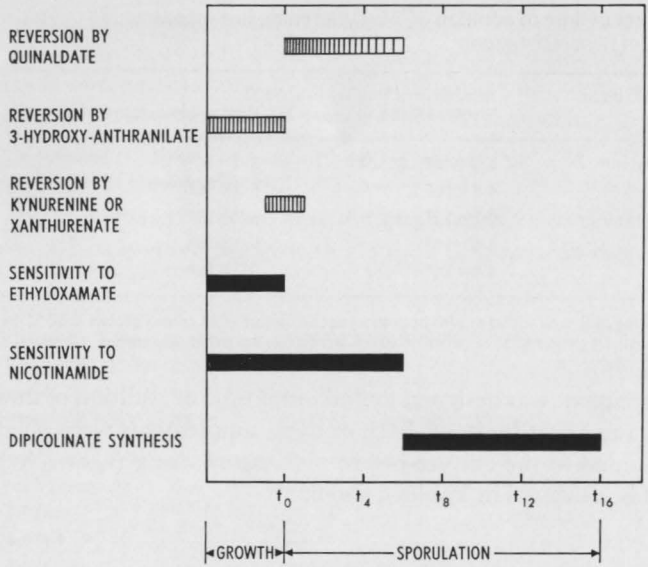

Figure 1 Inhibition of development of heat resistance of *Bacillus cereus* TR-2 spores and its reversal by tryptophan catabolites.

during growth phase, while NA could inhibit the heat resistance if added up to t_6. Quinaldic acid added between t_0 and t_6, to an EO inhibited culture, increased the number of heat resistance spores. However, the efficiency of reversion decreased as the sporulation progressed. On the other hand, hydroxyanthranilic acid was effective only if it was added during growth phase. It is interesting to speculate that quinaldic acid may be affecting some late reaction(s) involved in the development of heat resistance and a decrease in the reversing effect may simply be due to decreased permeability to this compound.

Two major lines of reasoning have been put forward to explain the resistance of bacterial spores to heat: (i) involvement of a dehydrated or hydrophobic spore cortex (Warth, Ohye and Murrell, 1962; Warth, Ohye and Murrell, 1963a,b; Murrell and Warth, 1964) and (ii) stabilization of spore biopolymers by cross-linking between macromolecules (Black and Gerhardt, 1962; Murrell amd Warth, 1964) or by chelation involving DPA, divalent cations and spore amino acids, and peptides (Fleming, 1963; Riemann, 1963; Tang, Rajan and Grecz, 1968). It may be that a complex involving DPA, divalent cations, amino acids, peptides and some other low molecular weight compound(s) may act as a protective barrier of low polarity, low hygroscopicity and low chemical activity. This protective barrier may mask the vital spore polymers from denaturation or inactivation.

Investigations on the presence of low molecular weight compounds in bacterial spores have shown the existence of DPA (Powell, 1953), glutamic acid, 3-phosphoglyceric acid and sulpholactic acid (Nelson, Spudich, Bonsen, Bertsch and Kornberg, 1969). The function of the last three components in a bacterial spore is still unknown. DPA is related to the development of heat resistance but it is not an essential step in this process (Murrell and Warth, 1964). It has been

shown that the two strains of *Clostridium perfringens* with almost the same level of DPA differed 48 times in heat resistance (Weiss and Strong, 1966). Furthermore, several workers have reported a relationship between the level of diaminopimelic acid and calcium and the heat resistance of bacterial spores of several species (Murrell and Warth, 1964; Weiss and Strong, 1966).

As it can be seen from this discussion, heat resistance is a result of multiple reactions operative in the cell during sporulation. An attempt to impart heat resistance or increased level of DPA by tryptophan catabolites in a DPA-less mutant of *B. cereus* (Wise, Sewanson and Halvorson, 1967) was unsuccessful. On the basis of present experiments, we can not state with any certainty which of the reaction(s) involved in heat resistance are associated with tryptophan catabolism. However, the evidence presented in this paper shows a correlation between development of heat resistance and tryptophan catabolism.

Acknowledgement

This research was supported by U.S. Public Health Grant AI-07287. Thanks are due to J. K. Raymond and M. W. Bumm for excellent technical assistance.

References

Black S. H., Hashimoto T. and **Gerhardt P.** 1960. Calcium reversal of the heat sensitivity and dipicolinate deficiency of spores formed 'endotrophically' in water. *Can. J. Microbiol.* **6** 213.

Black S. H. and **Gerhardt P.** 1962. Permeability of bacterial spores. IV Water content, uptake and distribution. *J. Bact.* **83** 960.

Church B. D. and **Halvorson H. O.** 1959. Dependence of the heat resistance of bacterial endospores on their dipicolinic acid content. *Nature* **183** 124.

Fleming H. P. 1963. Chelation properties of bacterial spore components and spore response of ionic environment. Ph. D thesis. University of Illinois, Urbana, Illinois, U.S.A.

Gollakota K. G. and **Halvorson H. O.** 1960. Biochemical changes occurring during sporulation of *Bacillus cereus*. *J. Bact.* **79** 1.

Gollakota K. G. and **Halvorson H. O.** 1963. Biochemical changes occurring during sporulation of *Bacillus cereus* T. II Effect of esters of organic acids on sporulation. *J. Bact.* **85** 1386.

Janssen F. W., Lund A. J. and **Anderson L. E.** 1958. Colorimetric assay for dipicolinic acid in bacterial spores. *Science* **127** 26.

Murrell W. G. and **Warth A. D.** 1964. Spore composition and heat resistance of bacterial spores. *In* Spores III Edited by L. L. Campbell and H. O. Halvorson. Amer. Soc. for Microbiol., Ann Arbor, Mich. 1–24.

Murrell W. G. and **Scott W. J.** 1966. The heat resistance of bacterial spores at various water activities. *J. gen. Microbiol.* **43** 411.

Nakata H. M. and **Halvorson H. O.** 1960. Biochemical changes occurring during growth and sporulation of *Bacillus cereus*. *J. Bact.* **80** 801.

Needham J. 1942. Biochemistry and morphogenesis. Cambridge University Press. pp. 116, 168–169.

Nelson D. L., Spudich J. A., Bonsen P. P. M., Bertsch L. L. and **Kornberg A.** 1969. Biochemical studies of bacterial sporulation and germination. XVII Small molecules in spores. *In* Spores IV. Edited by L. L. Campbell. American Society for Microbiology, Bethesda, Md., 59–71.

Powell J. F. 1953. Isolation of dipicolinic acid (pyridine-2,6-dicarboxylic acid) from spores of *Bacillus megaterium*. *Biochem. J.* **54** 210.

Riemann H. 1963. Germination of bacterial spores with chelators. Ph.D thesis. The Bangs, Copenhagen, Denmark.

Schmidt C. F. 1955. The resistance of bacterial spores with reference to spore germination and its inhibition. *Ann. Rev. Microbiol.* **9** 387.

Slepecky R. A. and **Foster J. W.** 1959. Alteration in metal content of spores of *Bacillus megaterium* and the effects on some spore properties. *J. Bact.* **78** 117.

Stewart B. T. and **Halvorson H. O.** 1953. Studies on the spores of aerobic bacteria. I The occurrence of alanine racemase. *J. Bact.* **65** 160.

Tang T. C. and **Grecz N.** 1965. Relation of dipicolinic acid to thermal and radiation destruction of spores of *Clostridium botulinum*. *Bacteriol. Proc.* **A37,** pp. 7.

Tang T. C., Rajan K. S and **Grecz N.** 1968. Mixed chelates of Ca (II)-pyridine-2,6-dicarboxylate with some amino acids related to bacterial spores. *Biophys. J.* **8** 1458.

Upreti G. C., Singh R. P., Verma J., Bhatia P. L. and **Gollakota K. G.** 1969. The effect of some alpha picolinic acid derivatives on growth and sporulation of bacilli. *Biochem. Biophys. Res. Communs.* **35** 611.

Warren S. C. 1968. Sporulation in *Bacillus subtilis*. Biochemical changes. *Biochem. J.* **109** 811.

Warth A. D., Ohye D. F. and **Murrell W. G.** 1962. Spore cortex formation, composition and heat resistance. (Abstr.) Intern. Congr. Microbiol., 8th, Montreal, pp.16.

Warth A. D., Ohye D. F. and **Murrell W. G.** 1963a. The composition and structure of bacterial spores. *J. Cell Biol.* **16** 579.

Warth A. D., Ohye D. F. and **Murrell W. G.** 1963b. Location and composition of spore mucopeptide in *Bacillus* species. *J. Cell Biol.* **16** 593.

Weiss K. F. and **Strong D. H.** 1966. Some properties of heat resistant and heat sensitive strains of *Clostridium perfringens*. Bacteriol. Proc. G99, pp. 32.

Wise J., Swanson A. and **Halvorson H. O.** 1967. Dipicolinic acid-less mutants of *B. cereus*. *J. Bact.* **94** 2075.

Accepted 8 January 1970