Quantitation of pH- and Salt-Tolerant Subpopulations from *Clostridium botulinum*

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Plating efficiencies of *Clostridium botulinum* 62A spores on media with variable pH (7.0 to 5.5) and salt (0, 1, 2, and 3%) levels revealed that only a very small subpopulation could give rise to colonies. The relative size of this subpopulation decreased by orders of magnitude with decreasing pH and increasing salt concentrations. Strong interactions of pH with salt were noted. For example, on a medium containing 2% salt at pH 5.5, colonies could be formed from only 1 in 100,000 spores. Proper monitoring of medium anaerobiosis was critical in obtaining reproducible results.

It is commonly accepted that interactions of extrinsic factors play important roles in protecting foods against toxin production by *Clostridium botulinum*. Seemingly small changes in one factor can seriously compromise the safety of a food product. For example, Hauschild (4) has noted that the trend to lower the salt content of cured meats has resulted in a decrease of the average brine concentration from 5.5 to 4.5%. This 1% decrease in salt concentration increases the probability of spore outgrowth and toxin production 100-fold (4). The current emphasis on salt reduction to decrease risks associated with hypertension will undoubtedly increase pressure on food processors to further lower the salt content of their products.

I have previously reported on the effects of pH-glucose interactions (10) and pH-NaCl interactions (11) on the growth of *C. botulinum* in broth cultures. The purpose of this communication is to report that, because of pH-salt interactions, colonies were formed from only a small fraction of a given spore population when plated on media containing various pH and salt levels.

MATERIALS AND METHODS

Botulinum assay medium (BAM) (9) was prepared with the addition of 0, 1, 2, or 3% salt and adjusted to pH between 7.0 and 5.5 with 1 N HCl. The pH of the solidified plates was checked with a digital meter (Sargent-Welch Scientific Co., Skokie, Ill.; model PAX 9000) equipped with slope control and a flat-surface combination electrode standardized against buffers at pH 4.00 and 7.00. The plates were then placed in an anaerobic chamber (Coy Laboratories, Ann Arbor, Mich.) which had an atmosphere of 5% CO_2–6% H_2–89% N_2. All subsequent operations and incubations were done in the chamber.

The use of media held in the chamber for at least 48 h (2) after a methylene blue anaerobic indicator (GasPak; BBL Microbiology Systems, Cockeysville, Md.) indicated anaerobiosis of the chamber did not yield consistently reproducible data (not shown). I subsequently found that, when media containing resazurin (1 mg/liter) were placed in the anaerobic chamber, as long as 4 days were required for the resazurin to be reduced to the leuco form. The use of plates held until the resazurin was reduced resulted in consistently reproducible results. This finding can be attributed to the fact that methylene blue becomes colorless at −50 mV (5), whereas resorufin (the product of the first reduction step of resazurin) is reduced to the leuco form at a redox potential of −110 mV (3). The 60-mV difference in redox potential required to reduce methylene blue and resazurin may be critical in experiments involving salt sensitivity (15).

Spores of *C. botulinum* 62A were prepared as previously described (9). Although heat activation is not required for maximum plating efficiency on the medium used, the spores were heat-shocked (10 min at 80°C) to kill any germinated spores. Heating spores at 80°C does not sensitize them to salt concentrations as high as 5.5% (7). The spores were diluted in 0.1% peptone water to 10^7/ml, and portions were spread onto plates of BAM. Incubation of the plates was at 30°C. Counts were done after 7 days. Plating efficiencies were calculated as the CFU per milliliter divided by the number of refractile spores per milliliter (as determined by counting in a Petroff-Hausser chamber under phase optics) multiplied by 100 to give a percentage.

Since clostridial spores germinate at salt concentrations that prevent vegetative growth (1), and the influence of pH and salt on spores or vegetative cells inoculated into liquid medium is similar (11), it is probable that the growth of vegetative cells rather than spore germination would be inhibited in this study. Therefore, vegetative cells of other *C. botulinum* strains were used to determine whether the influence of salt on plating efficiency was strain specific. Vegetative cells from overnight cultures in BAM broth were diluted to 10^8/ml and plated on BAM (pH 7.0) containing 4% salt. The strains used were *C. botulinum* 17409, 62A, 25763 (type A), 7949, 53B, and B-aphis (proteolytic type B), and A028 (type C). These strains were used because type A and proteolytic type B strains are generally considered to be more salt resistant than nonproteolytic types B, E, and F (8).

RESULTS AND DISCUSSION

Growth on the various media differed qualitatively. Most striking was the fact that colonies formed on plates containing salt were very small, circular, and convex, whereas colonies on the basal medium were flat, rhizoid, and spreading. Toxin-neutralized mouse bioassays of subcultures from colonies formed on media containing salt confirmed that they were type A *C. botulinum* (data not shown). Colonies were visible on plates at pH 7.0 through 6.0 and 0, 1, or 2% salt after 24 h of incubation. Two days were required for the formation of visible colonies on plates with 3% salt, and 3 days were required on plates at pH 5.5 at all salt levels.

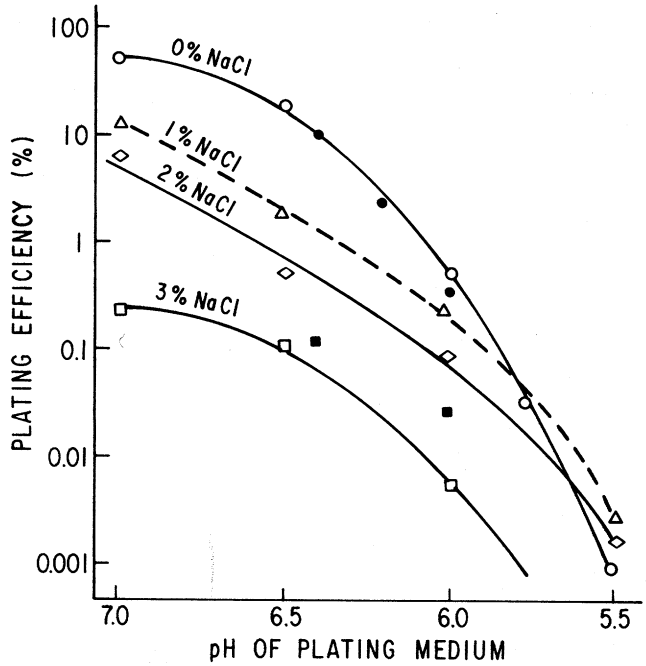

FIG. 1. Plating efficiency of *C. botulinum* 62A spores on botulinum assay medium containing 0% (○), 1% (Δ), 2% (◇), or 3% (□) salt. Closed symbols are from a replicate experiment conducted with a second spore crop.

The effect of both pH and salt on the extent of colony formation by *C. botulinum* 62A spores was dramatic (Fig. 1). Decreasing the pH in the absence of salt decreased the plating efficiency in a curvilinear fashion; this effect became more pronounced as the pH declined. This inhibition was caused by protons rather than Cl^- contributed by the HCl used to adjust the medium pH. The largest molar amount of Cl^- added (i.e., to adjust the pH to 5.5) was 15-fold less than the molar amount of Cl^- contributed by 1% NaCl, but medium at pH 5.5 with no exogenous salt was much more restrictive to colony formation than was medium with 1% salt at neutral pH. As the pH declined from 6.5 to 6.0 to 5.5, the percentage of spores that differentiated into vegetative cells capable of forming colonies dropped from 20 to 0.5 to 0.005%. Because plating media are often prepared to a target pH ± 0.2 units, this was an important finding; a plating medium prepared with a target pH of 6.8 could have plating efficiencies ranging from 20 to 60%. The addition of salt to media at neutral pH also reduced the portion of inoculated spores able to form colonies. For example, only 18 of every 10,000 spores could germinate and form colonies on plates which contained 3% salt at pH 7.0. These results appear to contradict results obtained in broth (11), in which decreasing pH in the absence of salt or the addition of salt to cultures at neutral pH had only a moderate effect on the maximum culture density. In the case of the broth system, it is probable that only a small fraction of the spores differentiated into vegetative cells capable of growth, but that these eventually produced turbidity, thereby masking the quantitative effect. These results are qualitatively similar but quantitatively different from those of Riemann (12). Although he did note that pH and NaCl together determine the probability of spore outgrowth, he observed no effect of pH between 7.0 and 6.0 nor salt at <4%. The difference may be due to the inherent dissimilarity between type E strains, which he used, and the type A and B strains used here. It may also be due to his use of a most-probable-number (broth) system. The quantitative insensitivity of broth systems has already been discussed.

The interaction between salt and pH on plating efficiency is shown in Fig. 1. Although media with a pH of 6.5 had a plating efficiency of 20%, the addition of 1% salt reduced the plating efficiency by a factor of 10. At 3% salt and pH 6.5, only 1 of 1,000 spores could form colonies. Nonlinear interactions of pH have been reported for salt in a comprehensive study of multiple interactions in a high-pH (6.3 to 7.2) cured meat system (14). The interactions in my system were also strongest above pH 6.2. Below that pH, the interaction of salt and pH decreased to the point at which, at pH 5.5, there was less than a threefold difference among the plating efficiencies of media containing 0, 1, and 2% salt. Roberts and Ingram (13) have shown that at a given salt or nitrite concentration, pH interactions can be critical. Vegetative cells of *C. botulinum* capable of growing in a meat system with pH 6.2, 3% salt, and 100 ppm nitrite could not grow if the pH was 6.0 (13).

The influence of salt on plating efficiency was reproducible and not strain specific. When vegetative cells were enumerated on a medium with 4% salt at pH 7.0, six of seven strains had plating efficiencies of less than 1% (Table 1). A second spore crop of strain 62A, plated on media with five combinations of pH and salt (Fig. 1, solid symbols) yielded results similar to those of the first spore crop. This suggests that the relative size of the spore subpopulation capable of germination and outgrowth was stable.

The data presented here quantify a fundamental fact about the growth of *C. botulinum* under restrictive conditions. That is, in a given population of cells, only a small fraction were capable of growing under what, in the context of botulinal inhibition, were only mildly restrictive conditions. Such a subpopulation explains the widely noted (6) inoculum effect by which large inocula can grow under more restrictive conditions than small inocula. Returning to Fig. 1 for an example, one would expect that 100 cells inoculated into media with a pH of 6.5 and 3% salt would not produce growth, but an inoculum of 10,000 cells would. In the former case, the "effective inoculum" of tolerant cells would be 0.1 cell. In the latter case, it would be 10 cells. Riemann (12) has suggested that the reduction, due to environmental conditions, of the number of spores capable of outgrowth might be applied to process lethality required of preserved foods. Hauschild (4) has argued that the expression of *C. botulinum* inhibition in cured meats as log $1/P$ (where P is the probability of toxigenesis from a given spore) provides a rational basis for comparing results of divergent challenge studies. The results reported here provide additional support for this approach.

In summary, this report shows that growth under various

TABLE 1. Effect of 4% salt on plating efficiency of *C. botulinum* vegetative cells on BAM at pH 7.0

Strain	Plating efficiency[a] (%)
17409	4.1
25736	<1.0
7949	0.8
53B	0.3
62A	0.2
B-aphis	0.9
A028	<1.0

[a] Plating efficiency = (CFU/ml ÷ cells/ml) × 100.

combinations of pH and salt can be attributed to a small number of resistant spores within the total population. The relative size of this subpopulation is determined by the pH and salt level of the growth medium.

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