

# Characterization of a Halo-Acid-Tolerant Variant of *Clostridium botulinum* B-aphis

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*Clostridium botulinum* B-aphis spores plated on medium containing 4% salt at pH 6.0 yielded colonies at a frequency of ca.  $1 \text{ in } 10^6$ . A subculture of one of these colonies, designated strain Ba410, was compared with the parent strain, B-aphis, for a variety of traits. After 7 days of incubation at 37°C, strain Ba410 grew in medium containing 7% NaCl, whereas strain B-aphis could not grow in salt concentrations greater than 5%. The strains also differed in cellular and colonial morphology. After exponential growth in the basal medium was completed, lysis of both strains was pH dependent; in media containing salt, lysis of Ba410 cells was pH independent. Strain Ba410 was more proteolytic than strain B-aphis in conditions of low pH and high salt, so that its toxin could be detected by the mouse assay. In a medium containing alanine and cysteine, the germination rate of B-aphis was  $0.77\% \text{ min}^{-1}$ , whereas that of Ba410 was  $0.14\% \text{ min}^{-1}$ ; 2% salt inhibited the germination of Ba410 but not B-aphis.

Current discussions of dietary sodium reduction in humans have attempted to balance the physiological benefit against possible risks from the increased probability of growth by food pathogens. The role of dietary sodium in human health and the implications of sodium reduction in muscle foods have been reviewed (23). The risks of potential increases in the probability of pathogenesis are being investigated in many laboratories. Because of the demand for rapid answers to problems in specific foods, much of this work concentrates on specific pathogens in a limited spectrum of foods. Hauschild (11), for example, has reviewed the influence of nitrite, pH, and salt on the probability of botulinal toxigenesis in cured-meat systems and has found that salt reduction may be more compromising to product safety than nitrite reduction.

In this laboratory, some basic studies have been conducted on the combined influence of salt and pH on the physiology of *Clostridium botulinum*, and it has been found that these factors interact in a complex fashion (19). Growth of *C. botulinum* under suboptimal, but nonlimiting, combinations of salt and pH has been attributed to the selection of tolerant subpopulations (20). The purpose of this study was to isolate a variant of *C. botulinum* capable of growth under such adverse conditions, characterize it, and determine whether it differed from the parent strain in a number of phenotypic traits.

## MATERIALS AND METHODS

**Organisms.** When strain B-aphis was plated on botulinum assay medium (see below) containing 4% salt at pH 6.0, ca.  $1 \text{ in } 10^6$  spores formed colonies. Several of these colonies were subcultured, and one, designated Ba410, was subjected to further study. This strain was confirmed as type B *C. botulinum* by specific neutralization of its toxin with type B antitoxin (Centers for Disease Control, Atlanta, Ga.). Stock cultures were maintained in cooked-meat medium (Difco Laboratories, Detroit, Mich.) at ambient temperature and transferred every 6 months. Spores were prepared as previously described (16), suspended in sterile deionized water, and stored in an anaerobic chamber at room temperature.

**Media and culture conditions.** The basal growth medium was botulinum assay medium (BAM) (16); for plating, 2%

agar and 0.0001% resazurin were added. NaCl was added on a weight-to-volume basis where noted before the adjustment of pH with 1.0 N HCl. After autoclaving (121°C, 15 min), pH values were within 0.1 unit of the target value. Details of the procedures used to study the influence of NaCl and pH on growth and lysis have been published elsewhere (19). Data presented in Fig. 1 and 2 are averages of duplicate cultures inoculated with  $10^6$  spores per ml. Colonial and biochemical characteristics were determined with modified McClung-Toabe egg yolk agar (5).

All media were prerduced in an anaerobic chamber (Coy Laboratories, Ann Arbor, Mich.) with an atmosphere of 5% CO<sub>2</sub>-6% H<sub>2</sub>-89% N<sub>2</sub>. All procedures, except microscopy and toxin testing, were carried out in the chamber. Incubations were carried out at 37°C unless otherwise noted. Growth in broth cultures was determined by measuring the absorbance at 610 nm with a colorimeter (Markson Scientific, Phoenix, Ariz.). Colony counts were carried out on plates that were found to be unchanged by an additional 48 h of incubation.

**Protease assay.** Protease activity was quantified in duplicate by the hydrolysis of casein and gelatin in a dual-substrate plate diffusion assay (17), with *Bacillus polymyxa* protease (type IX; Sigma Chemical Co., St. Louis, Mo.) used as a standard. Hydrolysis zone sizes after 24 h of incubation at 37°C were proportional to the logarithm of the applied protease activities.

**Detection of botulinal toxin.** Culture supernatants were obtained by centrifugation at  $8,740 \times g$  for 90 s in a Microfuge B (Beckman Instruments, Inc., Fullerton, Calif.). When protease assay results were  $<0.3 \text{ IU/ml}$ , portions of the supernatants were adjusted to pH 7 and trypsinized at 30°C for 1 h with 1:250 Bacto-Trypsin (Difco) at a final concentration of 0.1%. Samples were tested for botulinal toxin by injecting each of two Swiss white mice (weight, 15 to 20 g) intraperitoneally with 0.4 ml of the putative toxin. The mice were observed for symptomatic botulinal death for 72 h. Samples scored as toxic in these experiments produced symptomatic death of both mice within 24 h. Controls, which included boiled (10 min) portions of every sample, type B antitoxin-neutralized portions of selected samples, and 0.1% trypsin, were also tested for toxicity to mice.

**Germination kinetics.** The CTB germination medium described by Rowley and Feeherry (22) was modified by the addition of 4.5 mM L-alanine (Sigma) (8) and contained 32 mM L-cysteine hydrochloride (Sigma), 11.9 mM sodium bicarbonate (J. T. Baker Chemical Co., Phillipsburg, N.J.), 4.4 mM sodium thioglycolate (Difco), and 100 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Sigma) (22). The medium was made in the anaerobic chamber just before use by adding to the preweighed chemicals an appropriate volume of prerduced glass-distilled water which had been equilibrated to 37°C. The medium was adjusted to pH 7.0 with 3 M NaOH before filtration (pore size, 0.45 µm) sterilization.

Germination was followed as the loss of phase brightness (8, 21). The spore suspension (100 µl; 10<sup>8</sup> to 10<sup>9</sup> spores per ml) was heat activated (80°C, 30 min) in flint glass culture tubes (10 by 75 mm); activation in polystyrene culture tubes decreased germination. At time zero, 100 µl of double-strength CTB, which had been pre-equilibrated to 37°C, was added to the heat-activated spores. At specified times during the incubation, 10-µl aliquots were spotted on microscope slides preheated to 130°C on a heat block. Samples evaporated to dryness within 15 s. At the end of sampling, a drop of sterile distilled water was added to each slide, a cover slip was put in place, and the spores were examined microscopically under phase optics at ×600 to ×1,500 magnification. At least 200 individual spores were examined for each sample. Spores were characterized as ungerminated if they were phase bright or as germinated if they were phase dark or in the transition from refractile to nonrefractile (32). The percentage of germinated spores at each sampling time was plotted against time of incubation by mean least-squares regression, and from the slope, the germination rate in percent per minute was calculated. Results obtained from the heat-fixed spores were comparable to results obtained by immediately examining wet mounts (data not shown). These experiments were done in triplicate. The data presented are representative of a single experiment.

**Stability.** The stability of strain Ba410 was determined by inoculating cells into BAM at pH 6.0 with 3% salt or at pH 7.0 with no salt added and by sequentially transferring 10 µl into the same medium on days 2, 3, 4, 5, 8, 9, 10, 11, 12, 15, 16, 17, 18, 19, and 22 of the experiment. Cultures resulting from transfer 15 were streaked onto BAM agar containing 0 or 3% salt, and the colony morphology was determined after 48 h of incubation. The resultant cultures were also evaluated for salt sensitivity.

## RESULTS

**Morphology.** The colonial morphologies of strains B-aphis and Ba410 streaked onto BAM agar were different. In the absence of salt, B-aphis colonies were up to 5 mm in diameter after 7 days of incubation, flat with swirling rhizoid edges, and transparent to translucent. Their appearance was similar to that of toxigenic type E plated on blood agar (i.e., reference 24, plate 4b). When 3% NaCl was in the plating medium, the colonies were small (1 to 2 mm in diameter), circular, convex, and cream colored. Ba410 colonies were intermediate in size, circular with uneven edges, and slightly raised or umbonate, and they did not show a different morphology in the presence of salt. Ba410 colonies had a distribution of sizes and morphologies (i.e., ranging from slightly raised to umbonate) on both media; B-aphis colonies were much more uniform. On egg yolk agar, both strains had similar flat, rhizoid colonies with a pearly layer and precipitate edged with a clear zone, which is indicative of proteoly-

sis. The clear zone surrounding Ba410 colonies were larger than those of the B-aphis colonies.

In BAM broth culture, strain Ba410 cells were short (4.2-µm) rods, regardless of the salt content of the medium. B-aphis cells were, on the average, 7.8 µm long and, in the presence of salt, formed rods typically 32 µm and, in some cases, greater than 300 µm long. Aseptate filamentous (in excess of 600 µm in length) forms of strain Ba410 were observed in unrelated experiments.

**Salt tolerance.** Spores of strain B-aphis inoculated into BAM containing up to 5% salt produced turbid cultures within a week (Table 1). Growth of spores from Ba410 were not completely inhibited until salt levels of 8% were reached.

When the incubation time was extended to 4 weeks, additional B-aphis growth was observed in five tubes with >5% salt. This may have occurred because of the large inoculum used. It may have contained salt-tolerant spores at levels so low as to require several weeks for the attainment of measurable turbidity.

Samples incubated for 7 days were assayed for toxin. Botulinal toxin was detected in all untrypsinized samples from growth-positive tubes of Ba410. Only one of the five cultures of B-aphis in the 5% NaCl medium was positive for toxin, but toxin could be detected in the other four cultures after trypsinization. The identity of the lethal agent was confirmed as type B botulinal toxin by appropriate antitoxin-neutralized controls. Neither 7% NaCl nor trypsin was toxic for mice.

**Influence of medium pH and salt on culture characteristics.** B-aphis cells inoculated into 20 formulations of BAM at salt concentrations of 0 to 4% and pH values of 7.0 to 5.0 usually produced turbidity within 24 h (Fig. 1). Cultures in media with 4% salt, however, had longer lag periods. Growth in media with an initial pH of 5.0 was delayed in the absence of salt and was completely inhibited by salt concentrations ≥2%. Lysis (quantitated as the loss of optical density, but confirmed microscopically) of postexponential-phase cultures was pH dependent. Cultures in media with initial pH values of 5.5 or 6.0 exhibited less lysis and a slower lysis rate than cultures with initial pH values of 6.5 or 7.0. The pH dependency of lysis was not influenced by the salt content of the media. The combined effect of pH and salt was such that as the pH decreased, the optical density versus time curves became broader with lower maxima. As the salt concentration increased, the curves indicated decreased turbidity and were displaced to the right on the temporal axis.

The growth portions of the Ba410 optical density versus time curves (Fig. 2) were similar to those of strain B-aphis, but the lysis of cultures grown in the presence of NaCl differed. Although the cultures grown in the absence of salt

TABLE 1. Salt sensitivity of *C. botulinum* strains<sup>a</sup>

Strain	Incubation time (wk)	No. of positive tubes per total tested at the following percentages of NaCl:						
		3	4	5	6	7	8	9
B-aphis	1	5/5	5/5	5/5	0/5	0/5	0/5	0/5
	4	5/5	5/5	5/5	3/5	2/5	0/5	0/5
Ba410	1	5/5	5/5	5/5	5/5	3/5	0/5	0/5
	4	5/5	5/5	5/5	5/5	4/5	0/5	0/5

<sup>a</sup> Sensitivity was determined by inoculation of five tubes of BAM at each salt level with 10<sup>6</sup> heat-activated spores per ml and incubation at 37°C.

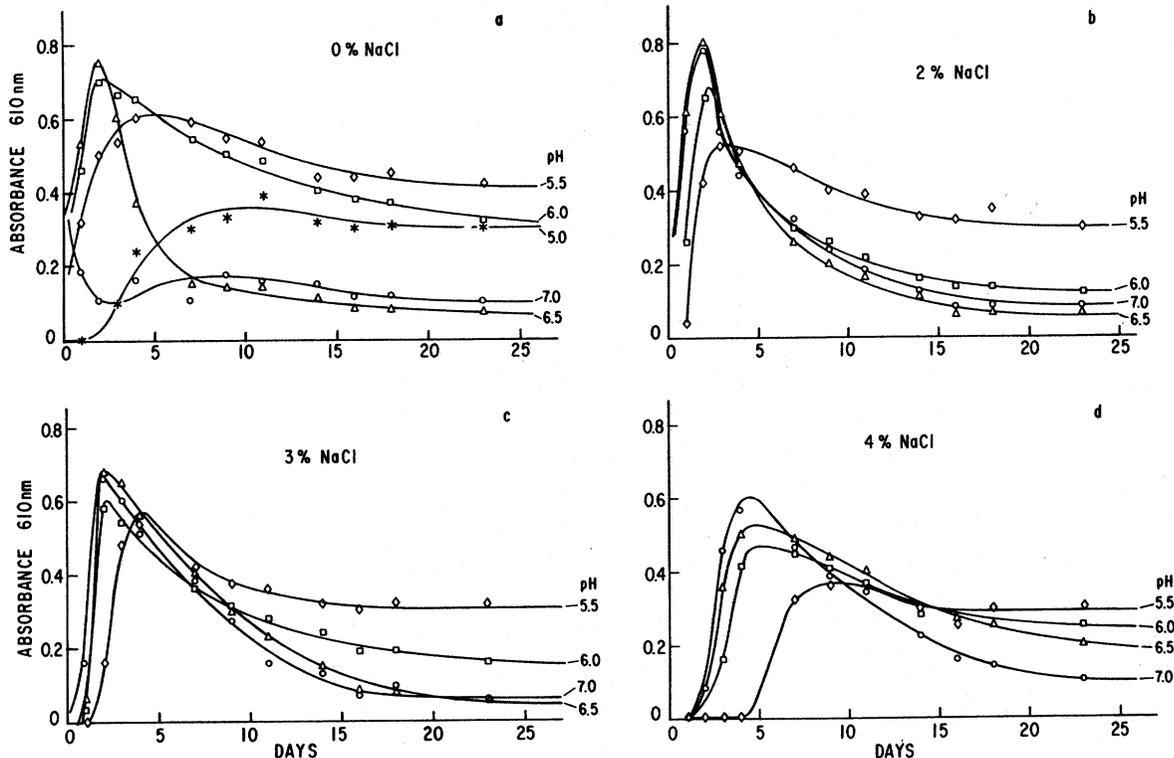


FIG. 1. Influence of pH and salt on optical density of *C. botulinum* B-aphis. A total of 10  $\mu$ l of an 18-h culture was inoculated into BAM with an initial pH of 7.0 (○), 6.5 (△), 6.0 (□), 5.5 (◇), or 5.0 (\*) to give an inoculated level of ca.  $10^6$  cells per ml. Absorbance was determined at times indicated during anaerobic incubation at 30°C.

exhibited the expected pattern of pH-dependent lysis, post-exponential cultures in media which contained salt underwent rapid and complete lysis, irrespective of the medium pH.

The data in Fig. 1 and 2 were reproducible; results from a duplicate experiment were similar, except that no growth occurred at pH 5.5 with 4% salt. Data on the toxicities, protease activities, maximum culture densities, and lytic characteristics of the cultures from the second trial are presented in Table 2. Of the 17 B-aphis cultures, 5 required trypsinization to detect the toxin. The requirement for trypsinization became evident at progressively higher pH values when media with progressively higher salt levels were examined. This requirement was related to low protease activities in cultures from media with a high salt content and low pH. Some samples with similar protease levels in the assay at pH 7 (i.e., B-aphis, 0% NaCl, and pH 5.5 and 5.0) differed in their requirement for an exogenous protease. Protease activity has been shown to drop with pH (7). Therefore, the protease in the sample with a lower pH would be less active *in situ* than under assay conditions. Protease activities of Ba410 cultures grown in the presence of salt were markedly higher than the activities of corresponding B-aphis cultures. None of the Ba410 cultures required trypsinization to detect toxin.

**Germination of B-aphis and Ba410 spores.** Although it was easy to obtain high numbers of clean B-aphis spores, yields of Ba410 spores were consistently 30 to 50% lower. In addition, the Ba410 preparations contained more exosporangial material, ghost cells, and debris and were difficult to clean. This made characterization of phase brightness technically difficult. Even though germination of B-aphis spores could be followed under  $\times 600$  magnification, it was fre-

quently necessary to use  $\times 1,000$  magnification and higher to characterize Ba410 spores.

B-aphis spores germinated rapidly and completely in media containing alanine and cysteine as germinants (Fig. 3a). Germination was linear ( $r = 0.99$ ), with a rate of  $0.77\% \text{ min}^{-1}$  for the first 60 min of incubation. A total of 88% of the spores germinated within 6 h of incubation, and 97% germinated after 24 h of incubation at 37°C. When spores were germinated in media containing 2% salt, only a slight influence was observed. The germination rate was again  $0.77\% \text{ min}^{-1}$ , but the data were not as linear ( $r = 0.94$ ). The extent of germination at 6 and 24 h was slightly lower, with 84% germination after 24 h.

Germination of Ba410 spores (Fig. 3b) was linear ( $r = 0.99$ ) for at least 6 h of incubation but proceeded at a slower rate ( $0.14\% \text{ min}^{-1}$ ) than B-aphis spores. After 24 h of incubation, only 46% of the spores were phase dark. Germination in the presence of 2% salt was inhibited. Since scattering of the data during the early period of germination resulted in a low regression coefficient (0.43), the slope of the germination curve was not quantitatively useful. However, 35% of the spores germinated within 24 h. Neither strain showed an increase in the number of germinated spores during 24 h of incubation in CTB, from which cysteine and alanine were omitted.

**Stability.** Ba410 cells sequentially passaged through BAM under selective pressure (i.e., pH 6.0, 3% salt) maintained their salt tolerance at 7% and their characteristic colony morphology, which, as previously noted, was the same on media with or without NaCl. Cells transferred through BAM at pH 7.0 in the absence of salt reverted to the parental colonial morphology and could grow only at NaCl concentrations  $\leq 5\%$ .

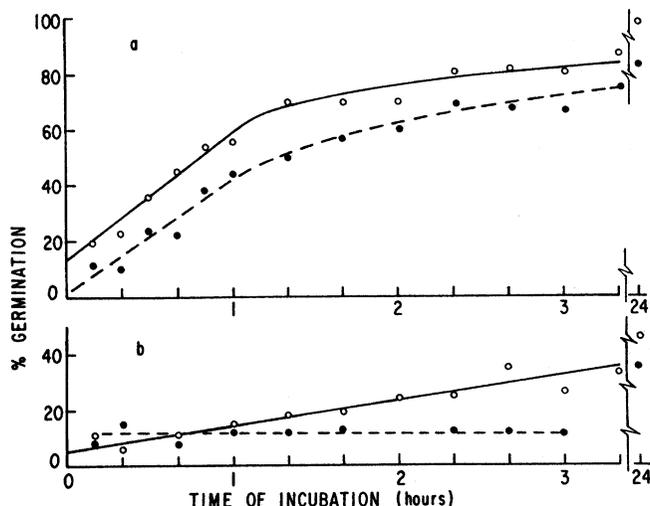


FIG. 3. Germination of *C. botulinum* B-aphis (a) and Ba410 (b) spores in CTB with (●) or without (○) 2% sodium chloride.

### DISCUSSION

pH-dependent lysis appears to be the norm for *C. botulinum*. Bonventre and Kempe (2) have demonstrated that lysis of strain JTD-IV is greater at pH 7.0 and 6.0 than at 5.5 and is under carbon source control (1). Lysis of strain 62A has a pH dependency (19) similar to that of strain B-aphis (Fig. 1). The partially purified autolysin from *C. botulinum* type A has *N*-acetylmuramyl-L-alanine amidase and hexosaminidase activities (25) and is active over a pH range of 6 to 8, with a maximum at pH 6.8 (13). The lysis of strain Ba410 at lower pH values in the presence of salt appears to be unusual. When strains Ba410, B-aphis, 62A, 17409, 2968, and 3121 were cultured in BAM containing 3% salt at pH 6.0 (data not shown), Ba410 was the only strain to undergo complete lysis. Salt apparently played some regulatory role in Ba410 lysis at low pH, since in the absence of salt, Ba410 exhibited a normal pH-dependent lysis pattern. The mechanisms for the positive effect of salt on Ba410 lysis at low pH may be indirect. For example, trypsin and nagarase have been shown to stimulate autolysin activity (13). A similar role may be played by Ba410 proteases, which are present at high levels in low pH media containing salt.

Most interest in botulin proteases stems from their important role in activating the progenitor toxin (3, 14). Thus, studies of the *C. botulinum* proteolytic system have concentrated on substrate specificity, pH optima, temperature optima, etc. The classification, purification, and characterization of botulin proteases have been reviewed by Tjaberg (27). Crude extracts from botulin supernatants contain polypeptides, dipeptidase, aminopolypeptidase (7), amidase, esterase (4), and proteinase (4, 7) activities. At least two distinct proteases have been purified (28). The proteases have maximum activity near pH 7, and activity drops rapidly as conditions become acidic (7, 28).

It was apparent from streaks on egg yolk agar that strain Ba410 had higher proteolytic activity than B-aphis. Quantitative assay of the endoproteases produced by both strains under variable salt and pH levels (Table 2) confirmed this. Because the supernatants were assayed at neutral pH, the data were indicative of the amount of protease produced. For strain B-aphis, more protease activity was found in cultures with low salt concentration, and there was little pH

effect. Strain Ba410 protease activity decreased with decreasing pH in the absence of salt. However, very high protease levels, comparable to those produced by other strains in cooked-meat medium (18), were found in BAM which contained salt. Because very little is known about the regulation of botulin proteases, the cause of these high protease activities is obscure. Although proteolytic type B strains are known to be more salt resistant than nonproteolytic type B strains (15), such a tentative linkage of salt tolerance to protease activity would have to be confirmed by further investigation.

The increased proteolytic activity of Ba410 cultures allowed detection of toxin in every culture. The results for B-aphis cultures were analogous to those for cultures grown in media with variable pH and glucose levels (18). Although strain B-aphis is phenotypically proteolytic, at low pH or high salt levels or both, there was insufficient protease activity for the detection of toxin. Trypsinization, which increases toxicity more than does a comparable activity of botulin proteases (29), resulted in the detection of additional toxic samples. These data again suggest that when no a priori assumptions about the influence of experimental variables on the expression of protease activity can be made, negative samples should be trypsinized and reassayed to reduce the probability of obtaining false-negative results in the mouse toxin test.

Although phase darkening is a late event in germination (22, 31), it is a widely used marker. The rapid germination response of strain B-aphis was comparable to that observed for cysteine-triggered germination of strain 62A (22), as well as *Clostridium sporogenes* PA3679h (26) and alanine-triggered germination of *C. botulinum* 12885A (8). Although phosphate has been proposed as a requisite cogerminant (30) and lactate is required for alanine-triggered germination of hypochlorite-injured spores from some crops (8-10), both B-aphis and Ba410 germinated well in CTB, which did not contain phosphate or lactate. Although 2% sodium chloride did not affect the B-aphis germination rate, it consistently caused a slight decrease in the extent of germination. In contrast, the germination rate of Ba410 spores was much lower than the rates reported for other strains. The Ba410 germination rate was drastically reduced by 2% salt, but the effect of salt on the (already low) extent of germination was much less. Qualitatively similar results were obtained with several spore crops from both strains. The observation that 2% salt affected germination was unexpected. Studies with *C. sporogenes* PA3679 indicate that 3 to 6% salt blocks cell division and that higher concentrations are required to prevent germination (6). The apparent contradiction may be due to the fact that in the earlier study gross effects were examined rather than rate effects, because overgrowth of the cultures by vegetative cells prevented the observation of individual spores at low salt concentrations, or because of differences between *C. botulinum* and *C. sporogenes*. It is also possible that in the presence of salt, factors supplied by complex media, but not by CTB, are required for germination. It is not clear why slow germination in the presence of salt would be advantageous to spores of a salt-tolerant strain of *C. botulinum*.

Both the genesis and instability of strain Ba410 can be explained by selection of either preexistent "commensal contaminants" (12) or genetic variants from the original B-aphis culture. These two hypotheses have been discussed with regard to apparent transformation in opaque sporulating (hypotoxigenic) and toxigenic cultures of *C. botulinum* types A, B, C (24), and E (12, 24). Both mechanisms explain the

isolation of salt-tolerant strains equally well. However, a commensal contaminant would have to remain associated with the culture through successive transfers under selective pressure to be available for subsequent growth under nonselective conditions (i.e., "reversion"). This is difficult to visualize. Because single point mutations occur as frequently in the reverse direction as in the forward direction, genetic variation would be a more plausible explanation for the reversion phenomenon. In either case, if only a few parental type cells were present, their faster growth in the absence of salt would cause them to dominate the culture after multiple transfers under permissive conditions. Further experimentation is necessary to distinguish between these mechanisms of population variability.

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