THE ACTION OF SUBTILIN ON HEATED BACTERIAL SPORES

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It has been reported previously that certain bacterial spores cannot be detected by cultural methods after a sublethal heat treatment in the presence of subtilin (Andersen and Michener, 1950; Andersen, 1952; Michener, 1953; Le Blanc *et al.*, 1953; Lewis *et al.*, 1954). In this report these observations are extended to other species and strains, especially of the genus *Bacillus*. The spores of the several strains that were examined in detail remained alive after heat and subtilin treatments but apparently died when they were brought under conditions that encourage germination.

MATERIALS AND METHODS

Spores were grown on nutrient agar slants as follows: *Bacillus subtilis* strain ATCC 9524, *B. subtilis* strain NRRL B-644, *B. subtilis* strain N. R. Smith 787,¹ *B. cereus*, *B. brevis* strain ATCC 8185, *B. pumilus* strain NRRL B-542, and *B. polymyxa* NRRL B-694. *B. subtilis* ATCC 9524 was also grown on a synthetic medium (Foster and Heiligman, 1949; salts mixture of Stokes and Woodward, 1943) for production of clean spores with a minimum of debris. Spores from this medium were used where indicated below. *B. subtilis* ATCC 6633 and an unidentified bacillus, 210-1, isolated at this laboratory from spoiled, subtilin-canned corn were grown on 5 ml layers of pea broth in unshaken flasks. *B. macerans* was grown on a medium consisting of 5 per cent oatmeal and 2 per cent CaCO₃ by Dr. W. S. Hale of this laboratory. The clostridium PA 3679² was grown on a liver-peptone medium. Those listed above were grown at 30 C. *B. stearothermophilus* FS 1518,

"WRRL stock"³ and "NCA stock"³ and *B. thermoacidurans* strain 43P³ were grown on nutrient agar at 52 C (Michener, 1953).

Water suspensions of the growth from the *Bacillus* cultures, containing 10⁶ to 10⁸ spores per ml and less than 0.1 per cent vegetative cells, could be stored at 4 C for months. They were used directly as inoculum when spores were to be treated while suspended in nutrient media. Spores for use in phosphate buffer were washed twice in the same buffer and suspended in it for storage. Clostridial spores were concentrated in their culture liquor. The resulting suspension contained about 10⁷ spores per ml and many vegetative cells. Spores were not heated except as indicated because heat activation may permanently remove the natural dormancy of spores (Powell, 1951; Curran and Evans, 1945).

The following media were used for suspension of spores during the experimental treatments: Difco nutrient glucose broth, pea broth, pea-pork broth (anaerobes), tryptone broth (*B. stearothermophilus*; Denny *et al.*, 1951), and phosphate buffer. Pea broth contained 33 per cent pea infusion; Na₂HPO₄, 3.3 g/L; added glucose, 6.7 g/L; and its pH was adjusted to 6.7. The pea infusion was prepared by steaming frozen peas in an equal weight of water for 15 min. Pea-pork broth was the medium described by Andersen (1951) but with agar omitted. The buffer was 0.025 M sodium phosphate, pH 7.0.

Spores were heated in 16 mm culture tubes (8 ml spore suspension per tube) in a water bath at 85 C or 100 C. The tubes required about 2.5 min to come to temperature equilibrium with the bath. The heating time given is the total time during which the culture tube was immersed in the bath.

Plate counts on preparations containing subtilin must be made at such a dilution that subtilin from the suspending medium will not be trans-

¹ Furnished as *Bacillus vulgatus* by P. A. Ark, Division of Plant Pathology, University of California, Berkeley (see Ark and Hunt, 1941).

² Cultures supplied by C. T. Townsend, National Canners Association, Berkeley, California. Putrefactive anaerobe 3679 is an unnamed strain which resembles *Clostridium botulinum*.

³ Cultures supplied by C. W. Bohrer, National Canners Association, Washington, D. C.

ferred to the plate in inhibitory concentrations. Under the conditions used here, subtilin has little or no effect on the counts at dilutions of 1:1,000 or more for *B. stearothermophilus* or 1:100 or more for the other organisms. Lower dilutions were not used in plating except when spores had been washed and resuspended in subtilin-free buffer. Plating was in Difco nutrient glucose agar except for the anaerobes (plated in pea-pork agar, Andersen, 1951) and *B. stearothermophilus* (plated in Difco glucose-tryptone agar).

The subtilin was produced (Garibaldi and Feeney, 1949) and purified (Fevold *et al.*, 1948) in this laboratory and was approximately 70 per cent as active as the most potent preparation (prepared by countercurrent distribution). Concentrations are stated in terms of the impure material.

For inactivation of subtilin (Dimick *et al.*, 1947; Sacks, 1954b), Bacto-trypsin B153 was diluted with sterile water as directed on the

container. The resulting solution was used in pH 7.0 phosphate buffer at the dilutions indicated below.

RESULTS

Subtilin appears to increase the lethality of heat. If spores are heated at 85 or 100 C in the presence of subtilin, the number which can subsequently be detected by plating is often lower than if no subtilin is present during the heat treatment. This was shown in experiments which were carried out as follows: Culture tubes containing pea broth with or without subtilin were inoculated with a known number of spores (usually between 10^6 /ml and 10^8 /ml). They were then heated in a water bath, after which samples were taken immediately for plate counts.

In 11 of the strains shown in figure 1 the count was reduced more by subtilin and heat together than by heat alone, while three other strains were not significantly affected by the presence of subtilin during the heat treatment. This effect

Figure 1. Percentage of surviving spores after heating in broth with and without subtilin. Subtilin concentrations: *Bacillus stearothermophilus* and *B. thermoacidurans*, 5 ppm; the clostridium PA 3679, 16 ppm; *Clostridium botulinum*, 20 ppm; all others, 10 ppm. Heat treatments: *B. stearothermophilus*, *B. thermoacidurans*, and PA 3679, 5 min at 100 C; *C. botulinum*, 2 min at 100 C; all others, 10 min at 85 C. Heating media: *B. stearothermophilus*, tryptone broth; anaerobes, pea-pork broth; all others, pea broth. Plating media: *B. stearothermophilus*, glucose-tryptone agar (Difco); anaerobes, pea-pork agar; all others, nutrient glucose agar (Difco). (Data for *C. botulinum* from Andersen, 1952.)

Figure 2. Effect of various subtilin concentrations on plate counts of previously heated *Bacillus subtilis* strain 9524 spores during incubation in buffer.

of subtilin has also been described by Le Blanc *et al.* (1953), who considered it to be a reduction in thermal death time brought about by the presence of the antibiotic during the heat treatment.

B. subtilis strain 9524 was chosen for many of the following experiments because its spores are easy to grow in quantity and are highly sensitive to the combined action of subtilin and heat.

Application of subtilin and heat need not be simultaneous. Although the preceding results were obtained with a simultaneous application of subtilin and heat, the same effect is obtained by treating spores of *B. subtilis* strain 9524 with subtilin after they have been heated. Tubes containing spores suspended in subtilin-free phos-

phate buffer were heated for 20 min at 85 C and incubated at 30 C for various periods up to 22 days. Subtilin was then added to give a concentration of 20 ppm. Plate counts made at intervals remained approximately constant as long as no subtilin was added, but the addition of subtilin always caused a drop of 90 per cent or more in the plate count in less than an hour. The effect of heat on subtilin sensitivity was shown in several other experiments in which subtilin was applied both to previously heated and to unheated spores. Under various conditions, the number of survivors was 2 to 10 times greater for unheated than for heated spores (see for example Lewis *et al.*, 1954, figure 2).

The effect of various subtilin concentrations on heated spores was also investigated. Spores

Figure 3. Effect of subtilin on spores of *Bacillus subtilis* strain 6633 during incubation at 30 C and at 65 C.

of *B. subtilis* strain 9524 were heated in buffer for 20 min at 85 C; then subtilin was added to give the desired concentrations. The resulting reduction in plate count and the rate with which it decreased were both dependent on the subtilin concentration (figure 2).

Survival of spores in the presence of subtilin. A

considerable proportion of the spores usually remains viable in the presence of subtilin if environmental conditions do not encourage germination. For example, out of 800,000 spores per ml of *B. subtilis* strain 9524 in buffer containing 10 ppm of subtilin, approximately 30 per cent withstood 5 min at 85 C and 10 per cent with-

TABLE 1

Effect of heat activation on sensitivity of spores of Bacillus subtilis strain 6633 to subtilin

Heat Activation	Subtilin Conc	Viable Cells per ml after the Following Incubation Periods at 30 C with Subtilin				
		0 hr	0.25 hr	0.5 hr	1 hr	2.5 hr
3 min at 85 C*	10	279,000	1,770	980	580	68
None	10	358,000		30,500	8,700	1,780
None	0	358,000			300,000	301,000

* Heat followed by 60 min incubation at 30 C in pea broth before addition of subtilin. The other treatments were not heated but were incubated similarly before addition of subtilin.

stood an additional 4 days at 30 C. In pea broth containing 10 ppm of subtilin, about the same number of spores withstood 5 min at 85 C, but none withstood the additional 4 days at 30 C. Spores of *C. botulinum* and of the clostridium PA 3679 resisted subtilin in pea-pork broth, but only if it lacked 0.09 per cent NaHCO_3 , which greatly accelerates germination (Andersen, 1951, 1952).

This suggests that spore germination is a prerequisite to the antibiotic action of subtilin. If so, spores should be resistant to subtilin if incubated at a temperature too high for germination. When spores of *B. subtilis* strain 6633 were incubated at 65 C in pea broth, subtilin had little or no effect on them although the heat caused a gradual reduction in plate counts. If the spores were incubated at 30 C, the counts decreased rapidly if subtilin was present, while without it rapid growth soon began (figure 3).

Sublethal heating (heat activation or "heat shocking") of spores is known to accelerate germination (Evans and Curran, 1943; Curran and Evans, 1945; Powell, 1950, 1951). If subtilin is active only after the spores have germinated, then heat-activated spores should show their sensitivity to subtilin more quickly than unheated spores. Table 1 shows this to be true for *B. subtilis* strain 6633, in which spores in pea broth were first heat activated and then incubated for 60 min before the addition of subtilin.

Germination of subtilin-treated spores. If spores are not affected by subtilin until they germinate, then it should be possible to observe early

changes characteristic of germination in subtilin-treated spores. Phase microscopy was used for this purpose. In order to observe spores during germination, nutrient glucose broth was inoculated with about 10^7 spores per ml and incubated. At intervals a drop of broth was placed on a warm microscope slide and mixed with a drop of 0.5 per cent agar to prevent Brownian movement. After the cover slip was added, the slide was cooled and examined with a dark-contrast phase objective.

Ungerminated spores of many cultures appear light under these conditions, with the periphery of the spore showing as a dark ring. When placed in a nutrient medium, they soon begin to change from light to dark until finally most of the spores are dark. The appearance of dark spores is followed typically by the appearance of vegetative cells, which may be seen either partially or completely emerged from the spore wall. That the change from light to dark represents a step in the germination process has been shown by Pulvertaft and Haynes (1951), who found that a light spore always becomes dark before pro-

TABLE 2

Darkening of washed spores of Bacillus subtilis strain 9524 after heating in phosphate buffer

	Percentage Darkened	
	50 ppm subtilin	No subtilin
Experiment I		
Before heating		5
After 30 min at 85 C	5	11
Ditto, plus 45 min with nutrients*	46	72
After 30 min at 85 C and 24 hr at 25 C	2	11
Ditto, plus 45 min with nutrients*	81	94
Experiment II		
Before heating		16
After 30 min at 85 C	4	10
Ditto, plus 60 min with 5 mM L-alanine†	75	86
After 30 min at 85 C and 20 hr at 30 C		28
Ditto, plus 60 min with 5 mM L-alanine†	73	90

* Beef extract, 3 g/L; peptone, 5 g/L; glucose, 10 g/L.

† See Hills (1950). Adenosine (Hills, 1950; Pulvertaft and Haynes, 1951) was ineffective.

ducing a vegetative cell. (Photomicrographs of dark and light spores appear in their paper.) The darkening of a spore indicates a change in refractive index (Bennett *et al.*, 1951). Changes in refractive index as an early step in germination have also been detected by other methods (Powell, 1951). It is not known whether the change from light to dark coincides with the loss of dipicolinic acid and other compounds as described by Powell and Strange (1953).

Spores suspended in buffer changed from light

to dark when they were brought into contact with nutrients which encourage germination, irrespective of the presence of 50 ppm of subtilin (table 2). Spores of *B. cereus* and *B. stearothermophilus* not only germinated but formed a few vegetative cells in the presence of 20 ppm of subtilin. Other experiments showed that continued vegetative growth and colony formation were not possible for these organisms at subtilin concentrations above 0.1 to 5 ppm. Thus, although these spores begin or complete the proc-

Figure 4. Effect of trypsin on subtilin-treated spores before and after addition of L-alanine. Phosphate buffer (pH 7.0, 0.025 M) containing 10^6 spores/ml of *Bacillus subtilis* strain 9524 was heated for 16 min at 85 C. After addition of 20 ppm of subtilin, the spores were incubated at 30 C for 50 min, during which time the plate count fell to 150,000/ml. The spores were then washed twice and suspended in the original volume of buffer. The washing took 45 min. The count was then about 12,000/ml as shown above. T = addition of trypsin (to give 1:100 dilution of Bacto-trypsin); A = addition of L-alanine (5 mM).

ess of germination in the presence of a relatively high concentration of subtilin, they are incapable of continued growth under these conditions.

Adsorption of subtilin on spores. It is shown above that subtilin affects the spores at the time of germination. Nevertheless large proportions of the spores heated or incubated in buffer containing subtilin failed to form colonies when plated in agar after high dilution (figures 1 and 2). This suggests that subtilin is adsorbed on spores but does not kill them until they start to germinate (Mondolfo and Hounie, 1951). In order to demonstrate adsorption of subtilin on spores, *B. subtilis* strain 9524 spores (grown on a synthetic medium and washed 3 times in buffer) were suspended at a count of 5×10^8 spores per ml in buffer containing subtilin, incubated one hour at 30 C, and centrifuged. The supernate was then assayed for subtilin by a cup plate method with *Arthrobacter citreus* (Sacks, 1954a) as test organism. Initial subtilin concentrations varied from 1 to 80 ppm. Within this range, the spores removed about 90 per cent of the subtilin from the buffer. Heat-killed spores (heated 30 min at 100 C), mildly heated spores (10 min at 85 C), and unheated spores behaved similarly.

Inactivation of adsorbed subtilin prior to spore germination. It should be possible to prevent the action of subtilin adsorbed on spores by removing or inactivating it before the spores start to germinate. Dimick *et al.* (1947) and Sacks (1954b) have shown that trypsin greatly reduces the activity of subtilin. For making heated *B. subtilis* strain 9524 spores incapable of subsequent growth, trypsin-treated subtilin was only 10 to 20 per cent as active as untreated subtilin. The subtilin was incubated in concentrations of 40 and 200 ppm with a 1:50 dilution of Bactotrypsin in phosphate buffer for 30 min at 25 C; the spores were heated, incubated 20 min at 30 C in buffer containing the treated subtilin, and plated.

Trypsin also inactivates subtilin which has been adsorbed on spores, and thus allows the spores to germinate and form colonies, but only if it is applied to the subtilin-treated spores before they have germinated. Spores of *B. subtilis* strain 9524 were heated and incubated in buffer containing subtilin, thus reducing the plate count by about 85 per cent. They were then washed twice in buffer and resuspended in buffer to give a plate count of about 10^4 /ml, as shown

at "zero" time, figure 4. Addition of trypsin to the washed spores was followed within 40 min by 5 to 8-fold increases in plate count (curves 1 and 2, figure 4). If no trypsin was added (curve 3), the count nevertheless rose slowly, probably due to slow destruction or elution of subtilin. After 24 hr, however, the number of spores which could form colonies was still increased by addition of trypsin. (The 24 hr response to trypsin was greater in another experiment.)

L-Alanine was added 40 min after washing to cause germination of the spores (Hills, 1950). This had no effect when trypsin was added simultaneously (curve 2), but when no trypsin was added, the plate count began to drop as spores germinated and were apparently killed by subtilin (curves 4 and 5). The addition of trypsin 40 min and 24 hr after the addition of alanine still caused an increase in plate count, apparently by inactivating the adsorbed subtilin on some spores which had not yet germinated.

Spores for this experiment were grown on the synthetic medium and appeared dark when examined with the phase microscope. Three similar experiments with light spores of the same strain grown on nutrient agar gave essentially the same result.

DISCUSSION

Since subtilin-treated spores have been shown to survive until they germinate, it is a reasonable assumption that spores exposed to subtilin and heat together (figure 1) and to heat followed by subtilin (figure 2) were alive at the time they were plated. Many of them had adsorbed enough subtilin, however, to kill them when they germinated in the nutrient agar in the plates.

It is not clear why heated spores are more sensitive to subtilin than unheated ones, but this phenomenon may be related to the fact that heat activation often accelerates germination. (Heat activation can occur in buffer (Powell and Strange, 1953) as well as in a nutrient medium as previously described.) Since subtilin is adsorbed by spores, it is also possible that heated spores adsorb more subtilin than unheated ones. This effect might not have been observed in the adsorption experiments reported here since the spores were so numerous that almost all of the subtilin was adsorbed.

Since these spores are not killed until they germinate, subtilin is not primarily sporocidal

in its activity. This is also true for streptomycin, chlortetracycline, and chloramphenicol, which permit germination of spores of *B. subtilis* and *B. anthracis* even when present in sufficient concentrations to prevent formation of visible colonies on plates (Pulvertaft and Haynes, 1951). According to Mondolfo and Hounie (1951), subtilin and tyrothricin appear to act on vegetative forms resulting from the germination of spores of *B. anthracis*, but streptomycin and penicillin act also on spores.

Although Cameron and Bohrer (1951) and Adams *et al.* (1951) suggested that subtilin is sporostatic, our work clearly shows that it does not in general prevent spore germination. This was also shown previously for *B. stearothermophilus* (Michener, 1953) and is probably true for *C. botulinum* (Andersen, 1952).

These experiments were so arranged as to determine the effect of subtilin on the majority of the spores which were inactivated by the combined subtilin and heat treatments. In general, a few spores survived these treatments. Since Sacks (1954b) has shown that subtilin can have a sporostatic effect on a small proportion of the spores of *B. maceans*, it is possible that subtilin also exerted a sporostatic effect on these few surviving spores.

Some cultures (for example *B. subtilis* 9524 grown on a synthetic medium) produce spores which are dark when examined with the phase microscope. Although these thus appear to have made a step towards germination, they retain their heat resistance and respond to subtilin in the same way as light spores. The reason for this is unknown, but perhaps the change from light to dark is a step towards germination which can occur early or late in the history of the spore, without increasing its sensitivity to heat or subtilin.

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SUMMARY

During short heat treatments at 85 C or 100 C, spores of 11 out of 14 strains of bacteria tested were more sensitive to a combination of subtilin and heat than to heat alone. Heat and subtilin need not be applied simultaneously, however, for

heated spores of *Bacillus subtilis* strain 9524 retained increased subtilin sensitivity for at least 22 days. Unheated spores were less sensitive to subtilin than heated ones. The effect of subtilin increased with concentration in the range of 2 to 20 ppm.

Spores which were incubated with subtilin under conditions which are unfavorable to germination (absence of nutrients, temperature too high) frequently survived and could be detected by plating. Under conditions favorable to germination, spores of *B. subtilis* did not survive incubation with subtilin. When germination was accelerated by heat activation, development of subtilin sensitivity was also accelerated. Many spores were observed microscopically to have begun the process of germination when suitable nutrients were supplied, irrespective of the presence of subtilin in a concentration sufficient to prevent vegetative growth and colony formation.

Ungerminated spores adsorbed subtilin from buffer. After they had been treated with subtilin, only a few would grow unless they were treated with trypsin to inactivate the adsorbed subtilin before they were placed in an environment which caused them to germinate.

It is concluded that subtilin is adsorbed on ungerminated spores, but that the spores are not affected by it until they are placed in an environment which causes them to germinate.

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