EFFECT OF SUBTILIN AND NISIN ON THE SPORES OF
BACILLUS COAGULANS1, 2

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The early literature on the effect of the polypeptide antibiotics subtilin and nisin on bacterial spores has been reviewed by Campbell and O'Brien (1955). Most of the early reports have indicated that subtilin acts as a sporostatic agent. Other reports have shown, however, that the spores of several bacterial species will germinate in the presence of subtilin and are subsequently killed by the antibiotic (Andersen, 1952; Michener, 1953, 1955; Sacks, 1955). This appears to be the main mode of action of subtilin on bacterial spores, although Sacks (1955) presented convincing evidence that subtilin was sporostatic for a small fraction of the spores of Bacillus macerans. Wheaton et al. (1957) found that subtilin was effective in preventing spoilage of tomato juice by Bacillus coagulans, although from their data they could not determine whether the antibiotic was sporocidal or sporostatic. Hirsch and Grinstead (1954), working with species of the genus Clostridium, reported that nisin could act, depending upon the test organism, either sporocidally or as an inhibitor of germination.

Subtilin and nisin have also been reported to reduce the thermal resistance of spores of a number of bacteria important in the spoilage of heat processed foods (LeBlanc et al., 1953; Lewis et al., 1954; O'Brien et al., 1956) with the greatest reduction occurring with nisin and the spores of B. coagulans (O'Brien et al., 1956). This reduction in thermal resistance is probably more apparent than real, however, since O'Brien and Titus (1955), Michener (1955), and O'Brien et al. (1956) have shown that this effect may be caused by carry-over of the antibiotic into the subculture medium used for recovery of the heated spores.

In the present paper, data are presented which show that subtilin and nisin do not reduce the thermal resistance of the spores of B. coagulans, nor do they prevent spor germination. The main mode of action of these antibiotics is the inhibition of the subsequent outgrowth (Campbell, 1957) of germinated spores.

EXPERIMENTAL METHODS

Materials. B. coagulans strain 43P was used throughout the study. Stock cultures of the organism were maintained by bi-weekly transfer on nutrient agar (Difco) slants and were stored at 5°C. Subtilin of 70 per cent potency was obtained from the Western Utilization Research Laboratory through the courtesy of Dr. J. C. Lewis. Crystalline nisin (40 × 10^6 reading units per g) was generously donated by Dr. H. B. Hawley of Aplin and Barrett Ltd., England. Antibiotic concentrations are given in terms of 100 per cent potency material. Trypsin (1:250) was purchased from Difco Laboratories.

Preparation and counting of spore suspensions. Spore crops of B. coagulans were grown as surface cultures in Roux bottles on the proteose peptone acid agar of Frank (1955) supplemented with 10 μg per ml of MnSO4·H2O (Amaha et al., 1956). After incubation for 2 weeks at 55°C the spores were harvested as described by Frank (1955) and washed 5 times with sterile 0.01 M potassium phosphate buffer, pH 7.2. Stock suspensions were prepared by suspending the washed spores in sterile distilled water in screw cap bottles containing glass beads and were stored at 5°C.

Spore suspensions virtually free of vegetative cells were prepared by diluting 2 ml of the concentrated stock suspension to 25 ml with sterile...
The diluted suspension was placed in the sterile cup of a Raytheon 9 kc sonic oscillator (cooled with running ice water) and treated for 25 min. The resultant suspension was centrifuged at 3000 × G for 10 min in a Servall centrifuge at 0 C, washed two times with sterile distilled water, resuspended in sterile distilled water in screw cap bottles containing glass beads, and stored at 5 C. Dark phase contrast microscopy revealed that the suspension was composed largely of ungerminated spores with only a few scattered vegetative cells. All of the experiments reported in this paper were carried out with sonically treated spore suspensions. The spore concentration of each suspension was determined as described by Frank and Campbell (1957).

**Determination of thermal resistance.** Spores of *B. coagulans* were exposed for various time intervals at 107.2 C in a thermostresimeter (Stumbo, 1948) in the absence and presence of the antibiotics. Antibiotics were used at a level of 28 µg per cup in a total volume of 0.02 ml. The sampling, dilution, and plating techniques employed were those described by Frank and Campbell (1957). Colonies were counted after incubation for 3 days at 37 C and the number of survivors calculated for each time exposure. The data obtained from each run were then plotted to obtain survivor curves. Composite survivor curves were constructed after at least 4 replications had been made.

**Determination of effect of antibiotics on heat activated spores.** Spores of *B. coagulans* were subjected to an activation treatment of 0.14 min at 104.4 C in the thermostresimeter in the absence and presence of antibiotics (28 µg per cup). The dilution and plating procedures were the same as those described by Frank and Campbell (1957). Colony counts were made after incubation for 3 days at 37 C.

**RESULTS AND DISCUSSION**

**Effect of subtilin and nisin on thermal resistance of spores of B. coagulans.** Composite survivor curves in the absence and presence of subtilin and nisin from 4 individual replications are presented in figure 1. Each point on the curve is the mean value obtained in 4 separate determinations. It may be seen that the curves in the absence and presence of subtilin are virtually superimposable and that they are curvilinear in nature, as previously reported for this organism by Frank and Campbell (1957). The number of survivors is greatly reduced, however, in the presence of nisin.
This finding indicates that either nisin does indeed reduce the thermal resistance of \textit{B. coagulans} spores or that nisin is more strongly bound by the spores than subtilin and is not removed by the dilution procedures employed. If nisin is bound to the spores it would be carried over into the subculture medium and would be available to inhibit germination or outgrowth of the survivors. This would result in an apparent reduction in the thermal resistance of the spores as noted by O'Brien et al. (1956).

Experiments were run to test this point by heating the spores in the presence and absence of nisin (28 \( \mu \text{g} \) per cup) and plating in proteose peptone acid agar at pH 8.0 containing 56 \( \mu \text{g} \) of sterile trypsin per ml. The results obtained in three separate experiments are presented as composite curves in figure 2 and show that the survivor curves in the absence and presence of nisin are superimposable when nisin is destroyed by trypsin after heating. These data clearly show that subtilin and nisin do not reduce the thermal resistance of spores of \textit{B. coagulans}. They further indicate that nisin is more firmly bound by the spores than subtilin.

\textbf{Effect of subtilin and nisin on heat activated spores of \textit{B. coagulans}.} It is evident from the

\begin{table}[h]
\centering
\caption{Effect of subtilin and nisin on heat activated spores of \textit{Bacillus coagulans}}
\begin{tabular}{l|c|c}
\hline
Condition & Expt No. & \\
\hline
No antibiotic & 11,570 & 10,800 \\
Subtilin & 11,800 & 10,460 \\
Nisin & 1,600 & 1,830 \\
No antibiotic + trypsin & 11,680 & 10,900 \\
Subtilin + trypsin & 11,300 & 10,280 \\
Nisin + trypsin & 11,480 & 9,840 \\
\hline
\end{tabular}
\end{table}

Spores were heat activated at 104 C for 0.14 min in the thermoreistometer in the absence and presence of antibiotics (28 \( \mu \text{g} \) per cup in a total volume of 0.02 ml). When trypsin was employed it was added to the proteose peptone acid agar recovery medium (adjusted to pH 8.0) at a concentration of 56 \( \mu \text{g} \) per ml. The values given for each experiment represent the mean values, obtained in 3 separate replications per experiment.

\begin{table}[h]
\centering
\caption{Effect of preincubation with subtilin and nisin on spores of \textit{Bacillus coagulans} prior to heat activation}
\begin{tabular}{l|c|c}
\hline
Condition & Expt No. & \\
\hline
No antibiotic, water washed & 10,450 & 9,950 \\
Subtilin, water washed & 10,780 & 10,200 \\
Subtilin, trypsin washed & 10,500 & 10,000 \\
Nisin, water washed & 3,250 & 2,920 \\
Nisin, trypsin washed & 10,000 & 9,400 \\
\hline
\end{tabular}
\end{table}

Spores were incubated in the presence and absence of the antibiotics (14 \( \mu \text{g} \) per ml) at 37 C for 1 hr at pH 5.3 in a total volume of 2.0 ml. Water washed spores were washed 7 times with 10 ml of sterile distilled water by centrifugation at 6000 X G. Trypsin washed spores were washed 3 times with 10 ml of sterile trypsin (560 \( \mu \text{g} \) per ml, adjusted to pH 8.0) followed by 4 washes with 10 ml of sterile distilled water. Aliquots (0.01 ml) were then heat activated at 104 C for 0.14 min. The dilution, plating, and incubation conditions were those described in the text. The values given represent the mean values obtained in 3 separate replications per experiment.

Data presented in table 1 that subtilin has no effect on the recovery of heat activated spores, since it is diluted out in the plating procedures employed. (If subtilin were not bound by the spores, the final concentration in the agar plates would be 0.07 \( \mu \text{g} \) per ml.) On the other hand, heat activation in the presence of nisin causes an apparent reduction in the spore count. However, when trypsin is added to the recovery medium the count is again restored to the level obtained in the absence of nisin. Similar data were obtained when spores were preincubated with subtilin and nisin prior to heat activation (table 2). These data show that subtilin and nisin are not sporocidal for this organism. It is suggested that nisin is firmly bound by the spores of this organism and perhaps inhibits spore recovery by inhibiting spore germination or outgrowth.

\textbf{Effect of subtilin and nisin on spore germination and outgrowth.} Spores of \textit{B. coagulans} were heat activated at 85 C for 5 min and inoculated into tubes of proteose peptone acid broth (pH 5.3; 5.0 ml of broth per tube) containing 0 and 500
TABLE 3

Effect of subtilin and nisin on spore germination and outgrowth

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Control</th>
<th>Nisin</th>
<th>Subtilin</th>
</tr>
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<tbody>
<tr>
<td>hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100% UG</td>
<td>100% UG</td>
<td>100% UG</td>
</tr>
<tr>
<td>4</td>
<td>35% G</td>
<td>20% G</td>
<td>30% G</td>
</tr>
<tr>
<td>8</td>
<td>80% G</td>
<td>65% G</td>
<td>75% G</td>
</tr>
<tr>
<td>24</td>
<td>V</td>
<td>90% G</td>
<td>95% G</td>
</tr>
<tr>
<td>48</td>
<td>V</td>
<td>92% G</td>
<td>95% G</td>
</tr>
<tr>
<td>→</td>
<td>V</td>
<td>V</td>
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</tr>
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UG, ungerminated refractile spores; G, germinated dark spores; V, vegetative cells; → trypsin (500 μg per tube) added, medium adjusted to pH 8.0. Spores heat activated at 85 C for 5 min. Incubation mixtures contained 4.0 ml of proteose peptone acid broth (pH 5.3), 1.0 ml of spores (3 X 10^8), 0 or 500 μg of nisin, and 100 μg of subtilin. The tubes were incubated in a 37 C water bath.

μg of nisin. The tubes were incubated in a 37 C water bath and observed for germination and outgrowth by dark phase contrast microscopy and methylene blue uptake. The results presented in table 3 show that the spores germinated in the presence of nisin. Outgrowth of the germinated spores, however, was inhibited by nisin until it was inactivated by the addition of trypsin. Similar results were obtained when spores were incubated in the presence of 100 μg of subtilin (table 3) confirming the reports of previous investigators that subtilin does not inhibit germination of bacterial spores (Andersen, 1952; Michener, 1953, 1955; Sacks, 1955).

ACKNOWLEDGMENTS

The authors wish to express their sincere thanks to Drs. J. C. Lewis and H. B. Hawley for their generosity in supplying the antibiotics used in this study.

SUMMARY

The data presented in this paper clearly show that subtilin and nisin do not reduce the thermal resistance of the spores of Bacillus coagulans. The antibiotics are not sporocidal or sporostatic but inhibit the outgrowth of germinated spores.

REFERENCES


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