The Bactericidal Action of Subtilin on *Bacillus stearothermophilus*

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During an investigation of the effect of subtilin on spores of *Bacillus stearothermophilus* (a thermophile that causes flat sour spoilage of canned vegetables), it was found that two stocks of the same strain responded differently to subtilin. One behaved as though subtilin were sporicidal. The behavior of the other gave rise to the suggestion that subtilin is sporistatic, and that heat revives the subtilin-inactivated spores (Cameron and Bohrer, 1951). This paper considers this difference in behavior and shows that subtilin inactivates (probably kills) germinating spores, but that many of the spores of one stock remain dormant under the experimental conditions and, as a result, remain insensitive to subtilin.

Methods

One stock strain of *B. stearothermophilus* FS 1518 was obtained originally from C. T. Townsend of the National Canners Association Laboratory, Berkeley, California. It had been kept at this laboratory for about two years, and for convenience is here designated the WRRL stock. The other (used by Cameron and Bohrer, 1951) was sent to us more recently by the National Canners Association Laboratory in Washington, D. C., and is designated here as the NCA stock.

Spores were grown on a thin layer of nutrient agar at 52 C as suggested by C. T. Townsend. After two days' incubation the growth from 50 cm^2 was suspended in about 5 ml of water to give approximately 10^7 spores/ml and stored in the refrigerator. These stock spore suspensions were not heated prior to use since heat activation may have long-lasting effects on spores (Curran and Evans, 1945; Powell, 1951). The initial count in each experiment described below thus includes a small proportion of vegetative cells. The spores were suspended in 8 ml of tryptone broth (in 16 mm culture tubes) and heated for 5 min in boiling water. The tryptone broth, as used by Denny *et al.* (1951) consisted of tryptone, 10 g/l; dextrose, 5 g/l; K_2HPO_4, 1.25 g/l; yeast extract, 1 g/l; and brom cresol purple, 0.04 g/l. Aliquots were removed for counts at intervals during the incubation (52 C) following the heat treatment. Those aliquots which were heated again were first diluted in fresh tryptone broth.

Counts were made on Difco Dextrose Tryptone Agar (48 hr at 52 C). Valid counts were not obtained at dilutions below 1 to 100 because of interference by subtilin (see table 2). Counts of 0 are therefore reported as <100.

Results and Discussion

The effect of incubation in the presence of subtilin in tryptone both on plate counts of spores of the NCA and WRRL stocks of *B. stearothermophilus* is shown in table 1. Similar behavior was observed in pea broth made anaerobic with 0.1 per cent sodium thioglycolate. The results with the NCA stock, in which many spores survived heating in the presence of subtilin, are similar to those of Cameron and Bohrer (1951) and Denny *et al.* (1951), who reheated their entire spore suspension after each period of incubation. A parallel experiment, duplicating their procedure exactly, gave essentially the same result.

Although they were resistant to subtilin during incubation in broth, spores of the NCA stock were unable to form colonies in dextrose tryptone agar at higher concentrations of subtilin than were spores of the WRRL stock (table 2).

Both smooth and rough colonies were found on plates of the NCA stock, and these retained their identity on subculturing. The rough colonies resembled those of the WRRL stock. The effect of incubation in the presence and absence of subtilin is shown for the smooth and rough components in table 3.

A high proportion of the spores of the smooth component, although initially able to germinate and produce colonies in dextrose tryptone agar, quickly became dormant on incubation in broth and could then produce colonies in agar only if they were plated immediately following a heat treatment. The necessity of heat activation for germination of spores is a common phenomenon (Powell, 1951; Curran and Evans, 1945). The phenomenon was observed regardless of the presence or absence of subtilin. The failure of the spores to germinate accounts for the relatively small effect of subtilin on the NCA strain shown in table 1 and reported by Cameron and Bohrer (1951). That most of the dormant spores were not inactivated by subtilin is shown by the 48 hr- and 6-day counts of table 3. In another experiment 5 per cent of the original number of spores survived 44 days of incubation in the presence of subtilin.

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Attempts to elucidate the effect of the broth in the induction of dormancy were not successful. The behavior was the same if yeast extract and phosphate (which were not present in the agar medium) were left out of the broth, or if the broth was aerated or spread in a shallow layer during incubation. Inclusion of agar in the broth (which was possible at the incubation temperature of 52 C) permitted twice as many spores to germinate without heating.

TABLE 1. *Effect of incubation with subtilin on plate counts of* Bacillus stearothermophilus *spores*

INCUBATION TIME	HEAT TREATMENT*	WRRL STOCK Subtilin (ppm): 50	WRRL STOCK Subtilin (ppm): 5	NCA STOCK Subtilin (ppm): 50	NCA STOCK Subtilin (ppm): 5
0 (initial count)†		770,000	770,000	1,050,000	1,050,000
0	5-min boiling	220,000	560,000	220,000	500,000
3 hr	none	~100	6,200	19,000	43,000
3 hr	5-min boiling	<100	1,700	150,000	180,000
9 hr	none	~100	1,500	12,000	37,000
9 hr	5-min boiling	<100	2,000	130,000	200,000
48 hr	none	~400	~100	64,000	68,000
48 hr	5-min boiling	~300	~400	120,000	170,000
8 days	none	<100	<100	120,000	83,000
8 days	5-min boiling	<100	<100	190,000	120,000

* All spores received the first heat treatment. The other heat treatments were given only to the aliquot being counted.
† Before addition of subtilin.

TABLE 2. *Effect of subtilin concentration on development of colonies from* Bacillus stearothermophilus *spores*

SUBTILIN CONCENTRATION (ppm)	COLONIES IN EACH PLATE, EXPRESSED AS PERCENTAGE OF SUBTILIN-FREE CONTROL*: WRRL Stock	NCA Stock
0.12	0	0
0.06	1	0
0.03	8	14
0.015	34	39
0.008	82	55
0.004	110	85
0	(100)	(100)

* Count after 2 days' incubation. Approximately 300 spores were seeded per plate. In plates with 0.1 per cent sodium thioglycolate, colony formation was prevented by only 0.015 ppm subtilin.

In the case of the rough component, only a small proportion of the original number of spores and vegetative cells were detected by plating after incubation for 1.5 hr in broth (table 3). Most of the survivors were heat sensitive and therefore were assumed to have germinated. Microscopic observations in other experiments showed that most of the spores germinated promptly. The broth appeared to exert an unexplained bactericidal action regardless of the presence or absence of subtilin. The bactericidal effect of subtilin (Sacks, 1952) was noted within a short time at 50 ppm (table 1, WRRL strain) and at 48 hr at 5 ppm (table 1, WRRL strain, and table 3, rough component).

The augmenting effect of subtilin on thermal death of spores observed for many species by LeBlanc *et al.* (1953) may be noted with FS 1518 (zero time, tables 1 and 3).

The presence of dormant spores can serve to maintain the viability of a culture in the presence of subtilin long enough for effective elimination of the antibiotic through its relatively poor stability in vegetable media at 52 C (Denny *et al.*, 1951). Spoilage of vegetables inoculated with the NCA strain of *B. stearothermophilus* FS 1518 and canned with subtilin (Denny *et al.*, 1951) probably is explained in this way. Other thermophilic flat sour bacteria have been reported by Williams and Campbell (1951) to be less inhibited by subtilin than FS 1518.

TABLE 3. *Effect of incubation with subtilin on plate counts of the smooth and rough components of* Bacillus stearothermophilus

TIME	HEAT TREATMENT*	SMOOTH COMPONENT SUBTILIN (ppm): 5	SMOOTH COMPONENT SUBTILIN (ppm): 0	ROUGH COMPONENT SUBTILIN (ppm): 5	ROUGH COMPONENT SUBTILIN (ppm): 0
0 (initial count)†		129,000	129,000	96,000	96,000
0	5-min boiling	80,000	121,000	57,000	123,000
1.5 hr	none	1,900	3,500	9,500	19,700
1.5 hr	5-min boiling	58,000	66,000	~700	~700
3 hr	none	2,200	1,900	8,600	8,700
3 hr	5-min boiling	50,000	49,000	~500	~800
7 hr	none	1,000	4,000	2,300	4,000
7 hr	5-min boiling	45,000	49,000	~700	~200
48 hr	none	1,900	millions	<100	millions
48 hr	5-min boiling	45,000		<100	
6 days	none	4,800		2,100	
6 days	5-min boiling	40,000		<100	

* All spores received first heat treatment. Other heat treatments given only to aliquot being counted.
† Before addition of subtilin.

SUMMARY

A stock of *Bacillus stearothermophilus* FS 1518 was found to contain a smooth and a rough component. A high proportion of the spores of the smooth component became dormant quickly in tryptone broth and could be detected only if they were plated immediately after

their dormancy had been interrupted by heating. Subtilin had no effect on these dormant spores. The spores of the rough component germinated quickly and the resulting vegetative cells were sensitive to subtilin. Evidence was not found for a sporistatic action of subtilin with either the smooth or the rough component.

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The Influence of Environmental Factors on the Molecular Size of Dextran

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Of the many types of polysaccharides produced by bacteria, dextran, which is formed from sucrose by *Leuconostoc mesenteroides*, is one of the best known. It is a polymer of D-glucose in which the glucose units are joined chiefly through the 1,6-glucosidic linkages with side chains joined through the 1,4-glucosidic linkages (Fowler *et al.*, 1937). The organism is generally found in fermenting vegetable material and sugar solutions (Hucker and Pederson, 1930). Tarr and Hibbert (1931) found that sucrose was the only suitable carbohydrate substrate for the formation of dextran. Hehre (1941) and Stacey (1942) were able to produce dextran by a cell-free enzyme system. The purified preparations of dextran derived by enzymatic synthesis proved to be identical both in chemical and serological properties to the dextran derived from sucrose broth cultures of living bacteria (Hehre and Sugg, 1942).

Since it was realized during World War II that it was almost impossible to supply the great demand for blood plasma needed for the treatment of shock resulting from serious loss of blood, dextran as a substitute for plasma was suggested by Gronwall and Ingelman in 1944.

Dextran obtained from the *Leuconostoc mesenteroides* fermentation consists of polymers ranging from several thousand to many millions and as such cannot be used for infusions because of adverse reactions in the body. Dextran having a molecular weight of 75,000 $\pm$ 25,000 with an intrinsic viscosity of 0.25 $\pm$ 0.05 g 0.05 g per 100 ml is the desired clinical material (U.S. Army, 1951). To obtain such a dextran, the "crude" material is first hydrolyzed to the desired size by means of acid (Renfrew and Cretcher, 1949) and then fractionally precipitated to yield the necessary homogeneous dextran preparation (Ingelman and Halling, 1950).

The chief drawback to these procedures for dextran production is the low yield. For economic as well as practical reasons it is desirable to increase the final yield to meet the needs arising from civil and military emergencies. This investigation deals with the preparation of large quantities of low molecular weight dextran by direct fermentation procedures. During the course of this research, a procedure has been devised by which it is possible to produce high yields of dextran within the clinical molecular weight range of 75,000 $\pm$ 25,000.

EXPERIMENTAL METHODS

Leuconostoc mesenteroides, strain 683 and a variant strain, 48-13 were used throughout this investigation. This variant was obtained after subjecting strain 683 to irradiation with radioactive phosphorous.

The medium used for the cultivation of *Leuconostoc*

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