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LETHAL HEAT INDUCES SINGLE STRAND BREAKS IN THE DNA OF BACTERIAL SPORES

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SUMMARY

Lethal heating induces DNA single strand breakage in bacterial endospores as detected by the alkaline sucrose gradient centrifugation technique. Heating of spores of Bacillus subtilis 168 at 90°C for 10, 30, and 60 min induced 6, 15, and 15 single strand breaks, respectively and inactivated 6%, 98.2%, and 99.974% of the spores. This is the first report to our knowledge identifying specifically single strand DNA breakage with lethal heat injury of bacterial spores.

INTRODUCTION

Heat injury of bacterial spores has been variously attributed to: i. denaturation of vital spore enzymes(1); ii. impairment of spore germination and/or outgrowth mechanisms (2); iii. damage to membrane resulting in leakage of cytoplasmic constituents expecially calcium-dipicolinic acid (3), increased sensitivity to inhibitory agents such as sodium chloride (4), and finally structural damage seen in the electron microscope as overt blistering and disruption of the integrity of spore membrane (5). Damage to the spore chromosome has also been inferred from the occurrence of heritable mutations induced by either dry heat (6,7) or wet heat (8,9). It was postulated that heat modified specific sites on the spore chromosome (10). So far however no overt DNA strand breaks could be detected in spores subjected to lethal heat injury (8,9), although in vegetative cells heat injury invariably induces single strand (11) and double strand (12) DNA breaks. Furthermore, the cell's own apurinic acid endonuclease was implicated as being responsible for the heat induced DNA breakage in vegetative cells (13).

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We have conducted several independent experiments using spores of four different organisms, two strains of Bacillus subtilis and two strains of Clostridium botulinum. Special care was taken that prior to heating the spores would not be subjected to freezing, lyophilization or prolonged storage and that spores would be harvested as soon as possible after sporulation since all these factors may affect the ability to undergo DNA breakage. In our hands, lethal heat consistently induced DNA breaks in these spores as detected by the alkaline sucrose gradient technique(14). The present paper reports our observations on single strand breakage of DNA and the associated loss of viability in spores of B. subtilis 168 subjected to heating at 90°C for up to 60 minutes.

MATERIALS AND METHODS

Bacillus subtilis strain 168 (wild type) (15) was grown in nutrient broth (Difco) on a shaker water bath at 37°C. Spores were harvested after 48 hr of incubation, washed twice with sterile distilled water, resuspended in 100 ml distilled water at 1 x 10^8 spores/ml and stored at 4°C until needed. Spore DNA was labeled by inoculating 2 ml of stock spore suspension into 20 ml Schaeffer's broth (16) supplemented with 2.5 uCi/ml methyl-[^{3}H]-thymidine and incubating in the 37°C shaker water bath for 16 hr. At this time satisfactory sporulation was obtained with sufficient incorporation of label into the spore DNA. Labeled spores were harvested by centrifugation, washed twice with distilled water and cleaned of vegetative debris by treatment at 37°C with 500 ug/ml lysozyme for 40 min followed by 1% sodium lauryl sulfate for 20 min (8). Cleaned spores were washed three times with sterile distilled water and suspended in 6.5 ml distilled water yielding a concentration of 1.5 x 10^8 spores/ml. Heating was conducted on 1 ml samples of spores suspended in distilled water in tubes immersed in a water bath at 90°C, and immediately cooled in ice water after the desired time of heating.

For alkaline sucrose gradient sedimentation studies 1.5 x 10^8 spores were converted to spheroplasts by the method of Sakakibara et al (17). The spheroplasts were pelleted and suspended in 0.3 ml of 0.5 N NaOH and 0.2 ml of this lysing solution was immediately layered on top of the alkaline (pH 12.6) 5-20% sucrose gradients and allowed to lyse for 30 min. Zone sedimentation of [^{3}H] DNA was done essentially as described by McGrath and Williams(14) except that centrifugation was at 35,000 rpm for 120 min (18). Heat survival of spores was assessed on the surface of plates of Schaffer's medium (16) solidified with 1.5% agar by the bent glass rod method.

RESULTS AND DISCUSSION

The [^{3}H] DNA from the unheated (control) spores showed consistently a bell-shaped sedimentation profile in alkaline sucrose gradients with a peak at a distance of 14/30 from the meniscus (Figure 1,A). The sedimentation

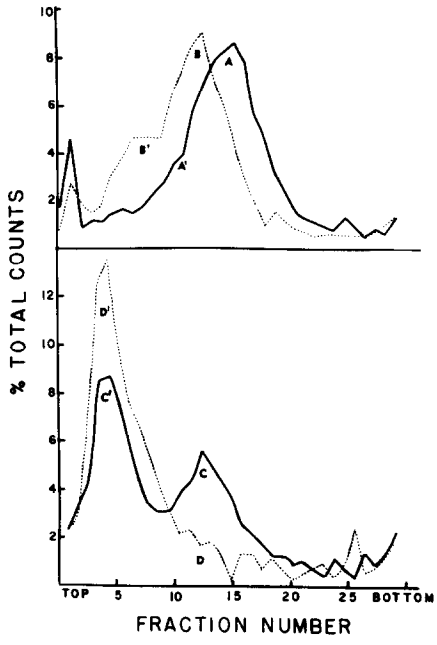

Figure 1. Effect of heating of spores of Bacillus subtilis 168 on sedimentation rates of their [^{3}H] DNA in alkaline sucrose gradients (14) centrifuged at 35,000 rpm for 120 min.

Symbols: A,A' - spores not heated (control); B,B' - heated 10 min at 90°C; C,C' - heated 30 min at 90°C; D,D' - heated 60 min at 90°C.

profiles of the [^{3}H] DNA from spores heated for 10 to 60 min at 90°C (Figure 1,B,C, and D) showed progressively increasing displacement toward the meniscus indicating that the DNA fragments became progressively smaller with increasing time of heating and sedimented at a slower rate. The DNA from spores heated for 10 min had a sedimentation peak at a distance of 12/30 from the meniscus, and the DNA profile showed a clearly discernable inflection at B' (Fig. 1) at a sedimentation distance of about 7/30. On heating for 30 min the main peak C (Fig. 1) showed no further displacement toward the meniscus, however, the size of peak C diminished significantly; at the same time a well defined second peak C' emerged at sedimentation distance 5/30 from the meniscus characteristic of a subpopulation of rather small DNA fragments comprizing some 65% of all [^{3}H] DNA recovered at this time. On heating for 60 min the main peak D virtually disappeared and

Table 1. Effect of heating at 90°C on spores of Bacillus subtilis 168: Loss of spore viability compared with fragmentation of spore [^{3}H] DNA as determined by alkaline sucrose gradient centrifugation (14,18)

Time of heating at 90°C (min)	DNA sedimentation peak in Fig. 1	Alkaline sucrose gradient centrifugation of DNA			Viable spore counts	% spore survival
		D[a]	MW, daltons[b]	# SSB[c]		
No heat (control)	A	14.2	3.3×10^7	---	1.6×10^8	100
10	B	12.0	2.1×10^7	0.57	1.5×10^8	94
	B'	6.9	4.9×10^6	5.75		
30	C	12.0	2.6×10^7	0.55	2.8×10^6	1.8
	C'	4.9	2.0×10^6	15.39		
60	D'	4.9	2.0×10^6	15.39	4.2×10^4	0.026

[a] Sedimentation distance $D = \Sigma XiYi/\Sigma Yi$ where Yi is the fraction of radioactivity in the i-th fraction at a distance Xi from the meniscus (18).

[b] Calculated using the formula $M_1/M_2 = (D_1/D_2)^{2.63}$ where M_1 and M_2 are the molecular weights of DNA at sedimentation distances D_1 and D_2 from the meniscus (13). For the calculation of the MW of unheated control, $M_1 = 16 \times 10^6$ daltons the MW of λ phage at pH 12.6 and $D_1 = 10.8$ (18).

[c] Number of DNA single strand breaks (SSB) calculated from the formula $SSB = \left[\frac{D_1}{D_2}\right]^{2.63} - 1$, where D_1 and D_2 are the average sedimentation distances of unheated and heated spores' DNA, respectively (19).

practically all DNA sedimented now with the second peak (D') at sedimentation distance 5/30 from the meniscus representing relatively small molecular weight DNA fragments obtained as the result of continued breakage of the spore DNA with progressively increasing time of heating.

Table 1 summarizes our data on DNA breakage and spore inactivation. DNA fragments ranged from 3.3×10^7 daltons in the case of unheated (control) spore DNA to 2.0×10^6 daltons in the case of spores heated for 30 and 60 min at 90°C. At the same time spore viability declined by some 10,000-fold, viz., from 1.6×10^8 spores/ml at the start to 4.2×10^4 spores/ml after 60 min at 90°C. On heating for 10 min essentially all DNA was found within main peak B (Fig. 1) which suffered only 0.57 SSB, and correspondingly most spores (94%) were able to recover from this injury (Table 1). However, on heating for 30 and 60 min, a precipitous increase in DNA breaks (15 SSB per DNA fragment) yielded increasing amounts of small DNA fragments in peaks C' and D' which were accompanied by a corresponding increase in the numbers of inactivated spores, i.e., spore survival after 30 and 60 min was only 1.8% and 0.026%, respectively.

This is the first report to our knowledge identifying specifically single strand breakage in spore DNA as the result of lethal heat injury. Occurence of DNA breakage in heated spores has been independently confirmed by other workers in our laboratory (Amin, Wild, and Hartman - work in progress). This evidence does not conflict with other postulated mechanisms of heat injury such as membrane damage, protein denaturation, etc. (1-5). It is clear, however, that DNA breakage must be considered as an important - if not the major - mechanism explaining the action of lethal heat on bacterial spores at the molecular level.

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