A *Bacillus subtilis* Mutant Requiring Dipicolinic Acid for the Development of Heat-resistant Spores

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A *Bacillus subtilis* mutant is described which forms heat-resistant spores only in the presence of external dipicolinic acid (DPA). The mutation, *dpa-1*, is localized in a new sporulation locus, linked to *pyrA*. The *dpa-1* strain is unable to synthesize DPA but can incorporate external DPA. The amount of DPA incorporated, the frequency of heat-resistant spores and their degree of resistance are all dependent on the concentration of external DPA. Spores of *dpa-1* strains exhibit normal resistance to most chemicals, including octanol and chloroform, but not to ethanol, pyridine, phenol and trichloroacetic acid. Complete resistance to the latter group depends on DPA. DPA incorporation is slow and apparently requires an energy supply but not protein synthesis. Direct involvement of DPA in the heat-resistance of the spores is suggested. Thin sections of DPA-less spores exhibit clearly visible cytoplasmic membranes and ribosomes. These structures are absent or less visible in the core of spores obtained with added DPA.

INTRODUCTION

Although bacterial spores are well known for their resistance to heat, desiccation and various chemical agents, the mechanism of such resistance is not well understood. Dipicolinic acid (DPA) has been thought to play an important role in heat resistance. Such a belief was mainly based on indirect arguments, reviewed by Gould & Dring (1974): DPA is present in all bacterial endospores, its synthesis closely precedes the appearance of heat resistance, and in several systems there is a quantitative correlation between DPA content and heat resistance. This view was, however, challenged by brief (and still unconfirmed) reports of mutants which form DPA-less but heat-resistant spores (Hanson *et al.*, 1972; Zytkevicz & Halvorson, 1972). Such results were inconsistent with those obtained with other mutants unable to synthesize DPA, the spores of which remained heat-sensitive unless external DPA was added (Sebald, 1968; Fukuda & Gilvarg, 1968). Clearly, detailed investigation of further DPA-less mutants is required.

A *Bacillus subtilis* mutant with spores resistant to chloroform but not to heat treatment has been described previously (Balassa & Yamamoto, 1970). In this paper we report that the heat resistance of spores of this mutant depends directly on the incorporation of added DPA. Our observations confirm the role of DPA in heat resistance and suggest a reconsideration of theories on the mechanism of spore resistance.

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Table 1. *Strains of Bacillus subtilis* used

Strain*	IPCC† no.	Genotype
CB22	7626	<i>dpa-1 leu-8 metB5 thr-5</i>
AB12	7635	<i>dpa-1 pheA1 trpC2</i>
AC28	7697	<i>dpa-1 trpC2</i>
168	7645	<i>dpa⁺ trpC2</i>
PM9	7647	<i>dpa⁺ metC3 pheA1 trpC2</i>
PM12	—	<i>dpa⁺ metC3 pheA1 pyrA1</i>

* Strain CB22 was obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced mutagenesis (Balassa, 1969). Strains AB12 and AC28 were obtained by transformation with CB22 and AB12, respectively, as donors. Strains 168, PM9 and PM12 are *Spo*⁺ *dpa*⁺ control strains described by Milhaud *et al.* (1978).

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METHODS

Strains. The strains used are listed in Table 1. Strains AB12 and PM9 are isogenic except for the markers indicated (it was found, however, that AB12 had lost its motility and was not transducible). The strains were maintained either at 4 °C on DN agar tubes or at -20 °C on DN agar tubes containing 15 % (v/v) glycerol (Balassa, 1969).

Media and culture conditions. Unless otherwise indicated, sporulating cultures were obtained by inoculating bacteria from a preculture into a nutrient broth sporulation medium at 37 °C (Schaeffer *et al.*, 1965; Balassa, 1969; Dod *et al.*, 1978). Growth was followed by measuring the absorbance at 550 nm. Time *t*₀ was defined as the end of exponential growth; *t*₁, *t*₂ etc. indicate sporulation times in hours after *t*₀.

Spore resistances. These were measured according to Milhaud & Balassa (1973). Undiluted samples of sporulating cultures (containing about 5 × 10⁸ cells ml⁻¹) were treated for 10 min at room temperature with 0.1 vol. toluene, benzene, octanol, chloroform, butanol, 90 % (v/v) ethanol or 90 % (v/v) pyridine, or at 4 °C with 5 % (w/v) phenol or 5 % (w/v) trichloroacetic acid (TCA). For heat treatment, unless otherwise indicated, samples were incubated at 80 °C for 10 min.

Genetic methods. Transformation and transduction experiments were done as described by Milhaud *et al.* (1978). *Dpa*⁻ transformants and transductants were obtained by a two step indirect selection method. Colonies were first selected for the loss of an auxotrophic marker. *Dpa*⁻ colonies were recognized among these colonies by their lighter yellow colour, and their phenotype was confirmed in liquid sporulation cultures.

Electron microscopy. This was done as described by Sousa *et al.* (1974) without post-staining of the sections.

Dipicolinic acid assay. Samples of sporulating cultures were centrifuged, resuspended in 2 ml H₂O, autoclaved for 15 min and centrifuged again. Then 1 ml of supernatant was mixed with 0.25 ml of the reagent described by Janssen *et al.* (1958). The results were compared with a standard curve of pure DPA (Koch-Light) assayed in the same conditions.

Protease activity. This was measured on azocasein as described by Dod *et al.* (1978).

RESULTS

DPA-dependent heat resistance of strain CB22 spores

When incubated in a sporulation medium, strain CB22 formed chloroform-resistant (Ch^R) spores with high frequency. The number of heat-resistant (T^R) spores was much lower, in general well below 1 % (Table 2). The phenotype of the strain with respect to spore resistances is therefore 'chloroform-resistant and heat-sensitive' (Ch^R, T^S). Strain CB22 appears to be an oligosporogenous one (Schaeffer *et al.*, 1965) in which only a small fraction of the cells complete maturation. This fraction varied from one culture to another: it was usually around 10⁻³ but in some experiments it reached 10⁻¹. These variations were probably due to variation between batches of nutrient broth: five independent subclones of CB22 gave similar frequencies of T^R spores in parallel cultures. There was no genetic heterogeneity in CB22: 10 clones obtained either from Ch^RT^S or from Ch^RT^R spores all showed the same sporulation frequencies and gave rise to identical strains.

Addition of DPA to the sporulating cultures (Table 2) allowed all the Ch^R spores to

Table 2. Heat resistance of *Dpa*⁻ spores

Cultures of the *dpa-1* strains were allowed to sporulate in the absence of DPA or with DPA added as indicated. After sporulation was completed, the numbers of spores surviving treatment with chloroform (Ch^R) and with heat (T^R) were measured in each culture.

Strain	Time of heat treatment	DPA absent		DPA added			
		No. of spores ml ⁻¹		Concn (μg ml ⁻¹)	Time*	No. of spores ml ⁻¹	
		Ch ^R	T ^R			Ch ^R	T ^R
CB22	<i>t</i> ₄₈	1.7 × 10 ⁸	1.2 × 10 ⁵	250	<i>t</i> ₀	2 × 10 ⁸	4 × 10 ⁸
CB22	<i>t</i> ₂₀	7 × 10 ⁷	2 × 10 ⁵	50	<i>t</i> ₆	3 × 10 ⁸	2.6 × 10 ⁸
CB22	<i>t</i> ₃₀	1 × 10 ⁸	6 × 10 ⁷	50	exp	3 × 10 ⁸	2 × 10 ⁸
AB12	<i>t</i> ₃₀	8 × 10 ⁷	6 × 10 ⁵	50	exp	4 × 10 ⁸	5 × 10 ⁸
AB12	<i>t</i> ₃₀	8 × 10 ⁷	6 × 10 ⁴	50	exp	7 × 10 ⁸	1 × 10 ⁹
AB12	<i>t</i> ₃₀	1 × 10 ⁸	1.5 × 10 ⁷	50	exp	6 × 10 ⁸	4 × 10 ⁸

* exp, DPA added during early exponential growth.

become heat-resistant. Therefore the phenotype of the strain should be 'chloroform-resistant and DPA-dependent heat-resistant spores' (Ch^R, Dpa^D-T^R), which will be abbreviated to DPA-less (Dpa⁻). Control experiments showed that the presence of DPA during the heat treatment of Dpa⁻ spores did not change their heat sensitivity. Sporulation of oligosporogenous strains is frequently temperature-sensitive, but this was not so with the Dpa⁻ phenotype of strain CB22: the frequency of Ch^R and T^R spores was the same in cultures grown at 30 °C or at 45 °C.

Genetic mapping of the *dpa-1* mutation

Strain CB22 was obtained by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine-induced mutagenesis in conditions where mutants were produced at high frequencies and multiple mutations could be frequent (Balassa, 1969). To demonstrate that the phenotype of CB22 spores is due to a single mutation it was transformed from CB22 into strain PM9 which sporulates normally. The frequency of Dpa⁻ transformants (2% of the transformants for an auxotrophic marker) was consistent with the Dpa⁻ phenotype arising from a single mutation, designated *dpa-1*. One of the transformants, AB12, was used in further studies of the *dpa-1* mutation. Its phenotype was similar to CB22 with respect to the spore resistances and the DPA effect (Table 2). However, we found that AB12 was not motile and not transducible, and so another strain, AC28, was derived from it by further transformation. This latter strain was used for genetic mapping, by PBS1-mediated transduction, of the *dpa-1* mutation.

The results of a cross between AC28 as donor and PM12 as recipient indicated linkage between *dpa-1* and *pyrA1* (35% cotransduction). Loose linkage was found between *metC* and *pyrA* (less than 2% cotransduction), but no linkage was detected between *metC* and *dpa-1*. Thus the gene order *metC-pyrA-dpa* is suggested, with a distance (100% co-transfer) of 65 units between *pyrA* and *dpa*.

Generally the *dpa-1* transformants and transductants had the same phenotype as CB22. Sometimes, however, intermediate phenotypes, sensitive to the addition of DPA but which sporulated even in its presence only at relatively low frequencies, were also found: these were not investigated further.

Characterization of *dpa-1* strains

Development of heat resistance is one of the last events in the process of spore maturation. One would expect, therefore, that the early steps of spore formation would not be affected by the *dpa-1* mutation. This seems to be the case with the synthesis of extracellular protease (Dod *et al.*, 1978) since *dpa-1* strains had normal protease activities. Wild-type spores are

Table 4. Heat resistance of *Dpa*⁻ spores

Sporulating cultures were heated as indicated.

Strain	Sporulation time	No. of spores ml ⁻¹ surviving:		
		80 °C 10 min	80 °C 30 min	90 °C 10 min
CB22 (<i>dpa</i> -1)	<i>t</i> ₃₀	1.5 × 10 ⁸	6 × 10 ⁴	7 × 10 ³
CB22 (<i>dpa</i> -1)	<i>t</i> ₂₀	1 × 10 ⁵	1.7 × 10 ²	0
AB12 (<i>dpa</i> -1)	<i>t</i> ₃₀	3 × 10 ⁶	4 × 10 ⁴	1.5 × 10 ⁴
168 (<i>dpa</i> ⁺)	<i>t</i> ₂₀	4 × 10 ⁸	4 × 10 ⁸	4 × 10 ⁸

Fig. 1. Development of chemical resistances during sporulation of strain CB22. Samples of a sporulating culture were tested every hour for resistance to toluene (■), octanol (●), chloroform (□), pyridine (▽) and to 80 °C for 10 min (○). The number of cells surviving each treatment is indicated.

resistant to various chemicals, and these resistances appear sequentially during spore development (Milhaud & Balassa, 1973). Mutants have been isolated whose spores are resistant to some chemical agents but not to others (Balassa *et al.*, 1974). We therefore measured the chemical resistances of *Dpa*⁻ spores to see if they were affected.

In the wild type, resistances to toluene, benzene, octanol, butanol and chloroform are the first to appear; *Dpa*⁻ spores were completely resistant to these solvents. In contrast, resistances which normally appear after chloroform resistance, i.e. to ethanol, pyridine, phenol and TCA, were attained only with decreasing frequencies by *Dpa*⁻ spores (Table 3). These frequencies, although varying from one experiment to another, were always higher than the frequency of *T*^R spores. On the basis of the decreasing frequencies, the following order of resistances is tentatively proposed: chloroform-ethanol-pyridine-phenol, TCA-80 °C for 10 min. Wild-type spores were equally resistant to all these agents (Table 3).

Treatment for 10 min at 80 °C does not detect the last step in spore formation. Appearance of this resistance is followed by further slow maturation leading to resistance of the spores to higher temperatures and for longer periods (Milhaud & Balassa, 1973). At *t*₂₀ wild-type spores survive for 60 min at 80 °C or at 90 °C. The *T*^R spores of *dpa*-1 strains were much less resistant (Table 4). Even in experiments where the frequency of *T*^R spores (detected by heating at 80 °C for 10 min) was exceptionally high (Table 4, line 1), incubation for a longer period or at a higher temperature killed most of the spores, allowing one to distinguish *dpa*-1 from wild-type cultures.

Fig. 2. Morphological development during sporulation of strain CB22 (*dpa*⁻; —) and strain 168 (*dpa*⁺; ---). The percentage of cells at morphological stage III (●, ○) and the percentage that had reached at least stage V (■, □) are indicated.

Kinetics of development of Dpa⁻ spores

The low frequency of heat-resistant spores could be due to delayed or slow sporulation of *dpa*⁻ strains in the absence of DPA. Mutants in which the development of chemical resistances is slowed down ('decadent' mutants) have been described (Balassa *et al.*, 1974, 1979). It was therefore of interest to follow in detail the kinetics of spore formation and the appearance of various chemical resistances in a *dpa*⁻ strain. The original CB22 strain was used. (This strain appears to be phenotypically identical to AB12 and preliminary experiments with the latter gave similar results.) Compared with the development of wild-type spores (Milhaud & Balassa, 1973), all the chemical resistances developed somewhat later in *dpa*⁻ cultures (Fig. 1). The development of heat resistance was particularly slow. When this was followed in sporulating cultures for 4 d, the number of spores resistant to chloroform and to heat (80 °C for 10 min) did not rise after *t*₂₄ and the spores appeared to be stable. The number of spores surviving a more exacting heat treatment (90 °C for 10 min) increased slowly but continuously from 40 spores ml⁻¹ after 1 d sporulation to over 10⁵ spores ml⁻¹ after 4 d. These results suggest that a slow, continuous maturation takes place in *dpa*⁻ spores; the mutation could be leaky.

The development of a Dpa⁻ culture was also followed by electron microscopy and compared with results obtained with the wild-type strain 168 (Sousa *et al.*, 1978). Samples of sporulating cultures were taken every hour and the number of cells at the different morphological stages was counted in thin sections as described by Sousa *et al.* (1978). In each sample 200 to 500 cells were counted. Since cells blocked at stages II and IV were relatively rare, only the frequencies of cells blocked at stages III and V or VI are shown in Fig. 2. There was only a slight delay in the appearance of stage III cells in the Dpa⁻ culture, and in both cultures the frequencies of stage III cells were highest at around *t*_{3.5}. In the CB22 culture, however, there was a significant 'trail' of cells which reached stage III much later (or which remained longer at stage III). Stages V and VI were difficult to distinguish in Dpa⁻ cells, so late spore development was followed in both cultures by measuring the frequency of cells which had reached at least stage V (stage V plus stage VI cells plus free spores). The appearance of stage V cells was clearly delayed in the Dpa⁻ culture (Fig. 2). These observations are consistent with those on the development of chemical resistances. Sporulation of Dpa⁻ strains appeared to be delayed and this delay was much more pronounced for later stages than for earlier stages. Such delays have been observed in more detail with 'decadent' mutants (Balassa *et al.*, 1979).

Table 5. Incorporation of external DPA into Dpa⁻ sporesDPA was added to nutrient broth cultures before t_0 . Spores were collected at t_{20} .

Strain	DPA added ($\mu\text{g ml}^{-1}$)	DPA in spores [$\mu\text{g (ml culture)}^{-1}$]
CB22 (<i>dpa-1</i>)	0	3
	50	18
AB12 (<i>dpa-1</i>)	0	3
	50	31
PM9 (<i>dpa</i> ⁺)	0	31
	50	33

Fig. 3

Fig. 4

Fig. 3. Dependence of the heat resistance of Dpa⁻ spores on the concentration of added DPA. Various concentrations of DPA were added to CB22 cultures at t_2 . The heat resistance of the spores was tested at t_{20} . The number of spores resistant to chloroform was the same in all the cultures.

Fig. 4. Concentration dependence of DPA incorporation. Various concentrations of DPA were added to CB22 cultures at t_1 . The incorporation of DPA was measured in spores collected at t_{20} .

Incorporation of DPA into *dpa-1* spores

The inability of *dpa-1* spores to attain heat resistance and their response to added DPA suggested that *dpa* strains were unable to synthesize (or accumulate) DPA. This was indeed the case (Table 5): *dpa-1* spores contained 6 to 10 times less DPA than wild-type spores. The small residual amounts of DPA could explain the presence of rare T^R spores in *dpa-1* cultures. This would suggest a heterogeneity in the distribution of residual DPA among *dpa-1* spores. [The colorimetric assay for DPA is known to be specific (Janssen *et al.*, 1958). No further attempt was made to identify chemically the residual amounts.] Addition of DPA to the cultures was followed by its incorporation into the spores. Spores of *dpa-1* cultures formed in the presence of external DPA contained normal or almost normal amounts (60 to 100%) of DPA. The DPA content of wild-type spores, however, was not influenced by external DPA (Table 5).

The effect of added DPA on the development of heat resistance in *dpa-1* spores was concentration-dependent. For low external concentrations of DPA (from 0 to 2.5 $\mu\text{g ml}^{-1}$) there was a linear correlation between the logarithm of the T^R spore count and the concentration of DPA (Fig. 3). All the spores became heat-resistant in the presence of 5 $\mu\text{g DPA ml}^{-1}$. When incorporation of DPA into the spores was followed chemically, a linear correlation was found between the concentration of DPA added and the amount of DPA

Table 6. *Incorporation of DPA, added at different times during sporulation, into Dpa⁻spores*

DPA (250 $\mu\text{g ml}^{-1}$) was added to a CB22 (*dpa-1*) culture at the times indicated. Incorporation of DPA and heat resistance (80 °C, 10 min) were measured at t_{20} (or, when DPA was added at t_{20} , at t_{48}).

	No DPA added	DPA added at:				
		t_0	t_2	t_4	t_6	t_{20}
DPA incorporated ($\mu\text{g ml}^{-1}$)	6	27	32	25	26	19
No. of heat-resistant spores ml^{-1}	2.0×10^4	2.2×10^8	1.7×10^8	1.9×10^8	2.4×10^8	1.2×10^8

Table 7. *Sporulation of strain CB22 in mixed cultures*

Cultures of CB22 (*dpa-1*) and of 'wild-type' strain 168 (*dpa*⁺) were mixed during exponential growth in the proportions indicated. After plating viable and heat-resistant cells at t_{20} , colonies of CB22 were counted on the basis of their white colour (distinct from the brown colonies of 168). Over 50 % of the strain 168 cells formed heat-resistant spores in the mixed cultures.

	Proportion of CB22 in the initial cultures					
	1.0	0.9	0.8	0.5	0.2	0.1
Percentage of viable CB22 cells at t_{20}	100	86	78	57	17	8
No. of viable CB22 cells ml^{-1} at t_{20}	—	1×10^9	1.2×10^9	6×10^8	1.7×10^8	1×10^8
No. of heat-resistant CB22 spores ml^{-1} at t_{20}	2×10^4	3×10^7	1.1×10^7	6.5×10^7	1.3×10^8	1.2×10^8

incorporated (Fig. 4). Saturating concentrations were much higher (about 50 $\mu\text{g ml}^{-1}$) than for the development of heat resistance (Fig. 3). Thus the heat resistance of spores is not a linear function of the amount of DPA incorporated.

In wild-type cells DPA synthesis starts at about stage III and all the spores become heat-resistant before t_7 . It was possible therefore, that there might be a limited period for the incorporation of DPA into *dpa-1* forespores. This is not so, however, since addition of DPA at any time between t_0 and t_6 allowed all the spores to become heat-resistant and to incorporate normal amounts of DPA (Table 6). Even when DPA was added to sporulating cultures at t_{20} , long after the completion of spore formation, all the spores became heat resistant and their DPA contents reached about 60 % of that of the wild type. This observation allowed us to follow incorporation of DPA indirectly by measuring the number of T^R spores after late addition of DPA. As soon as DPA was added to t_{20} cultures (containing Ch^R spores which were presumably mature in all respects but for their DPA content), the number of T^R spores started to increase. On the basis of the earlier results (Fig. 3), this increase corresponded to the incorporation of small amounts (less than 5 $\mu\text{g ml}^{-1}$) of DPA. Incorporation of DPA was very slow, and the maximum number of T^R spores was not reached until 5 to 10 h after its addition. It is not clear if integrity of the sporangium is required for DPA incorporation since in *dpa-1* cultures the majority of the forespores remained enclosed in the sporangium even at t_{20} . One might expect DPA incorporation to be faster if it is added at 'physiological times' (i.e. t_3 to t_6); preliminary observations suggest that this is not so.

Conditions for DPA incorporation

Incorporation of DPA was temperature-dependent since no T^R spores developed when the culture was kept, after addition of DPA, at 4 °C. It was completely inhibited by KCN and by chloroform but not by chloramphenicol (streptomycin had a weak effect). Since

Table 8. Effect of DPA on the 'late chemical resistances'

DPA ($50 \mu\text{g ml}^{-1}$) was added to strain AB12 (*dpa-1*) during exponential growth. Spore resistances were tested at t_{30} .

	No. of spores ml^{-1} resistant to:				
	Chloroform	Pyridine	Phenol	TCA	80°C
DPA absent	1×10^8	5×10^7	2×10^7	6×10^5	1.5×10^7
DPA present	6×10^8	5×10^8	3×10^8	3×10^8	4×10^8

Fig. 5. Heat resistance of spores obtained in the presence of limiting concentrations of DPA. At t_1 DPA was added to CB22 cultures at ($\mu\text{g ml}^{-1}$): $\circ$, 25; $\bullet$, 10; $\blacksquare$, 2.5; $\square$, 0. Samples were taken at t_{20} and tested directly for heat resistance at 80°C (a) and 90°C (b). Survival is expressed relative to the number of cells resistant to chloroform.

chloramphenicol inhibits protein synthesis in the forespores until spore maturation (see Balassa, 1966), these results suggest that DPA incorporation is an active process requiring energy but not protein synthesis. DPA was incorporated not only in nutrient broth cultures but also into cells resuspended in Tris buffer. The presence of bivalent ions (Ca^{2+} , Fe^{2+} or Mn^{2+}) was apparently not required for DPA incorporation.

Sporulating cultures excrete DPA into the medium (Sebald, 1968). A syntrophy-like phenomenon, i.e. sporulation of *dpa-1* cells in mixed cultures with *dpa+* cells, might therefore be expected. This was indeed the case: CB22 cells developed heat-resistant spores when grown together with strain 168 cells (Table 7). In cultures containing only 10% wild-type cells, the frequency of *dpa-1* T^{R} spores had increased from 2×10^{-5} to 3×10^{-2} . This frequency increased with the proportion of *dpa+* cells and all the CB22 spores developed T^{R} spores in cultures where the strain 168 cells were predominant. From these results, the amount of DPA excreted by strain 168 cells during sporulation is estimated to be about 5 to $10 \mu\text{g ml}^{-1}$, i.e. one-sixth to one-third of the DPA contained in the spores.

Properties of *dpa-1* spores obtained in the presence of DPA

Spores obtained from *dpa-1* strains in the presence of excess DPA appeared to be phenocopies of wild-type spores. To ascertain whether they were truly identical or still retained some properties of *dpa-1* spores, their heat resistance was measured in the presence of DPA. Spores of CB22 or AB12 obtained with excess DPA survived as well as wild-type spores at 80°C for up to 120 min or at 90°C for up to 30 min. There was therefore no indication that these spores were less heat resistant. Addition of DPA during sporulation also restored the 'late chemical resistances' (to pyridine, phenol and TCA) of *dpa-1* spores (Table 8).

Table 9. *Morphology of Dpa⁻ spores*

DPA was added, when indicated, to 100 $\mu\text{g ml}^{-1}$. In each sample 60 to 200 spores were examined for the fine structure of the spore core, i.e. visibility of the ribosomes and of the cytoplasmic membrane.

Strain	DPA added	Percentage of spores with the core fine structure:		
		Visible	Intermediate	Invisible
AB12 (<i>dpa-1</i>)	None	92	8	0
AB12 (<i>dpa-1</i>)	$t_{1.5}$	31	50	19
AB12 (<i>dpa-1</i>)	$t_{7.5}$	19	60	22
PM9 (<i>dpa⁺</i>)	None	0	0	100

One should not conclude from these observations that resistance of the spores is an all-or-none phenomenon. Intermediate resistances could be obtained by adding limiting concentrations of DPA. The heat resistance of spores obtained without DPA and in the presence of low concentrations of DPA was measured at 80 °C and at 90 °C (Fig. 5). The rare *dpa-1* spores resistant to 80 °C for 10 min were still sensitive to longer treatment or to incubation at 90 °C. Spores obtained in the presence of 2.5, 10 or 25 $\mu\text{g DPA ml}^{-1}$ showed intermediate resistances. Both the initial rate of decay and the resistant fraction increased in spores obtained with increasing concentrations of DPA. It seems that the curves of Fig. 5 derive from the two phenomena – increasing resistance of the majority of spores and heterogeneity of the population with the more resistant spores becoming more frequent.

Morphology of Dpa⁻ spores

Earlier electron microscopic observations (Balassa & Yamamoto, 1970) failed to detect any significant morphological difference between normal and CB22 spores; in these experiments thin sections of spores were post-stained with uranyl acetate followed by lead citrate. In the present work the fine structure of Dpa⁻ spores was examined in thin sections that had not been post-stained. Spores of the isogenic strains AB12 and PM9 were compared. No difference was found in the spore envelopes, but the appearance of the spore core was completely different in the two preparations. Dpa⁻ spores of AB12 exhibited a well-defined cytoplasmic membrane and evenly distributed ribosomes in the cytoplasm (Fig. 6a). Neither of these structures was seen in wild-type spores (Fig. 6b) which had the typical structureless, ill-defined appearance of mature spores. When spores of the *dpa-1* strain were obtained in the presence of excess DPA, the appearance of the spore core was changed. The spore population was heterogeneous: in some spores none of the spore core structures were visible (Fig. 6c), some looked like the Dpa⁻ spores and some had an intermediate appearance, with the cytoplasmic membrane and the ribosomes still more or less visible but blurred (Fig. 6d; Table 9). This heterogeneity is probably related to the heterogeneity of the spores obtained in the presence of DPA with respect to heat resistance.

Fig. 6. Thin sections of spores sampled at t_{20} : CX, cortex; IC, inner coat; OC, outer coat. All bar markers represent 0.25 μm .

(a) Spore of AB12 (*dpa-1*) produced in the absence of DPA. The ribosomes and the cytoplasmic membrane are clearly visible.

(b) Spore of PM9 (*dpa⁺*) produced in the absence of DPA. The spore core has the typical structureless appearance of mature spores.

(c) Spore of AB12 (*dpa-1*) produced in the presence of DPA (50 $\mu\text{g ml}^{-1}$, added at $t_{1.5}$). The spore core has a structureless appearance as in (b).

(d) Spore of AB12 (*dpa-1*) produced in the presence of DPA (as in c). The ribosomes of the spore are visible but not so clearly as in (a).

DISCUSSION

Strain CB22 carries a mutation that prevents the development of heat-resistant spores unless external DPA is added to the cultures. Sporulating CB22 cells are unable to synthesize normal amounts of DPA. Although many sporulation mutations prevent synthesis or accumulation of DPA (see below), few of them exhibit the 'DPA-dependent conditional heat resistance' phenotype. Sebald (1968) has described a *Clostridium histolyticum* mutant with similar properties: the mutant spores failed to become heat-resistant in the absence of DPA. Addition of DPA resulted in the slow development of heat resistance. This effect was concentration-dependent and saturation was attained with $5 \mu\text{g DPA ml}^{-1}$. Even with excess DPA, however, only a fraction of the population attained resistance. Fukuda & Gilvarg (1968) isolated a *Bacillus megaterium* mutant lacking the condensing enzyme involved in the synthesis of dihydrodipicolinic acid. This mutant, grown in the presence of lysine and diaminopimelic acid, required DPA for the development of heat-resistant spores. Spores obtained without DPA were heat-sensitive, devoid of Ca^{2+} and exhibited a lower buoyant density. *Bacillus cereus* mutants of similar phenotype have also been briefly mentioned (Wise *et al.*, 1967), but genetic studies were not possible with these mutants. To our knowledge, *B. subtilis* mutants of this type have not previously been found.

The *dpa-1* mutation described here seems to concern directly the synthesis of DPA. The strain grows normally in synthetic media and does not require lysine and/or diaminopimelic acid. Transport or retention of DPA are not likely to be affected since external DPA is incorporated and concentrated. Thus it is likely that the last step of DPA synthesis is affected and that the mutation could be in the structural gene of DPA-synthetase. However, the possibility of a regulatory mutation is not excluded. Dpa^- cells form heat-resistant spores with low frequency and contain low amounts of DPA. These properties, and the slow increase in spores resistant to 90°C , suggest either that the mutation is leaky or that DPA is slowly formed by spontaneous oxidation, in a non-enzymic manner.

The Dpa^- phenotype is due to a single mutation located in a new locus, *dpa*. The locus is linked to *pyrA*. This is one of the rare instances where the primary biochemical defect of a sporulation locus is known. No precise mapping of *dpa* with respect to the neighbouring sporulation genes was done. Among the loci situated in the same region, a stage III locus, *SpoIIIE*, seems to map close to *dpa*. Two late mutations are also in the same region (*SpoVD* and *SpoVE*; Piggot & Coote, 1966). The phenotype of these mutants appears to be sufficiently different from the Dpa^- phenotype to be sure that they belong to different loci. There is no indication at present of an operon-like organization involving *dpa*.

Synthesis of DPA is under the genetic control of sporulation genes related by one-directional pleiotropic interactions (Balassa, 1969, 1971). As a general rule, no early sporulation mutants are able to produce DPA. The phenotype of the Dpa^- strains, with spores which are heat-sensitive but apparently mature in every other respect, suggests that DPA synthesis could be the last step (or one of the last steps) of the pleiotropic sequence. The actual situation is more complex: among the late mutants, blocked at stage IV and V and affected in the development of the cortex and/or the spore coats, some fail to synthesize DPA, others are unable to accumulate or to retain DPA and excrete it into the medium, while a few contain normal amounts of DPA (Sebald, 1968; Fitz-James & Young, 1969; Coote, 1972). It seems that, although prior functioning of the majority of sporulation genes is required for DPA synthesis, the end of the pleiotropic sequence is branched. However, one could still consider that the *dpa-1* mutation blocks sporulation later than any other known sporulation mutation.

The principal characteristics of Dpa^- spores are their heat sensitivity and the lack of DPA. Although no comparison of Dpa^- spores with vegetative cells was made for the rate of heat inactivation at lower temperatures, from extrapolation of the results of Fig. 5 it seems likely that Dpa^- spores are more resistant than vegetative cells, due either to the low

amount of DPA present or to other spore structures. Preliminary experiments (with Dr H. Ohlenbusch) show that Dpa^- spores are also lighter and contain less calcium than wild-type spores. However, they attain normal resistance to treatment with organic solvents, including octanol and chloroform. This confirms previous suggestions (e.g. Ryter, 1965; Milhaud & Balassa, 1973; Sousa *et al.*, 1978) that resistance to these solvents is due to the spore envelopes (essentially the spore coats). 'Late chemical resistances', e.g. to ethanol, pyridine, phenol and TCA, are incomplete or absent from the Dpa^- spores. Observations with this mutant confirm the order of resistances deduced from kinetic studies with the wild type (Milhaud & Balassa, 1973). They suggest that the latter resistances are, at least in part, due to a mechanism common to that of heat resistance and which involves DPA. [As suggested previously (Milhaud & Balassa, 1973), the action of ethanol and pyridine could involve dehydration of the spore.] Addition of DPA leads to the recovery of the 'late chemical resistances.'

Incorporation of external DPA does not apparently require protein synthesis, but it appears to be an energy-dependent active process. This is not surprising since DPA concentration in the spore can attain 15% of the dry weight (Gould & Dring, 1974) and accumulation against such a high concentration gradient is likely to require energy. Incorporation into the forespore must be preceded by the crossing of two or three membranes: the cytoplasmic membrane of the mother cell, the forespore membrane and, if its integrity is preserved at the time of DPA incorporation, the outer spore membrane which has opposite polarity. This might explain the slow accumulation of DPA. The role of the sporangium in the uptake of DPA was not investigated. It should be noted that in normally sporulating cells DPA synthesis apparently occurs in the sporangium and not in the forespore (Andreoli *et al.*, 1975).

The fact that DPA can be incorporated into spores in the absence of protein synthesis, and that spores formed in this way are stable and maintain their resistance, suggests that the structure(s) and/or metabolites necessary for the retention of DPA are formed during sporulation even if DPA is not present. High amounts of calcium do not seem to be required.

Heat resistance is not an all-or-none phenomenon. Although excess DPA seems to permit complete recovery of heat resistance, non-saturating concentrations give rise to spores with intermediate heat resistance. The relationship between DPA content and heat resistance is not linear, which is what one would expect if heat resistance is due to a multimeric DPA-containing structure. This aspect requires further investigation, in particular by measuring the DPA content and heat resistance of homogeneous spore fractions (e.g. purified by density gradient centrifugation). Dpa^- spores seem to constitute a particularly useful model system for studying the accumulation and retention of DPA and of calcium. Spores containing varying amounts of DPA (and perhaps of calcium) can be obtained. A very sensitive bioassay can also be developed since quantities as low as $0.5 \mu\text{g DPA ml}^{-1}$ can easily be detected by measuring the number of T^B spores.

The properties of Dpa^- spores provide additional information for the discussion of the possible role of DPA and of the mechanism of heat resistance in bacterial spores. The status of this problem was reviewed by Gould & Dring (1974). Observations with Dpa^- spores of *Cl. histolyticum* and *B. megaterium* (see above) provided evidence for the direct involvement of DPA in heat resistance. On the other hand, several mutants lacking DPA but producing heat-resistant spores have been briefly reported (Hanson *et al.*, 1972; Zytkevich & Halvorson, 1972). There is apparently a contradiction between these results and those obtained with other mutants including the *dpa-1* strains. In the present study the observed correlation between the amounts of DPA incorporated and the heat resistance of the spores directly implicates DPA in the heat resistance of the spores. The properties of the abnormal $Dpa^- T^B$ mutants mentioned above might be explained by: (i) an undetected technical artefact; (ii) the presence of a factor interfering with the DPA assay; (iii)

accumulation in the mutant of a structural analogue of DPA (e.g. over-production of sulpholactic acid); (iv) a different mechanism of heat resistance in these strains.

DPA is localized in the spore core (Leanz & Gilvarg, 1972). If, as seems likely, this is also true for external DPA incorporated in the spore, our results suggest that heat resistance is directly due to a biophysical change which occurs in the spore cytoplasm, and depends on the presence of high amounts of DPA. If this is so, the spore cortex and the spore coats are not directly involved in heat resistance, although they might play a role in the transport, accumulation or retention of DPA. Our observations are difficult to reconcile with the expanded cortex theory (Gould & Dring, 1975). The preliminary observation that DPA incorporation can take place and confer heat resistance in the absence of calcium needs further confirmation. Electron microscopy confirmed that the spore population obtained in the presence of DPA is heterogeneous. It also suggests that the typical unstructured appearance of mature spores is directly due to the presence of DPA and not to a permeability barrier of the spores. Silva (unpublished) has observed, by measuring the speed of gelification of albumin and by ultrastructural studies of vegetative *Bacillus* cells, that DPA competes with OsO_4 fixation. This finding may well explain the appearance of the DPA-containing mature spore core.

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