REIMPOSITION OF HEAT-RESISTANCE ON GERMINATED SPORES OF BACILLUS CEREUS BY OSMOTIC MANIPULATION

by

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SUMMARY

The heat resistance of spores of Bacillus cereus, as measured by heating aliquots of germinating spore suspensions in water, decreased about 7,500-fold during germination. In contrast, when heated in solutions of sucrose at concentrations above c. 1.5 M germinated spores completely regained resistance. Neither sodium chloride nor glycerol protected germinated spores at concentrations equi-osmolal with the sucrose.
The observations are consistent with the hypothesis that the heat resistance of ungerminated bacterial endospores depends upon dehydration of the central core. Rehydration normally occurs during germination and is accompanied by a greatly decreased resistance to heat. However, heat resistance can evidently be completely regained by reimposing dehydration on the core by osmosis, e.g. by resuspending the germinating spores in sufficiently high concentrations of a non-permeant solute like sucrose, thus recreating conditions approximating those in the ungerminated spore.

The heat-resistance of bacterial endospores is thought to result in part from dehydration of the central core (1,2). Gould and Dring (3,4) recently suggested that this dehydration is maintained by the osmotic activity of expanded polyanionic peptidoglycan polymer, and positively charged counterions in equilibrium with it, in the cortex, which is the thick layer that surrounds the core and lies beneath the spore coat. The hypothesis was supported by experiments in which spores with defective (leaky) coats became heat-sensitive in the presence of multivalent cations that could neutralize peptidoglycan anionic groups and thus replace the soluble counterions. Such activity would be expected to reduce the osmotic pressure in the cortex and allow partial rehydration of the enclosed core. Furthermore, the heat resistance of the sensitized spores could be re-established by suspending them in sucrose solutions

at concentrations above about 2M prior to heating (4). Presumably the sucrose solutions osmotically dehydrated the partly rehydrated cores and osmotic pressures of approx. 40-50 atmos. were needed to do this.

During germination, hydration and swelling of the core is known to occur. If the hypothesis that spore heat resistance results from osmotic dehydration of the core is correct, it follows that it should be possible to re-establish heat resistance almost completely in just-germinated spores by osmotically reimposing dehydration on the recently rehydrated core. This paper reports experiments in which such re-establishment of heat-resistance has indeed been shown to occur.

EXPERIMENTAL

Spores of Bacillus cereus T. were grown on potato glucose yeast extract agar, cleaned as described by Gould (5) and activated by heating in water at 70° for 30 min immediately prior to use. Germination was initiated by adding spores (final conc. 10^8/ml) to germination medium which consisted of : L-alanine (10mM), inosine (10mM), O-carbamyl-D-serine (10mM), chloramphenicol (10 μg/ml), Tris (hydroxymethyl aminomethane)-HCl (10mM; pH 7.4). During incubation at 30° aliquots (1 ml) were removed at intervals and pipetted rapidly into water or solutions of sucrose, glycerol or sodium chloride (9 ml) preheated to 60° or 90°. After heating for various periods, samples (1 ml) were removed, dilutions made, and the heat-resistant survivors enumerated conventionally in poured plates of plate count agar (Oxoid) incubated at 30° for 24 h.

RESULTS

The loss of resistance of spores during germination was rapid as measured by heating aliquots of germinating suspensions in water at 60° for 10 min (Fig. 1), reaching about 98% inactivation after only 2 min incubation in the germination medium. Suspension in solutions of sodium chloride (0.9 M) or glycerol (1.8 M) only slightly affected heat sensitivity, but suspension in an approx. equi-osmolal solution of sucrose (1.8 M) markedly increased the heat resistance of the germinated spores. After 2 min of germination, for example, of the 98% of spores which had germinated and were inactivated by heating in water, nearly all survived and had, therefore, apparently regained resistance, when heated in sucrose (Fig. 1). On continued incubation in the germination medium (e.g. at

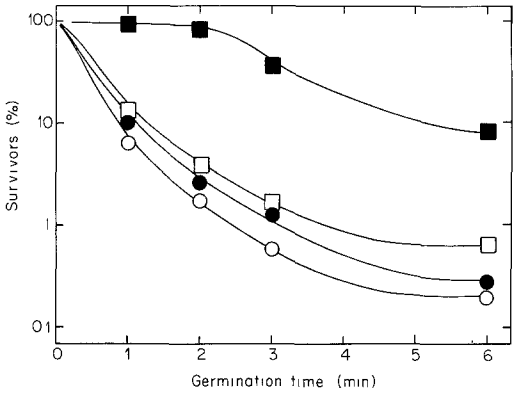

Fig. 1. Heat resistance of germinating spores. Spores were germinated at 30° as described in Methods. At intervals samples were withdrawn and pipetted into hot (60°) water (○), sodium chloride (0.9 M: ●), glycerol (1.8 M: □) or sucrose (1.8 M; ■) and left for 10 min before diluting and enumerating survivors.

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3 and 6 mins in Fig. 1) the protection of the germinated spores afforded by sucrose decreased.

Germinating spores were suspended in different concentrations of sucrose at 60°. Concentrations above about 1.5 M were found to be necessary to reimpose resistance to heat, whereas concentrations below 1 M were ineffective (Fig. 2).

Measurement of the rates of inactivation of germinated spores at various temperatures indicated that the protection afforded by sucrose was very great. Table 1 summarizes data showing that spores germinated for 1.5 min then heated at 90° in 2 M sucrose were about 37,000 times more heat resistant than those heated in water. Furthermore, the heat resistance of germinated spores in sucrose was so enhanced as to even exceed the heat resistance of ungerminated spores.

DISCUSSION

It has recently been suggested that the high heat resistance of bacterial spores

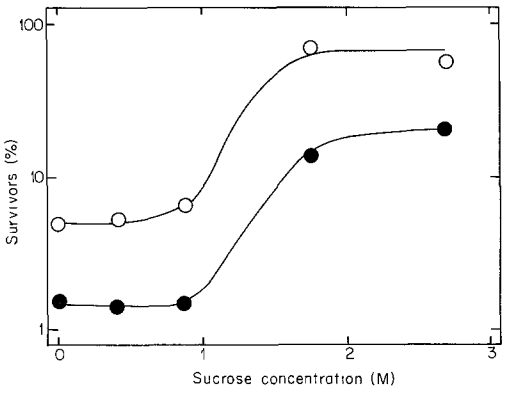

Fig. 2. Effect of sucrose concentration on heat resistance of germinated spores. Spores were germinated for 2 min (○) or 10 min (●), as in Fig. 1, and then heated at 60° for 10 min in water or in the indicated concentrations of sucrose.

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results from dehydration of the central core brought about through the osmotic activity of the surrounding cortex (4). A necessary condition of this expanded cortex hypothesis of spore heat resistance is that the core contains only low concentrations of low M.W. components in solution. Otherwise, osmotic dehydration brought about by the cortex would be relatively ineffective. Analyses have indicated that dipicolinic and glutamic acids and (in some spores) sulpholactic and phosphoglyceric acids (6) make up the major low M.W. pool components. With the high level of calcium which is also present in the dormant spore, these weak acids will be mostly in insoluble form, so that the necessary condition is probably satisfied.

During germination, hydration and swelling of the spore core occurs rapidly and is followed by an increase in the concentration of low M.W. pool components through transport of substrates and ions (7), initiation of metabolism (8), and selective proteolysis of spore components (9). The germinated spore and vegetative forms, consequently normally contain high levels of soluble low M.W. components and, therefore, should be less dehydrated by non-permeant solutes like sucrose than the spore core. However, <u>early</u> during germination, insufficient time will have elapsed for this increase in intracellular solute

Table 1. Protection by sucrose of the heat-inactivation of germinated B. cereus spores.

Organisms	D-value* (min) at 90° of cells heated in:	
	Water	Sucrose (2M)
Ungerminated spores	7.5	31
Germinated spores+	0.001‡	37

* D-value is the time of heating necessary to inactivate 90% of the population.
+ Spores were germinated for 1.5 min at 30° as described in Methods.
‡ Inactivation of germinated spores in water at 90° was too rapid to measure, so the D-value quoted was estimated by extrapolation from that at 55° (which was 3.6 min) assuming a 10-fold decrease per 10° rise in temperature.

levels to occur, so that the core in the recently germinated spore should be almost as osmotically dehydratable as that in the ungerminated spore.

The results recorded above suggest that this is so; in fact, the heat resistance of germinated spores was raised so greatly in sucrose as to exceed that of original ungerminated spores. The results also indicate that the minimum osmotic pressure necessary to dehydrate the core sufficiently to reimpose heat resistance is about 35 atmos. (i.e. equiv. to about 1.5 M sucrose), a value close to that inferred by Gould & Dring (4) to operate in the expanded cortex of the ungerminated spore. Germinated spores made heat resistant in this manner will have excreted all their dipicolinic acid and an approximately equimolar amount of calcium (10), indicating that these components are not indispensable for the full expression of spore heat resistance. Indeed, dipicolinic acid - negative mutant spores, low in calcium, are now known which

nevertheless retain full heat resistance (11). Following germination, extensive changes in macromolecular components normally occur, accompanying the changes in low molecular weight pool components referred to above. However, enzymes and other macromolecules in the recently germinated spores used in the present experiments will probably still be chemically spore-type (12) since too little time will have elapsed since the initiation of germination for extensive proteolytic modification, or new synthesis to occur (13), and the chloramphenicol included in the germination medium will have prevented the initiation of new protein synthesis.

The data reported above and previous experiments in which ungerminated spores were reversibly made heat sensitive by osmotic manipulation (4) strongly support the hypothesis that the principal factor responsible for the enormous heat resistance of bacterial endospores is simple osmotic dehydration of the central core. The extent of resistance that can be achieved in this manner is not surprising when one considers that even the heat resistance of vegetative forms of some bacteria (e.g. Salmonella typhimurium) (14) can be increased about 700-fold by osmotic dehydration (plasmolysis) in solutions of sucrose. The osmoregulatory mechanism that has evolved to maintain dehydration in the core of the dormant endospore, however, whatever the environmental conditions, is remarkable, and may also operate in other resistant and dormant biological structures.

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