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Effect of Sodium Nitrite, Sodium Chloride, and Sodium Nitrate on Germination and Outgrowth of Anaerobic Spores[1]

CHARLES L. DUNCAN[2] AND E. M. FOSTER
Department of Bacteriology and Food Research Institute, University of Wisconsin, Madison, Wisconsin 53706

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The effects of meat-curing agents on germination and outgrowth of putrefactive anaerobe 3679h (PA 3679h) spores were studied in microcultures. Nitrite concentrations up to 0.06% at *p*H 6.0 or between 0.8 and 1% at *p*H 7.0 allowed emergence and elongation of vegetative cells but blocked cell division. The newly emerged cells then lysed. With more than 0.06% nitrite at *p*H 6.0 or more than 0.8 to 1% at *p*H 7.0, the spores lost refractility and swelled, but vegetative cells did not emerge. Even as much as 4% nitrite failed to prevent germination (complete loss of refractility) and swelling of the spores. Sodium chloride concentrations above 6% prevented complete germination (i.e., the spores retained a refractile core). In the presence of 3 to 6% sodium chloride, most of the spores germinated and produced vegetative cells, but cell division was often blocked. Sodium nitrate had no apparent effect on germination and outgrowth at concentrations up to 2%.

Germination and outgrowth of bacterial spores include five sequential steps: (i) germination (becoming nonrefractile, stainable, and heat-sensitive); (ii) swelling of the germinated spore; (iii) emergence of the new vegetative cell; (iv) elongation; and (v) cell division.

Although many substances are known to block the development of spores into actively multiplying vegetative cells, the stage of germination or outgrowth at which a particular substance is effective is known only in a few cases. Gould (5) determined the effects of a number of common food preservatives on growth from spores of six *Bacillus* species, but he failed to include any of the clostridia. Most previous work (2, 6, 7) has dealt with growth of vegetative cells, and Gould's study was the first attempt to define the effects of chemical preservatives on spore germination and outgrowth.

In an earlier report (3), we described the end result, as shown by colony formation, when sodium chloride, sodium nitrate, and sodium nitrite were allowed to act on an anaerobic spore-former during and after heating The purpose of the work reported here was to define the point in the developmental process at which the curing salts act to prevent outgrowth.

MATERIALS AND METHODS

Spores of putrefactive anaerobe 3679 strain h (PA 3679h) were prepared as previously described (3). Liver Veal Agar (Difco) containing various concentrations of sodium chloride, sodium nitrate, or sodium nitrite was used as the growth medium at *p*H 6.0 or 7.0. Sodium nitrite was added to the base medium from a freshly prepared, filter-sterilized 10% solution immediately before use. The other salts were added before sterilization.

Microcultures were prepared by layering approximately 1 ml of the appropriate agar medium on the surface of a sterile glass slide (25 × 75 mm). The prepared slides were stored in petri dishes to maintain sterility. A loopful of spore suspension was smeared onto the surface of a sterile glass cover slip (24 × 50 mm) and allowed to dry in a petri dish. Then, the cover slip was inverted and carefully pressed onto the hardened agar surface. Air spaces along the sides of the cover slip were filled with additional medium, and the edges of the cover slip were sealed with Vaspar. The slide cultures were incubated at 30 C and examined at intervals up to 6 days with a Zeiss phase-contrast photomicroscope.

RESULTS

The normal development of PA 3679h spores from dormancy to active multiplication is illus-

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[2] Present address: Department of Foods and Nutrition, University of Wisconsin, Madison, Wis. 53706.

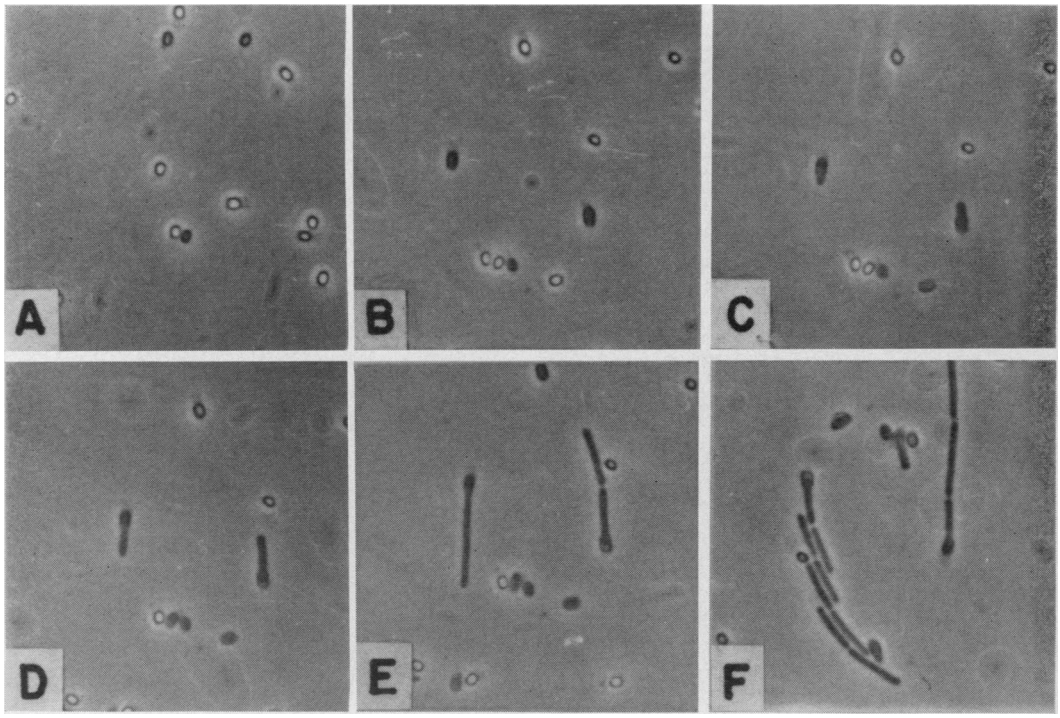

FIG. 1. *Stages in germination and outgrowth of PA 3679h spores in Liver Veal Agar microculture (pH 7.0) at room temperature. (A) At 5 hr, several spores have lost refractility; (B) 7 hr, swelling of spores; (C) 10 hr, emergence of vegetative cell from end of spore; (D) 13 hr, elongation of vegetative cell; (E) 15 hr, cell division; (F) 18 hr, continuing growth. (B) through (E) show the same microscopic field. Original magnification, × 1,070.*

trated in Fig 1. With this organism, emergence follows lysis of one end of the swollen germinated spore. This contrasts with some of the *Bacillus* species, whose spores undergo lysis or rupture of one side of the spore case.

The time intervals shown in Fig. 1 obviously are not representative of every spore observed, because outgrowth was not a synchronous process. The variability in response of individual spores is readily seen.

Effect of sodium nitrite. Preliminary experiments showed that 0.01 and 0.09% sodium nitrite completely inhibited colony formation at *p*H 6.0 and 7.0, respectively. The photographs in Fig. 2 reveal two points of inhibition in the outgrowth process. Even as much as 4% nitrite did not interfere with loss of refractility (Fig. 2A) and swelling (Fig. 2B). However, the process stopped there if the nitrite concentration was as much as 0.06% at *p*H 6.0 or 0.8 to 1% at *p*H 7.0. At lower nitrite levels, the vegetative cells emerged (Fig. 2C) and elongated (Fig. 2D), but cell division did not take place even at levels of 0.01% (*p*H 6.0) or 0.09% (*p*H 7.0). Elongated cells that did not multiply eventually lysed, leaving the empty spore coats (Fig. 2E).

Effect of sodium chloride. With more than 6% sodium chloride, most of the spores underwent only partial germination; that is, they lost part of their refractility but were not completely phase-dark (Fig. 3A and B). This condition persisted for at least 10 days.

At salt concentrations of 1 to 3%, overgrowth of the slide cultures made it impossible to observe inhibition of individual spores. However, concentrations between 3 and 6% were sufficiently inhibitory to prevent complete overgrowth, thereby allowing observation of the effects of salt. The variability in response of individual spores is evident, since no single concentration of salt completely blocked all of the spores at a particular developmental stage. Instead, cells which were inhibited at each of the transitional stages—emergence (Fig. 3C), elongation (Fig. 3D), or cell division (Fig. 3E)—could be found in the same slide culture at salt concentrations between 3 and 6%. Cells that did not develop

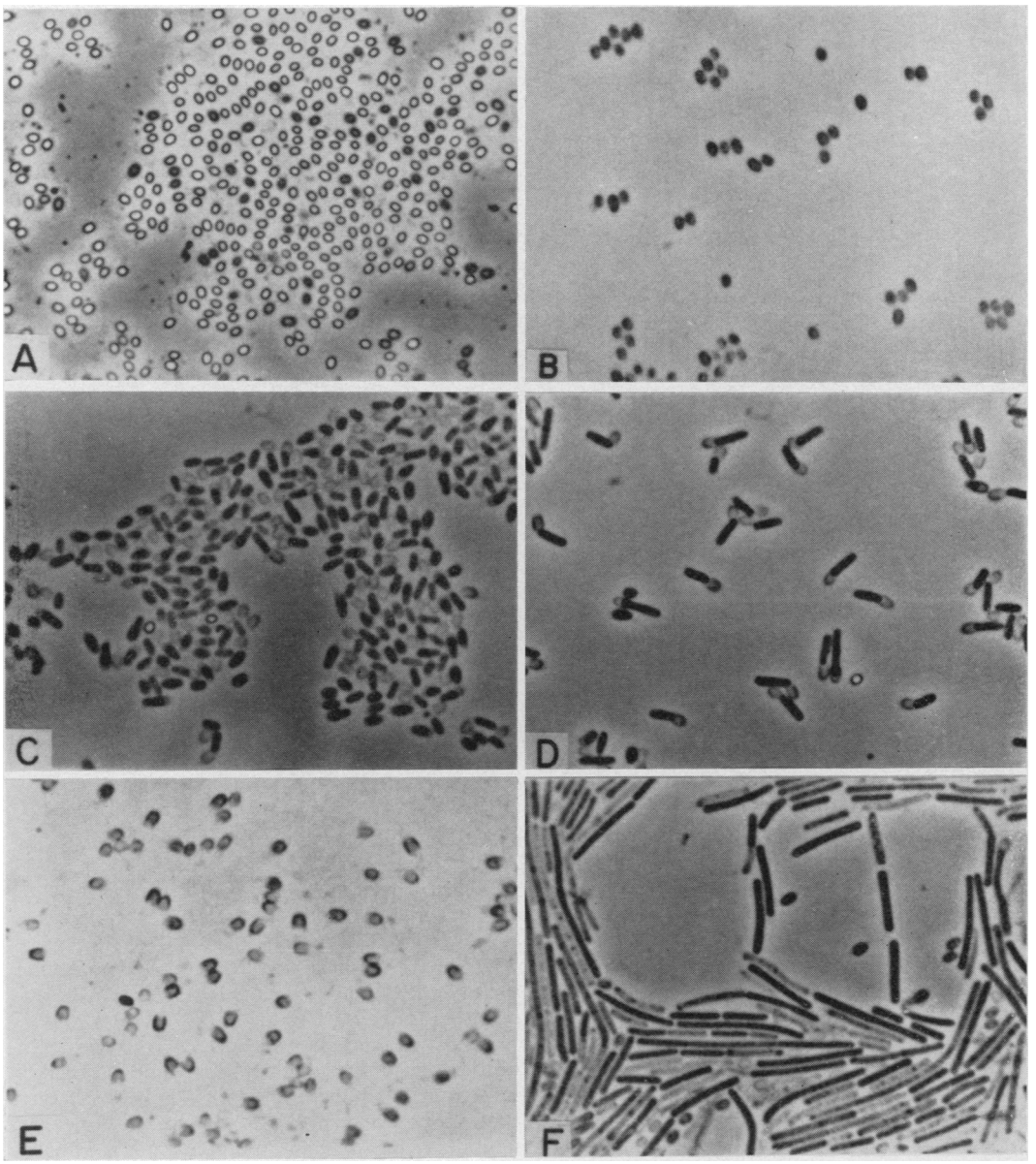

FIG. 2. *Effect of sodium nitrite on germination and outgrowth of PA 3679h spores in microculture at 30 C. Loss of refractility (A) and swelling of the spores (B) took place at all concentrations of nitrite up to 4%. Further development depended on nitrite level and pH. Emergence (C) and elongation (D) occurred at concentrations up to 0.06% at pH 6.0 and 0.8 to 1.0% at pH 7.0, but the process did not go further even with nitrite levels as low as 0.01% (pH 6.0) or 0.09% (pH 7.0). Under these conditions, the newly emerged cells lysed, leaving the spore coats behind (E). The normal pattern of growth without nitrite is shown in (F). Original magnification, × 1,070.*

beyond elongation eventually lysed, leaving the empty spore coats (Fig. 2E).

Effect of sodium nitrate. Overgrowth by vegetative cells at all concentrations of nitrate up to 2% masked any visible effects on individual spores. Since this concentration is at least 20 times that normally employed in commercial practice, no further tests were conducted with sodium nitrate.

A summary of the stages which, in the transformation of a spore into a vegetative cell, are inhibited by various concentrations of sodium

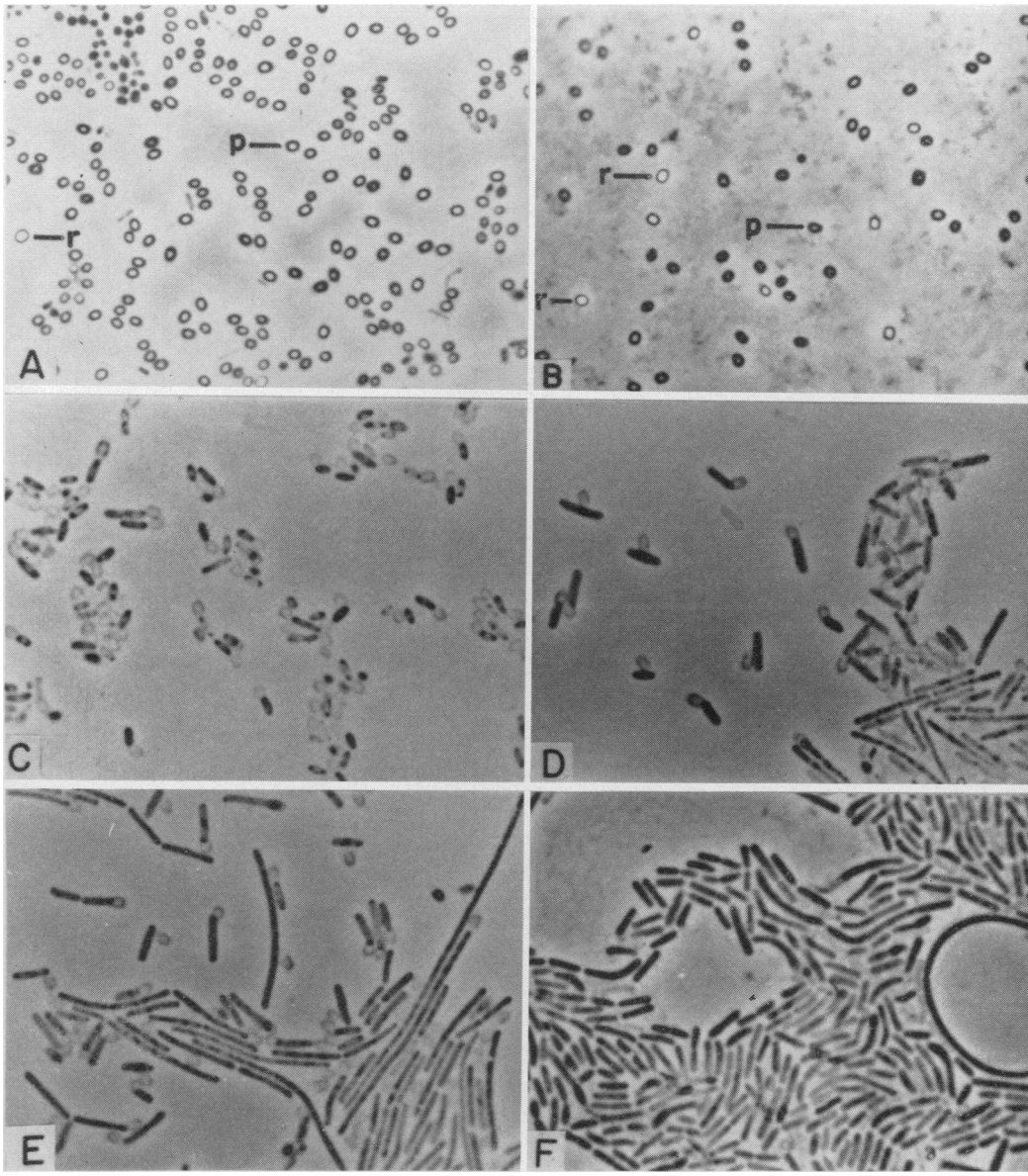

FIG. 3. *Effect of sodium chloride on germination and outgrowth of PA 3679h spores in microculture at 30 C. Complete loss of refractility was inhibited by more than 6% sodium chloride (A, B). Various stages of partial germination (p) are shown with completely refractile (r) spores. Variability among spores is reflected in the inhibition of emergence (C), elongation (D), or cell division (E) in some of the population at concentrations of 6% and less sodium chloride. No specific concentration completely blocked all of the spores at a particular developmental stage. The normal pattern of growth without sodium chloride is shown in (F). Original magnification,* × *1,070.*

nitrite and sodium chloride is presented in Fig. 4. In all cases, the end result of inhibition at a particular stage subsequent to swelling was lysis of the newly emerged or elongated cell.

DISCUSSION

With the exception of Gould (5), previous workers have studied the effects of sodium nitrite primarily on vegetative cells. Gould reported

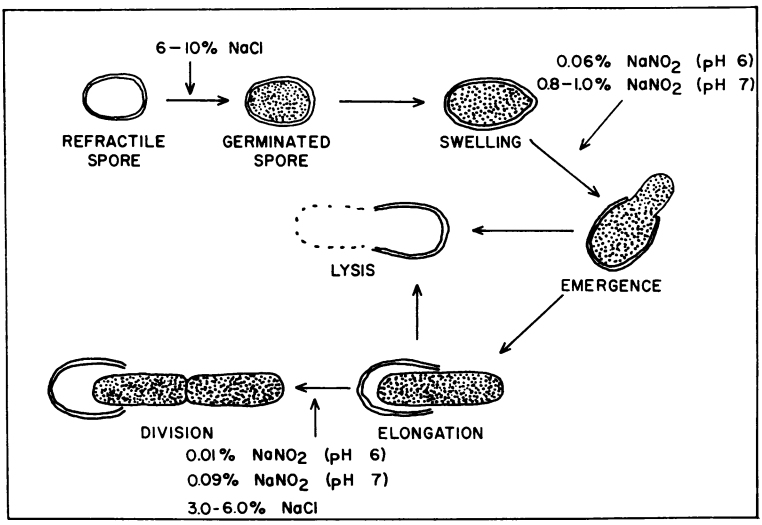

FIG. 4. *Summary of the effects of sodium nitrite and sodium chloride on the germination and outgrowth of PA 3679h spores. The points of inhibition are indicated by arrows perpendicular to those designating the normal process. Inhibition of stages prior to cell division results in lysis of cells.*

that spores of several *Bacillus* species germinated, though at reduced rates, in less than 0.03% sodium nitrite at *p*H 6.0. Development was arrested immediately after germination; i.e., before lysis or rupture of spore coats had occurred. Higher concentrations of nitrite (0.075 to 0.25%) prevented germination altogether at *p*H 6.0. Toxicity was three to five times greater at *p*H 6.0 than at *p*H 7.0.

By contrast, nitrite levels as high as 4% failed to inhibit germination of PA 3679h spores in this study. Furthermore, low concentrations of nitrite allowed emergence and elongation of some of the cells before inhibition occurred.

Gould also reported that up to 8% sodium chloride did not prevent germination of spores of any of the organisms studied. Concentrations ranging from 4 to 7% inhibited outgrowth, whereas concentrations of 10 to 15% progressively reduced and finally prevented germination. These results are comparable to the ones reported here. However, partial germination of PA 3679h spores occurred in 6 to 10% salt.

Partial germination of PA 3679h spores was first reported by Uehara and Frank (Bacteriol. Proc., p. 36, 1965). At hyperoptimal incubation temperatures (above 45 C), germination in the presence of L-alanine was only partial in that most of the spores appeared not to be completely darkened (the core was refractile). Partial germination also was observed when the germination system included D-alanine, a competitive inhibitor of L-alanine-induced germination. These results were interpreted to indicate that L-alanine-induced germination occurred in at least two stages.

Normally, spore germination is considered to be a rapid irreversible process, which is relatively insensitive to the environment (as compared to growth of vegetative cells). The present results confirm those of Uehara and Frank in suggesting that germination of PA 3679h spores may occur in two stages, since the addition of sodium chloride to the medium allowed only partial germination.

The results of these and other studies in our laboratory (3, 4) may help to explain the importance of nitrite in the preservation of canned cured meat products. Nitrite actually stimulates spore germination, especially under acid conditions at elevated temperatures (4). During normal heat processing, therefore, nitrite may induce spores to germinate and thus make them susceptible to thermal inactivation.

Spores that survive the heat treatment may germinate, but outgrowth is blocked by the residual nitrite in the product. At *p*H 6.0, as little as 0.01% nitrite allowed emergence of vegetative cells but prevented cell division. Under these conditions, the vegetative cells lysed.

Canned cured meat products usually contain 4 to 6% brine (i.e., 4 to 6% sodium chloride in the aqueous phase). Although this concentration did not prevent outgrowth of all PA 3679h spores, it did inhibit some of them and its effectiveness should be even greater with heat-injured spores

(3). Therefore, the salt concentrations used in practice may contribute significantly to the preservation of canned cured meat products. As with nitrite, newly emerged vegetative cells whose further development is blocked by 4 to 6% sodium chloride eventually will lyse.

ACKNOWLEDGMENT

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