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Alkalotolerance of *Yersinia enterocolitica* as a Basis for Selective Isolation from Food Enrichments

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Alkalotolerance of *Yersinia enterocolitica* measured in solutions of potassium hydroxide with 0.5% sodium chloride was influenced by the cell suspension medium, temperature, and growth phase. The rate of cell destruction (Δ log N per minute) was five times greater at 30°C than at 20°C. Differences in the degree of cell destruction at various concentrations of potassium hydroxide were related to pH and not to osmolarity. The addition of peptones to potassium hydroxide provided a protective effect that was greater for cells suspended in Trypticase soy broth than for those suspended in phosphate-buffered sorbitol-bile salts broth. Log-phase cells were less alkalotolerant than cells in the stationary phase of growth. A modified procedure for alkali treatment, using peptone-supplemented 0.5% potassium hydroxide–0.5% sodium chloride and the addition of a pH 6.6 buffer after treatment to prevent further cell destruction, was used to observe a marked difference in alkalotolerance between *Y. enterocolitica* and other gram-negative bacteria. Despite this difference, alkali treatment was not highly successful for recovery of *Y. enterocolitica* from enrichments of seeded foods in comparison with selective enrichment in bile-oxalate-sorbose broth.

Aulisio et al. (1) first described a technique for the separation of *Yersinia enterocolitica* from background organisms present in food enrichments by treatment with potassium hydroxide before streaking the medium onto MacConkey agar. The method was based on an apparent difference in alkalotolerance between *Y. enterocolitica* and other gram-negative bacteria, thereby increasing the probability of selecting *Y. enterocolitica* from similarly appearing colonies on the isolation agar medium. The procedure was reported to improve isolation rates for both seeded and indigenous strains of *Y. enterocolitica* in foods, as well as to reduce the time required for recovery. A subsequent report has appeared describing the benefit of alkali treatment for recovery of *Y. enterocolitica* from feces (8).

The sparse literature available on microbial life under alkaline conditions deals primarily with alkalophilic, rather than alkalotolerant, organisms (2, 4). The observation by Aulisio et al. (1) of a difference in alkalotolerance between *Yersinia* spp. and other gram-negative bacteria appears to be original and presents an appealing basis for selective isolation. The work reported here was undertaken to examine in more detail the parameters that influence alkalotolerance in *Y. enterocolitica* to standardize the test procedure in a way that would prevent the destruction of *Y. enterocolitica* while reducing the density of background organisms present in food enrichments.

MATERIALS AND METHODS

Alkaline solutions. The alkaline solution described by Aulisio et al. (1) was prepared by diluting a 50% stock solution of KOH and 1% sterile NaCl to give final concentrations of 0.5% KOH and 0.5% NaCl (KOH). Solutions with lower concentrations of KOH were maintained with the concentration of NaCl at 0.5%. To examine the effect of osmolarity, solutions with less than 0.5% KOH were balanced with KCl to give osmolarities equivalent to 0.5% KOH. A modification of KOH was prepared by adding 0.06% Phytone (BBL Microbiology Systems) and 0.34% Trypticase (BBL Microbiology Systems), using autoclaved stock solutions (KPT).

Test organisms. Six human isolates of *Y. enterocolitica* were used in these studies: E661 (O:8), E663 (O:8), E739 (O:3), E752 (0:3), E758 (O:5,27), and E771 (O:5,27). All strains were virulent by the test for autoagglutination (3). Strains were maintained in phosphate-buffered 1% peptone–40% glycerol at −20°C. Subcultures were prepared on brain heart infusion agar incubated at room temperature for 48 h. Cells were washed off the agar with phosphate-buffered saline (PBS).

Other gram-negative organisms, all originally isolated from foods and selected for comparing relative alkalotolerance, were subcultured from peptone-glycerol (−20°C) to Trypticase soy broth and incubated at room temperature for 48 h.

All dilutions for colony counts were prepared in PBS with 0.02% peptone, and counts were completed

by spread plate inoculation of brain heart infusion agar incubated at 32°C for 20 to 24 h.

Enrichment media. Cultures grown on brain heart infusion agar and suspended in PBS were diluted (10^{-2}) in one of four enrichment media: buffered sorbitol-bile broth (BSB) (5), Trypticase soy broth (TSB), PSB preenrichment broth (7), or yeast extract-rose bengal broth (6).

Alkali treatment. Cells of *Y. enterocolitica* suspended in PBS were transferred (100 μl) to enrichment broth (9.9 ml) and allowed to stabilize for ca. 30 min. The enrichment broth suspension was then diluted, and a colony count was completed to determine the initial cell density. The broth suspension (0.5 ml) was transferred to KOH (4.5 ml), mixed well, and held for 5 min. In the initial experiments, dilutions and counts were made promptly after the exposure time. Thereafter, the procedure was modified by the addition of an equal volume (5 ml) of PBS (pH 6.6) with 0.02% peptone, a technique which lowered the pH sufficiently to stop further cell destruction. The degree of cell destruction by alkali treatment was expressed as the difference in the logarithms of the initial and final cell densities (Δ log N).

Food enrichments. Food homogenates were prepared with canned beef stew and one of the four enrichment media described above (1:10 [wt/vol]). The homogenate was seeded with one strain of *Y. enterocolitica* at approximately 10 cells per g of food and a background composed of 10 other gram-negative bacteria at ca. 10^6 cells per g of food. A total of 100 ml of the homogenate (10 g of food) was incubated at either 22, 10, or 2°C. At various time intervals a sample was removed, equilibrated at room temperature, and subjected to alkali treatment in KPT with either a 5- or 1-min exposure time. In addition, 0.1 ml of the preenrichment medium was transferred to 10 ml of bile-oxalate-sorbose (BOS) selective enrichment broth which was incubated at 22°C (6). Recoveries from BOS were attempted after 3 and 5 days of incubation by streaking the enrichment broth onto cefsulodin-irgasan-novobiocin (CIN) agar. The identity of two presumptive colonies was confirmed after subculture by slide agglutination with specific antiserum.

RESULTS

Titration of KOH concentration. The first experiments were undertaken to determine the maximum concentration of KOH at which there would be no destruction of *Y. enterocolitica* (the "no effect" concentration) since this effect is counterproductive to the purpose of the technique. Six strains of *Y. enterocolitica* were individually suspended in four different enrichment media, and after stabilization at room temperature for 30 min, they were transferred to solutions of KOH with concentrations ranging from 0.25 to 0.50%. Counts were promptly completed after a 5-min alkali treatment. The Δ log N was plotted against the KOH concentration, and a linear regression analysis was used to determine the no effect concentration of KOH, which was 0.17%. The data showed considerable scatter, with the Δ log N at 0.50% KOH ranging from 2.45 to 6.26; at 0.25% KOH the Δ log N ranged from 0.05 to 2.71. Some of the subsequent experiments were designed in part to help explain the basis for this poor reproducibility. All further experiments were performed with the addition of a pH 6.6 buffer after exposure to KOH to prevent continued cell destruction, a technique which removed one possible explanation for the low reproducibility.

Osmolarity and pH. An experiment was designed to determine whether the reduced cell destruction observed at lower concentrations of KOH was due to pH or osmolarity. Solutions of 0.50 and 0.25% KOH were prepared in 0.5% NaCl. One solution of 0.25% KOH was balanced osmotically with the 0.50% solution by the addition of KCl (3.3197 g/liter). Cells of strain E661 (0:8) were suspended in BSB and introduced into the alkaline solutions as previously described. The Δ log N for the 0.25% unbalanced solution was 1.58 (±0.10), and for the balanced 0.25% solution it was 1.79 (±0.43). The pH of these solutions was 12.34 and 12.40, respectively. The Δ log N for the 0.50% KOH solution with a pH of 12.67 was 3.11 (±0.27). The results indicated that differences observed in alkalotolerance at different concentrations of KOH were related to pH and not to osmolarity.

In a second experiment, strain E661 was suspended in BSB and exposed to four different

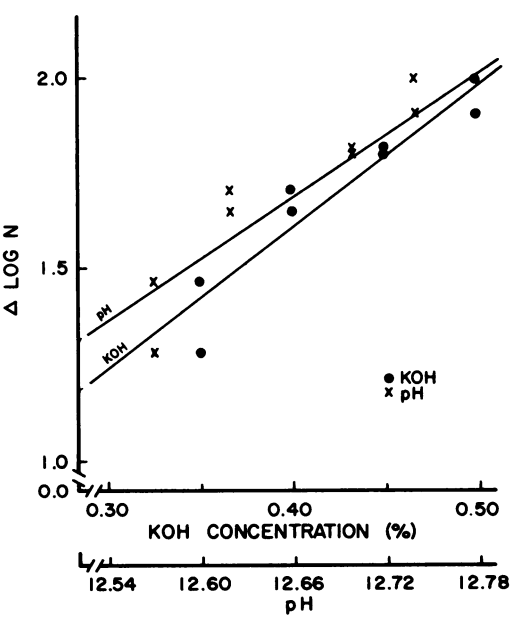

FIG. 1. Relationship of alkalotolerance of *Y. enterocolitica* (E661, O:8) suspended in BSB to a concentration of KOH and pH observed with 5-min exposure at room temperature. Solutions with less than 0.50% KOH were balanced osmotically with KCl. Δ log N = log N_0 − log N_5.

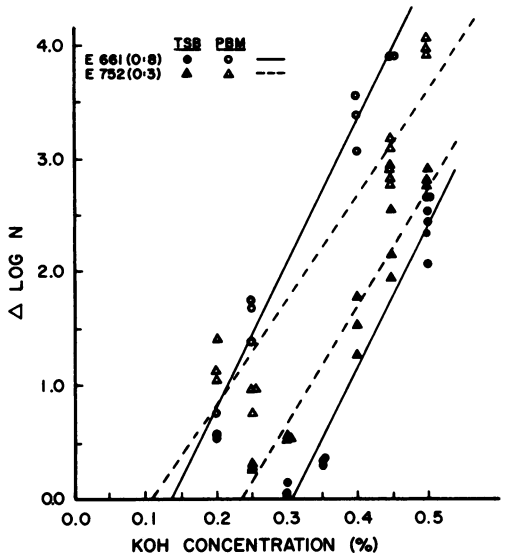

FIG. 2. Effect of strain and suspending medium on alkalotolerance of *Y. enterocolitica* observed with 5-min exposure at room temperature. Δ log N = log N_0 − log N_5. PBM is identified in the text as BSB.

concentrations of KOH for 5 min. All solutions with less than 0.50% KOH were balanced osmotically with KCl. The results (Fig. 1) confirmed that the degree of cell destruction was related to pH which, in turn, varied with the KOH concentration. The difference in pH between a Δ log N of 2 (i.e., 99% destruction) and the no effect concentration was about 0.5 U.

Enrichment medium. Two strains of *Y. enterocolitica* (E661 and E752) were examined for alkalotolerance after suspension in two different enrichment media, TSB and BSB. The results (Fig. 2) indicated very little difference between strains but a large difference between media. Destruction was far greater for cells suspended in BSB than for those suspended in TSB, with no effect concentrations of 0.11 to 0.14 and 0.24 to 0.30%, respectively. The difference in alkalotolerance was not the result of a difference in pH caused by the addition of enrichment medium.

The protective effect of peptones in TSB was examined further by KPT. Higher concentrations of peptones in KOH were not possible because of precipitation. The Δ log N with KPT for cells suspended in TSB was 0.28 (±0.13), compared with 1.88 (±0.43), the Δ log N with KOH, which indicated a distinct protective effect by peptones. The pH of KPT with added TSB (10%) was 12.85, and for KOH with TSB it was 12.91; this indicated that the protection was not likely due to a lower pH alone. The Δ log N for cells suspended in BSB was 1.78 (±0.08) with KPT and 2.25 (±0.48) with KOH, indicating less protection than that observed with TSB. Consequently, the elimination of *Y. enterocolitica* destruction during alkali treatment by peptone supplementation of KOH was not totally attainable because of the influence of enrichment medium. Nevertheless, all further experiments were completed with KPT.

An experiment was then undertaken to determine whether the bile salts in BSB accounted for greater destruction during alkali treatment. The Δ log N was 0.34 (±0.01), 2.70 (±0.35), and 1.88 (±0.09) for cells suspended in TSB, BSB with

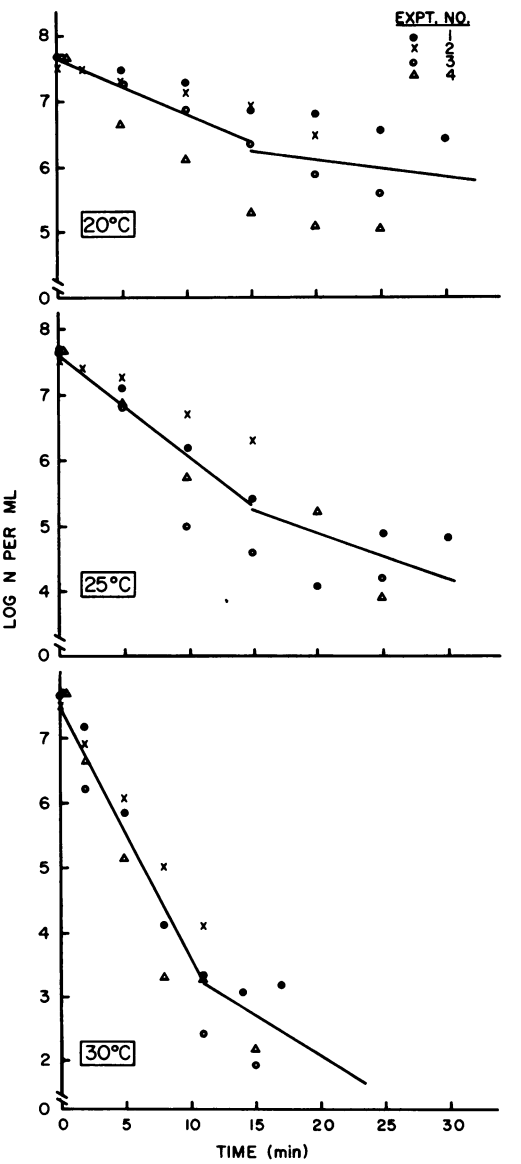

FIG. 3. Influence of temperature on rate of cell destruction under alkaline conditions. *Y. enterocolitica* (E661, O:8) was suspended in TSB and diluted in 0.40% KOH–0.50% NaCl.

TABLE 1. Comparison of alkalotolerance in log- and stationary-phase cells of gram-negative bacteria[a]

Organism	Δ log N for cells in:	
	Log phase	Stationary phase
Y. enterocolitica O:3	2.95	0.61
Y. enterocolitica O:5,27	3.22	1.04
Y. enterocolitica O:5,27	2.16	0.80
C. freundii	5.55	3.42
A. hydrophila	>5.34	3.97
P. fluorescens	>5.38	4.74

[a] Grown in TSB at 23°C.

bile, and BSB without bile, respectively. This showed that the bile in BSB did decrease alkalotolerance; however, removal of the bile did not increase alkalotolerance to the same degree as that observed in cells suspended in TSB.

Temperature. To examine the influence of temperature on the rate of cell destruction during alkali treatment, strain E661 was suspended in TSB and then transferred to 0.40% KOH in 0.5% NaCl after equilibration in a water bath to the experimental temperature (20, 25, or 30°C). Samples were removed at time intervals and neutralized with pH 6.6 buffer, and colony counts were completed. The experiment was repeated four times at each temperature. The survivor curves (Fig. 3) show a biphasic presentation and suggest an exponential death rate during the first phase ($r_{20} = 0.74$, $r_{25} = 0.87$, and $r_{30} = 0.96$). Alkalotolerance decreased markedly between 20 and 30°C, with destruction rates (Δ log N per minute) of 0.083 and 0.397, respectively.

It is likely that temperature was an important influence on the poor reproducibility observed in initial trials, which were completed under various room temperatures. The stricter control of temperature (22 to 25°C) and the addition of a "quenching" buffer provided good reproducibility within an experiment. However, the reproducibility between experiments with the same test strain was, for some reason, not as good.

TABLE 2. Injury in *Y. enterocolitica* (E661, O:8) after alkali treatment

Cell treatment	% Injury observed with[a]:	
	MacConkey agar	CIN agar
PBS suspension from brain heart infusion agar culture	2.2	10.8
PBS suspension diluted 1:10 in BSB; held for 30 min at 23°C	0.0	36.0
BSB suspension diluted 1:10 in KPT; held for 5 min at 23°C	49.8	91.7

[a] Relative to count on brain heart infusion agar. Media were incubated at 32°C for 24 h.

TABLE 3. Relative alkalotolerance of gram-negative bacteria[a]

Organism	Δ log N[b]
Yersinia enterocolitica O:8 (E661)	0.93
Yersinia enterocolitica O:8 (E663)	1.40
Yersinia enterocolitica O:3 (E739)	0.61
Yersinia enterocolitica O:3 (E752)	0.88
Yersinia enterocolitica O:5,27 (E758)	1.04
Yersinia enterocolitica O:5,27 (E771)	0.80
Acinetobacter calcoaceticus subsp. *anitratus*	4.03
Acinetobacter calcoaceticus subsp. *lwoffii*	>4.01
Aeromonas hydrophila	3.97
Citrobacter freundii	>4.73
Citrobacter amalonaticus	3.34
Klebsiella pneumoniae	2.88
Enterobacter agglomerans	4.00
Enterobacter agglomerans	3.56
Proteus vulgaris	2.26
Pseudomonas aeruginosa	4.03
Pseudomonas putida	>5.37
Pseudomonas fluorescens	4.74
Pseudomonas aeruginosa	5.39
Providencia rettgeri	3.55
Providencia alcalifaciens	4.13
Serratia rubidaea	5.70
Citrobacter freundii	3.42

[a] Grown in TSB at 23°C for 48 h.

[b] The mean of results for *Y. enterocolitica* strains was 0.94. The mean of results for the other gram-negative organisms was >4.10.

Growth phase. Three strains of *Y. enterocolitica* and three strains of other gram-negative bacteria (*Citrobacter freundii, Aeromonas hydrophila*, and *Pseudomonas fluorescens*) were inoculated into TSB and incubated at room temperature. The cultures were examined for alkalotolerance during the log and stationary (48 h) phases of growth. The results (Table 1) showed that log-phase cells were far less alkalotolerant than stationary-phase cells.

Injury after alkali treatment. Strain E661 (O:8) was suspended in PBS, and counts were completed with brain heart infusion, MacConkey, and CIN agars (five replicates). The PBS suspension was then diluted (10^{-2}) in BSB, and after 30 min at 23°C, counts were completed again. The BSB suspension was then diluted in KPT (10^{-1}), and counts were completed after a 5-min exposure at 23°C. The results (Table 2) showed that considerable cell injury was produced by alkali treatment. Consequently, a highly selective medium such as CIN agar is not desirable for use in this procedure.

Alkalotolerance of gram-negative bacteria. A total of 6 test strains of *Y. enterocolitica* and 17 strains of other gram-negative bacteria, all originally isolated from foods, were grown in TSB to the stationary phase (23°C, 48 h). Each organism was examined for alkalotolerance by using KPT

TABLE 4. Recovery of *Y. enterocolitica* strains from seeded[a] beef stew enrichments by alkali treatment

Enrichment		Recovery after alkali treatment (KPT for 5 min)											
		BSB			PSB[b]			TSB			YER[c]		
Temp (°C)	Time (days)	E661	E739	E758	E661	E739	E758	E661	E739	E758	E661	E739	E758
32	1	−			−[d]			−[d]			−[d]		
22	1	−[d]	−		−[d]	−	−[d]	−[d]	−		−[d]	−	
	2	−[d]			−[d]			−[d]			−[d]		
10	3	+[d]	−	−	−[d]	−	−[d]	−[d]	−[d]	−[d]	−[d]	−	−
	5	+			−			−			−		
	7	±			−			−			+		
2	7	−[d]	−	−[d]	−[d]	−[d]	−[d]	−[d]	−[d]	−[d]	−[d]	−[d]	−[d]
	10	−			−			−			−		
	14	+	−	−	−	−	−	−	−	−	−	−	−

[a] Seeded with 6.4×10^5, 1×10^7, and 8.7×10^6 cells of strains E661, E739, and E758, respectively, per g, with corresponding densities of 10 species of gram-negative bacteria of 5.0×10^5, 1.6×10^6, and 3.4×10^6 cells per g.
[b] PSB, Preenrichment broth.
[c] YER, Yeast extract-rose bengal broth.
[d] Recovered after selective enrichment in BOS at 22°C for 3 to 5 days. Selective enrichment was not attempted at 10°C for 5 and 7 days and at 2°C for 10 and 14 days.

and a 5-min treatment at 23°C. The results (Table 3) showed a marked difference in alkalotolerance between *Y. enterocolitica* and other gram-negative bacteria.

Food enrichments. A preliminary experiment was completed to evaluate the effect of food on alkali treatment. A 10% beef stew homogenate was prepared with BSB and seeded separately with *Y. enterocolitica* (E661, O:8) and *Enterobacter agglomerans*. The Δ log N for *Y. enterocolitica* was 2.08 (±0.43) in BSB and 1.22 (±0.57) in the homogenate. For *E. agglomerans* the Δ log N was 3.56 (±0.21) in BSB and 3.80 (±0.50) in the homogenate. The presence of food did not significantly alter alkalotolerance; instead, it appeared to offer a further protective effect for *Y. enterocolitica*.

Recovery of *Y. enterocolitica* from enrichments of seeded foods was attempted by alkali treatment and selective enrichment in BOS broth. Recovery of *Y. enterocolitica* by using either a 5-min exposure (Table 4) or a 1-min exposure (Table 5) in KPT was infrequent. In contrast, the use of BOS selective enrichment after preenrichment recovered every test strain except E771.

DISCUSSION

Y. enterocolitica does show far greater alkalotolerance than other gram-negative bacteria. However, several conditions were identified in this study that influence alkalotolerance, including the medium, temperature, and growth phase, and diminish the reliability of alkali treatment for separation of *Y. enterocolitica* from food enrichments. The enrichment of a food that initially contains a low number of *Y. enterocolitica* relative to the background flora will produce a population in which other organisms have

TABLE 5. Recovery of *Y. enterocolitica* strains from seeded[a] beef stew enrichments

Enrichment in TSB		Recovery after alkali treatment[b]			Recovery after selective enrichment in BOS		
Temp (°C)	Time (days)	E663 (23)[c]	E752 (14)	E771 (33)	E663	E752	E771
10	3	−	−	−	+++	+++	−
2	4	−	−	−	+++	+	−
	7	−	−	−	+++	+++	−
	10	−	+	−			−
	14	−	−	+			−

[a] Seeded with 10 gram-negative bacteria at 7.8×10^5 cells per g.
[b] Treated with KPT for 1 min.
[c] Seeded density per gram of food.

reached the stationary phase of growth before *Y. enterocolitica*. A lower number of *Y. enterocolitica*, more likely to occur at higher temperatures at which antagonistic effects appear to be greater, and the lower alkalotolerance of log-phase cells will result in a population ratio with alkali treatment that is not conducive to recovery. Alkali treatment can be successful only if *Y. enterocolitica* attains a population equal to or near that of the background flora, something which appears to occur only with enrichment at low temperatures for prolonged times.

The addition of peptones to the KOH solution reduced destruction of *Y. enterocolitica*, particularly when suspended in TSB. The addition of a buffer after alkali treatment stopped further cell destruction. Despite these improvements, when the technique was evaluated for recovery of *Y. enterocolitica* from food enrichments in which the initial ratio of background organisms to *Y. enterocolitica* was approximately 10^4:1, it was not highly successful. The use of BOS selective enrichment broth and CIN agar (6) was considerably more reliable. The marked difference in alkalotolerance between *Y. enterocolitica* and other gram-negative bacteria presents an appealing basis for selective isolation. However, the variable conditions associated with food enrichments prevent the precise standardization of the procedure that appears to be essential for obtaining predictable results.

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LITERATURE CITED

1. **Auliso, C. C. G., I. J. Mehlman, and A. C. Sanders.** 1980. Alkali method for rapid recovery of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from foods. Appl. Environ. Microbiol. **39**:135–140.
2. **Grant, W. D., and B. J. Tindall.** 1980. The isolation of alkalophilic bacteria, p. 27–38. *In* G. W. Gould and J. E. L. Corry (ed.), Microbial growth and survival in extremes of environment. Academic Press, London.
3. **Laird, W. J., and D. C. Cavanaugh.** 1980. Correlation of autoagglutination and virulence in yersinae. J. Clin. Microbiol. **11**:430–432.
4. **Langeworthy, T. A.** 1978. Microbial life in extreme pH values, p. 279–315. *In* D. J. Kushner (ed.), Microbial life in extreme environments. Academic Press, London.
5. **Mehlman, I. J., C. C. G. Aulisio, and A. C. Sanders.** 1978. Problems in the recovery and identification of *Yersinia* from food. J. Assoc. Off. Anal. Chem. **61**:761–771.
6. **Schiemann, D. A.** 1982. Development of a two-step enrichment procedure for recovery of *Yersinia enterocolitica* from food. Appl. Environ. Microbiol. **43**:14–27.
7. **Vidon, D. J. M., and C. L. Delmas.** 1981. Incidence of *Yersinia enterocolitica* in raw milk in eastern France. Appl. Environ. Microbiol. **41**:355–359.
8. **Weissfeld, A. S., and A. C. Sonnenwrith.** 1982. Rapid isolation of *Yersinia* spp. from feces. J. Clin. Microbiol. **15**:508–510.