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Sporulation Mutations Induced by Heat in Bacillus subtilis

Abstract. *When spores of* Bacillus subtilis *strain Marburg are heated (90° to 100°C) in a vacuum for 9 to 12 hours and then plated, numerous mutants are obtained, and very few spores are killed. Disproportionately large numbers of these mutants exhibit abnormal sporulation.*

When spores of *Bacillus subtilis* strain Marburg were heated in a vacuum, large numbers and wide varieties of mutants were obtained. In contrast to most phenomena of mutagenesis, where mutation is often accompanied by extensive killing of cells or spores, we observed little or no killing under conditions which yielded significant numbers of mutants. At 100°C for 9 hours, more than 1 percent of the original spores were consistently recovered as viable mutants; in some cases as many as 5 percent were obtained. Although there was a variety of mutants, almost all showed some type of abnormal sporulation. There was no apparent correlation between sporulation and auxotrophic mutation.

Table 1. Viable and mutant counts of spore pellets heated at 100°C (series 1) and at 90°C (series 2) for 9 to 12 hours.

Average count per pellet ($\times 10^6$)			Total mutants (No.)	Population recovered as mutants (% of control)
Not heated viable	Heated			
	Viable	Mutant		
		Series 1		
163	34.3	4.6	55	2.82
237	92.7	12.0	72	5.08
136	46.8	3.3	13	2.43
239	37.0	8.3	50	3.47
114	43.1	4.7	57	4.12
207	53.3	11.5	69	5.55
		Series 2		
180	86	3.17	19	1.76
151	105	1.63	13	1.08
137	128	1.33	16	0.97
156	102	0.80	24	0.51

Spores were prepared from cells cultured at 30°C on modified SMM (*1*) containing 0.1 percent glucose and 0.1 percent glutamate. The cultures were shaken during the growth period. After 4 days, samples (1 ml) were collected in conical, glass centrifuge tubes, and the spores were centrifuged at 2600*g* for 15 minutes. The supernatant was decanted, and the pellets were dried for 2 hours at room temperature. Pellets were heated in a vacuum for specified temperatures and times, usually 90° to 100°C for 9 to 12 hours. As a control, some pellets were left at room temperature for the same length of time. The pellets were then cooled, suspended, diluted in the growth medium (omitting all carbon sources), and plated on potato dextrose agar (Difco) fortified with $MnSO_4$ (to 0.01 percent) and adjusted to *p*H 7. After 4 days at 37°C, wild-type colonies displayed a characteristic morphology and brown pigmentation on this agar, whereas mutants defective in sporulation showed morphological differences and variation or lack of pigmentation. All colonies were examined for auxotrophy by plating on glucose-salts agar, and suspected colonies were further checked individually. Absence of growth after a 2-day incubation period was the criterion for auxotrophy.

In Fig. 1, the logarithms of the numbers of survivors and mutants are plotted as functions of heating temperatures for 9 hours. In Fig. 2, the logarithms of the numbers of survivors and mutants are graphed as functions of heating time at 100°C. Colony formation is exponentially inactivated. The sharp increase in the number of mutants between 4 and 6 hours was unexpected because of the constant rate of killing observed.

When pellets were heated to 100°C or less for 9 hours, there were large numbers of mutants with relatively little killing. In some experiments, 1 to 5 percent of the original spores were recovered as viable mutants (Table 1); this was the maximum survival and mutation observed. Variations in the age of the spores or their growth temperature can yield less survival and mutation. Several of the experiments at 90° and 100°C were analyzed further. The mutants were originally identified by auxotrophy or differences in morphology or pigmentation, but after extensive microscopic examination almost all exhibited abnormal sporulation. In the experiments at both 90° and 100°C, 23 to 30 percent of all mutants had less than 1 percent wild-type sporulation, of which 78 to 80 percent (or 18 to 23 percent of total mutants) were asporogenic by examination under phase

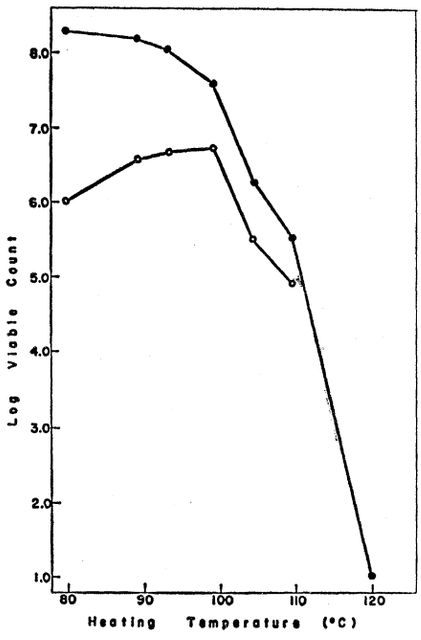

Fig. 1. Logarithm of the viable count of survivors (●———●) and mutants (○———○) as heating temperature increases. All heating periods are 9 hours.

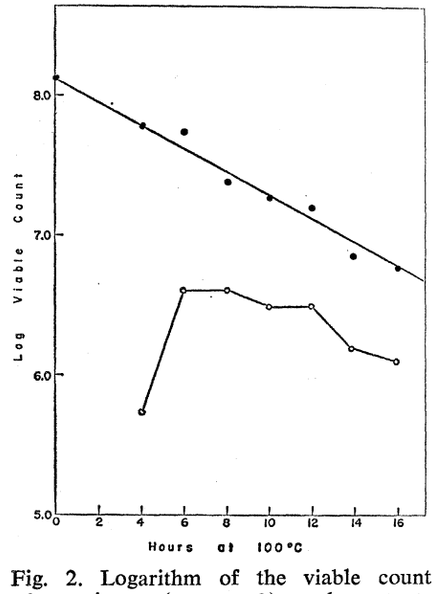

Fig. 2. Logarithm of the viable count of survivors (●———●) and mutants (○———○) as heating period increases. All heating temperatures are 100°C.

contrast. Only 4 percent of the total mutants were asporogenic after heating at 85°C for 15 minutes (no spores per 10^9 cells). In the experiments at 90°C, only 1.7 percent of all mutants were auxotrophic, whereas 12 percent of all mutants were auxotrophic in the 100°C experiments. This difference is in contrast to the similarity of sporulation mutant types at the two temperatures.

Many sporulation mutants have been obtained by this heating procedure. Almost all mutants can form a complete spore, but the sporulation frequency in most is lower than in the wild type. These mutants are oligosporogenic (OSP) (*2*), and their sporulation frequencies vary from ½ to 10^{-9}. The OSP mutants also have a range of cellular and colonial morphologies, as well as differences in pigmentation, at virtually every degree of sporulation observed. Another characteristic of the OSP mutants is that either spores or vegetative cells of any one mutant form cultures sporulating at identical frequencies. Thus a mutant which forms one heat-resistant spore per 10^7 vegetative cells produces spores which germinate and yield cultures with only one heat-resistant spore per 10^7 vegetative cells. Similar OSP mutants have been obtained when vegetative cells of *Bacillus subtilis* were treated with sublethal concentrations of acridine orange (*3*).

Zamenhof and co-workers, using a different heating procedure (shorter times and higher temperatures), have shown that temperatures above 100°C can produce large numbers of auxotrophic mutants and considerable killing of cells and spores (*4*). They have proposed that these mutations are due to depurination. Our results at temperatures greater than 100°C are consistent with their hypothesis, since we observed that spores were killed at a logarithmic rate and that many auxotrophic and sporulation mutants were among the survivors. At lower temperatures we observed virtually no killing and very few auxotrophs, whereas sporulation mutants were still present in significantly high numbers. This result suggests that there may be two mechanisms by which heat causes mutation. Depurination, resulting in random chromosomal mutation, could account for the data obtained at temperatures greater than 100°C, but probably does not explain the low killing and the predominance of sporulation mutants at lower temperatures. This phenomenon may be due to the elimination of (or damage to) a number of cytoplasmic factors (*5*). Possibly our data are relevant to the finding that spore DNA differs from vegetative DNA in some physicochemical properties (*6*).

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Human Synovial Fluid: Detection of a New Component

Abstract. *A new component has been detected in synovial fluid by the agar double-diffusion technique. This component is closely related immunologically to the proteinpolysaccharides of cartilage, and is the first probable degradation product of the cartilage matrix to be consistently identified in human synovial fluid.*

Synovial fluid is the viscous fluid which bathes the adjacent articular cartilage. The role of synovial fluid in the metabolism of articular cartilage is not well understood. Articular cartilage is relatively avascular, and nutrients may gain entrance to cartilage from the synovial fluid (*1*); also metabolic products might be removed from cartilage by way of the synovial fluid. It is possible to produce precipitating antibodies to the proteinpolysaccharides of cartilage. In my study synovial fluids were examined by immunological methods to determine whether degradation products of the proteinpolysaccharides of cartilage were released into synovial fluid.

Synovial fluids were obtained from diseased knee joints of five patients with gout, 18 with classical rheumatoid arthritis (*2*), three with septic arthritis, two with psoriatic arthritis, 15 with acute rheumatic fever, two with lupus, and one with pigmented villous synovitis. Synovial fluids were also obtained immediately after death from five young subjects with apparently normal knees. Fluids were stored at 4°C.

Proteinpolysaccharides were isolated from human articular cartilage by rapid homogenization in water (*3*). Rabbits were immunized with either the light (PP-L) or heavy (PP-H) proteinpolysaccharide of cartilage as described (*4*). The antiserums were absorbed with human serums (*4*) or plasmas. The antiserums were absorbed to remove antibodies produced to trace amounts of serum proteins which occasionally contaminate PP-L and PP-H (*4*). Agar double diffusion was performed with 1 percent agar (*5*) in barbital buffer (0.075*M*, *p*H 8.6). The PP-L and PP-H were digested with testicular hyaluronidase (*4*) before the above studies were made. Zone electrophoresis of several inflammatory synovial fluids were performed at 4°C on blocks (10 by 30 cm) of polyvinyl chloride in phosphate buffer (ionic strength 0.075, *p*H 7.4) at a voltage gradient of 15 volt/cm for 16 hours. After electrophoresis, the content of each segment (1 cm) of the block was eluted, analyzed for hexuronic acid, and after concentration (ten times) tested by agar double diffusion with the above antiserums. Synovial fluid from several patients was filtered through a Millipore cellulose filter (0.1 μ) at 4°C without stirring (*6*). Most of the hyaluronate was retained on the filter. The filtrate was placed on a column (50 by 4 cm, void volume 180 ml) of Sephadex G-200 (*7*) and eluted with a phosphate buffer (0.06*M* phosphate, 0.34*M* NaCl, *p*H 6.4) with an upward flow (20 ml/hour) provided by a peristaltic pump (*8*). The eluate from the column was monitored by continuously recording optical density at 254 mμ. Each fraction (10 ml) from column was concentrated (ten times) and was analyzed by agar double diffusion with antiserum to PP-L or PP-H.

After absorption with human serums (or plasmas) the antiserums to PP-L or PP-H produced one precipitin line with all 51 human synovial fluids (Fig. 1). In general, the antiserum to PP-H produced more intense precipitin lines with synovial fluid than the antiserum to PP-L did. This reaction with synovial fluid took place without prior digestion of the synovial fluid with testicular hyaluronidase. The precipitin line formed by this component of synovial fluid fused completely (Fig. 1) with