Influence of pH, Temperature, Curing Agents, and Water Activity on Germination of PA 3679 Spores

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ABSTRACT

The influence of pH, temperature, water activity, and curing agents on germination of spores of Clostridium sporogenes (PA 3679) was examined. The most influential factor was pH; least germination occurred at pH 5.5, and most at pH 7.0, the highest pH tested. Germination occurred over a temperature range of 4 to 55 C, with maximal germination at 35 and 45 C. NaNO₂ was more inhibitory than NaNO₃ and NaN₃ at pH 7.0 at the levels used. At pH 5.5 and 6.0, NaN₃ stimulated germination.

Bacterial endospores are notable because of their resistance to heat and chemicals that may be used for inactivation of bacteria. Bacterial spores are usually present in foods; and, under favorable conditions, these unwanted spores can germinate and multiply to cause spoilage and, in some instances, to create a health hazard. Control of germination and development of growth from bacterial spores in foods have been approached from two viewpoints. One is to induce germination that produces a spore or cell of increased susceptibility to adverse environments. Second is the concept of preventing or inhibiting germination, which eliminates the possibility of growth of the organism.

The purpose of this work was to determine the influence of environmental conditions including pH, temperature, water activity, and curing agents used for meat products on germination of a spore-forming putrefactive anaerobe. Knowledge of the influence of these factors on germination of sporeforming bacteria may yield insights into the control of spores in food, particularly in those foods depending on nitrates and nitrites for preservation.

MATERIALS AND METHODS

The organism used in this work was Clostridium sporogenes ATCC 7955; it is also referred to as Putrefactive Anaerobe 3679 NCA strain or PA 3679. Stock cultures were maintained in Cooked Meat medium (Difco) stored at 5 C. Spores were produced by using the technique of Uehara et al. (15) except that the medium used for sporulation consisted of 6.0% trypticase (BBL) and 0.1% glucose. The pH of the medium was adjusted to 7.0. Inoculated flasks were incubated at 35 C for 55 to 60 h at which time 80 to 90% of the cells had sporulated. Cells were collected by centrifugation and washed with sterile, deionized water. After the final wash, the pellet of cells was resuspended in distilled water and stored overnight at 5 C to allow for some lysis of vegetative cells. After an additional wash, further lysis of vegetative cells was induced by treatment with lysozyme as described by Finley and Fields (5). After a final wash, the spores were suspended in sterile, deionized water at a concentration of approximately 2.0 × 10^9 spores/ml. Approximately 99% of the spores in the suspension were refractile.

Media for determining the degree of germination were prepared from Brain Heart Infusion (BHI) broth to which 0.1% sodium metabisulfite had been added; the metabisulfite inhibited outgrowth without altering germination of spores (7). Various combinations of 4.0% sodium chloride, 0.1% sodium nitrate, and 0.02% sodium nitrite in this broth were examined for influence on germination. Other interacting factors included in the study were water activity (a_w) at levels of 0.99, 0.97, and 0.95 and pH levels of 7.0, 6.5, 6.0, and 5.5. The media containing 4.0% sodium chloride and 4.0% sodium chloride plus 0.1% sodium nitrate plus 0.02% sodium nitrite had a_w levels of 0.97 and could not be included in comparisons of combinations having an a_w of 0.99.

The a_w was calculated by first determining the freezing point of the various broths by using the graphical method of Shoemaker and Garland (12). This information was then used in the equations given by Daniels et al. (1) to calculate a_w. When needed, glycerol was added to each solution to attain the desired a_w.

Five different incubation temperatures (4, 25, 35, 45, and 55 C) were evaluated for their influence on germination of spores in each of the 52 different combinations. The order in which the 260 observations (52 combinations of additives and pH times 5 temperatures) were made was randomized to eliminate possible biases due to aging effect on spores. The total time used for each replication did not exceed 10 days; therefore, spores used in each replication were not more than 10 days old. Three replications were made with different batches of spores. Each tube of medium was brought to the specified temperature before incubation.

RESULTS AND DISCUSSION

Germination of spores of C. sporogenes PA 3679 occurred in Brain Heart Infusion (BHI) broth at all pH
levels in the range of 5.5 to 7.0; the least amount of germination occurred at pH 5.5, and the greatest at pH 7.0 (Fig. 1). An optimal pH for germination was not established because pH levels above 7.0 were not used. Duncan and Foster (4) reported that pH 6.0 was optimal for rapid germination of spores of PA 3679h in a phosphate buffer containing sodium nitrite; on the other hand, an alkaline pH of 8.5 or higher was optimal in systems in which L-alanine was used to induce germination (6,13,14).

The pH of the system plays a dominant role in the control of germination of spores of PA 3679 in BHI broth. A comparison of values of the mean squares in Table 1 show that temperature of incubation, curing agents, and water activity also played statistically significant roles, but to a lesser degree than pH. Replications in which different batches of spores were used did not differ significantly from each other.

Temperature and pH interacted in such a manner that, at pH 5.5, the level of germination increased linearly as the temperature was increased from 25 C to 55 C (Fig. 2). At pH 6.0, however, little variation occurred in the amount of germination as the temperature was increased from 35 to 55 C. Germination was least at 25 C for all pH levels. At pH 6.5 and 7.0, the percentage of germinated spores was maximal when the temperature was 35 and 45 C. Germination of PA 3679 spores occurs over a wide range of temperatures, with the optimum rate of germination occurring at 35 and 45 C when the pH is favorable. These findings are similar to those of Mehl and Wynne (9) who observed germination of PA 3679 spores over a temperature range of 25 to 45 C and reported that maximal germination occurred between 40 and 45 C and that germination was slow and limited at 20 C. This relationship between pH and temperature emphasizes the need for reporting pH when stating maximal germination of spores.

Figure 1. The effect of pH on germination of PA 3679 NCA spores.

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Figure 2. The effect of pH and temperature on germination of PA 3679 NCA spores.
Inhibition of germination by curing salts can be attributed to changes in water activity and to the inherent inhibitory activity of curing salts (Fig. 3, 4). At levels of 4.0% NaCl, a\textsubscript{w} becomes a dominant factor in preventing germination. When glycerol was added to the system instead of NaCl to adjust the systems to identical a\textsubscript{w} levels, the combination of the curing salts produced a reduction in germination exceeding that of any of them alone.

Addition of 4.0% NaCl to the medium inhibited outgrowth for 24 h under all conditions tested, but inhibited germination only in a few instances in which pH and temperature played dominant roles. At 25°C, addition of 4.0% NaCl produced the most extensive inhibition of all combinations of salts. When the pH was equal to or less than 6.0, little additional inhibition on spore germination was produced by addition of NaCl or by reducing the a\textsubscript{w} of the medium. Mundt et al. (10) observed germination of 90% of the spores of C. sporogenes in the presence of 8.0% NaCl at pH levels down to 5.3 in a temperature range of 4.4 C to 35 C, but outgrowth did not occur under these conditions. Duncan and Foster (3) used a microculture technique to demonstrate that germination of spores of PA 3679h occurred in the presence of 3 to 6% NaCl; vegetative cells appeared under these circumstances, but cell division was blocked. Germination of unheated spores of such anaerobes as Clostridium roseum and Clostridium botulinum is completely inhibited in tryptone solution containing 10% NaCl (6). Relatively high concentrations of NaCl are needed to completely prevent germination of spores of putrefactive anaerobes; the actual amount of salt required depends on the medium, the strain or species, and other experimental conditions.

The pH of the medium influenced the degree to which the various curing salts affected germination (Fig 3). At pH 7.0, nitrate and nitrite suppressed germination to a similar extent, but not to the extent observed in the presence of NaCl; a\textsubscript{w} was probably an important factor in the presence of 4.0% NaCl. The combination of the three salts was most effective for inhibiting germination at pH 7.0. At pH 5.5 and 6.0, the amount of germination was similar in all combinations except for NaNO\textsubscript{2} alone, which, under these conditions, enhanced germination. Duncan and Foster (2, 3, 4) have reported stimulation of germination in the presence of 0.02% NaNO\textsubscript{2} and found that levels of NaNO\textsubscript{2} up to 2.0% were incapable of completely inhibiting germination or outgrowth of PA 3679h spores. These workers postulated that, in cured meats, NaNO\textsubscript{2} induces germination, thus making the spores susceptible to subsequent heat processing.

In the presence of 0.02% NaNO\textsubscript{2}, more extensive germination occurred at 45 and 55 C than at the lower temperatures. Duncan and Foster (4) also have stated that nitrite-induced germination is accelerated at high incubation temperatures.

Germination occurred at a\textsubscript{w} levels of 0.95 to 0.99, but the extent of germination decreased as the a\textsubscript{w} was decreased (Fig. 4). The medium containing 0.02% NaNO\textsubscript{2} and having an a\textsubscript{w} of 0.99 yielded the greatest percentage of germinated spores. Least germination occurred in the medium containing all three curing salts.
and having an a_w of 0.95. No outgrowth occurred within 24 h in media with an a_w of 0.95. In media having identical a_w values of 0.97, germination occurred to a greater extent when only glycerol was used to adjust a_w than when NaCl was used.

After 24 h of incubation, approximately 5% of the tubes (38 tubes of 780) showed outgrowth (Table 2). Of

<table>
<thead>
<tr>
<th>Medium</th>
<th>a_w</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Number of tubes showing growth</th>
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<tr>
<td>I. After 6 hours of incubation:</td>
<td></td>
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<tr>
<td>Basal</td>
<td>0.99</td>
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<tr>
<td>Basal + 0.1%NaNO_2</td>
<td>0.99</td>
<td>7.0</td>
<td>35</td>
<td>1</td>
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<tr>
<td>Basal + 0.02%NaNO_2</td>
<td>0.99</td>
<td>7.0</td>
<td>35</td>
<td>2</td>
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<tr>
<td>II. After 24 hours of incubation:</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0.99</td>
<td>7.0</td>
<td>35</td>
<td>1</td>
</tr>
<tr>
<td>Basal + 0.1%NaNO_2</td>
<td>0.97</td>
<td>7.0</td>
<td>35</td>
<td>3</td>
</tr>
<tr>
<td>Basal × 0.02%NaNO_2</td>
<td>0.99</td>
<td>7.0</td>
<td>35</td>
<td>3</td>
</tr>
<tr>
<td>Basal</td>
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<td>7.0</td>
<td>35</td>
<td>1</td>
</tr>
<tr>
<td>Basal + 4%NaCl</td>
<td>0.97</td>
<td>7.0</td>
<td>35</td>
<td>3</td>
</tr>
<tr>
<td>0.1%NaNO_3 + 0.02%NaNO_2</td>
<td>0.97</td>
<td>7.0</td>
<td>45</td>
<td>3</td>
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<tr>
<td>NaNO_2</td>
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<td>7.0</td>
<td>55</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.97</td>
<td>6.5</td>
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<tr>
<td></td>
<td>0.97</td>
<td>6.5</td>
<td>45</td>
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<td></td>
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<td>7.0</td>
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<td>0.95</td>
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<td></td>
<td>0.95</td>
<td>6.5</td>
<td>45</td>
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</table>

Number of tubes from three replications showing growth.

all treatments, growth occurred most frequently in the presence of 0.02% NaNO_2; indeed, 15 instances of growth were observed in the presence of nitrite as compared with two instances in the control group. Sodium nitrite not only stimulated germination, but also reduced the lag time for initiation of growth. Temperatures optimal for germination also favored outgrowth. A satisfactory explanation for these occurrences is not obvious, but perhaps the greater number of germinated spores in the presence of NaNO_2 increased the chance of growth occurring. Riemann (11) has emphasized that curing agents and heat are much more effective in controlling growth when the numbers of organisms are small. However, this explanation is lacking when it noted that least germination of spores occurred in the presence of NaCl plus NaNO_3 plus NaNO_2 and that 17 out of 120 of the tubes containing this combination showed outgrowth. Additional information is needed on the interactions of numbers of cells, pH, curing agents, time, and growth before a satisfactory explanation can be made.

At 4 °C, germination was quite slow or absent, depending upon the pH. After 24 h, germination occurred to a limited extent in all media with a pH of 7.0.

At pH 5.5, no or very slight germination was evident. No outgrowth was observed at 4 °C within 24 h.

In summary, of the various parameters studied, pH of the medium was the most influential on germination of spores of PA 3679, followed in order of importance by incubation temperature, water activity, and curing salts. Maximal germination was observed at pH 7.0, the highest pH used. Germination occurred over a wide temperature range of 4 to 55 °C, with 35 to 45 °C being optimal. Addition of 4.0% NaCl caused greater reduction in germination than did 0.1% NaNO_3 or 0.02% NaNO_2 at pH 7.0; addition of 0.02% NaNO_2 stimulated germination at pH 5.5 and 6.0. The combination of the three salts produced the greatest inhibitory effect on germination at pH 6.5 and 7.0. No outgrowth occurred within 24 h in media containing 4.0% NaCl. Outgrowth occurred most frequently at temperatures of 35 °C and 45 °C when the pH was 6.5 and 7.0.

REFERENCES