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Antagonism by Gram-Negative Bacteria to Growth of *Yersinia enterocolitica* in Mixed Cultures

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The inhibition of the growth of *Yersinia enterocolitica* by other gram-negative bacteria in mixed cultures at 32°C was not the consequence of a depletion in essential nutrients, an unfavorable change in pH or oxygen tension or the production of toxic metabolic products. The inability of *Y. enterocolitica* to attain its potential maximum population in mixed cultures appeared instead to result from "metabolic crowding," which occurred when the faster-growing antagonistic organism reached stationary-phase density. Lowering the incubation temperature, a technique commonly used in "cold" enrichment for isolation of *Y. enterocolitica*, tended to equalize growth rates and thereby allowed *Y. enterocolitica* to achieve a higher population.

When a bacterium is introduced into an environment that is suitable for cell multiplication and provides an excess of all required nutrients, the organism will divide until reaching a stable population density in what is called the stationary phase. This limitation on the population is commonly attributed to an accumulation of toxic metabolic products, the exhaustion of essential nutrients required for growth, or a change in pH (5, 8, 10). This, however, was not the conclusion of Bail (2), who in 1929 suggested that the maximum number in the stationary phase ("M concentration") was set by an inherent attribute of the organism and the necessity of a certain amount of physical or "biological" space. Another hypothesis suggests that cell multiplication depends on the maintenance of a critical minimal concentration of food per unit of surface or volume (7). Multiplication may also depend on a certain critical rate of nutrient supply, which is dependent in turn on a concentration diffusion gradient that declines as the cell density increases.

In mixed populations, one organism may grow unrestricted but exert an antagonistic and inhibitory effect on the growth of another and thereby prevent the second organism from attaining its maximum potential population. The antagonism by lactic bacteria toward growth of psychrotrophs (1) and potential foodborne pathogens (4, 13) in mixed cultures has been studied the most thoroughly. These antagonistic interactions become extremely important when an attempt is made to isolate a specific organism from materials containing a varied microbial flora. The usual technique is to provide an enrichment system that will encourage the growth of the organism being sought (the target organism) to the exclusion or limitation of all others. It is neither necessary nor reasonable to expect total inhibition of all background organisms, rather to expect only that the target organism will grow to a density that will provide a significant proportion of the colonies presented on the selective-differential isolation agar medium used after enrichment.

Isolation of the enteropathogen *Yersinia enterocolitica* from food, water, and stools has commonly been accomplished by the use of "cold" enrichment (9, 11, 14), a procedure that imposes a lengthy time period. The only means for reducing this time is by use of a higher incubation temperature. This would require an enrichment medium that either is sufficiently selective or would eliminate any antagonistic effects of background organisms. Such an enrichment medium is not yet available.

The work reported here was undertaken to describe the antagonism of other gram-negative bacteria towards the growth of *Y. enterocolitica* in mixed cultures that appears from previous studies (12) to be temperature dependent. The ultimate objective was to provide a basis for the development of an enrichment medium that could be used at higher incubation temperatures and thereby reduce the time required for isolation of *Y. enterocolitica*.

MATERIALS AND METHODS

Test strains. One strain of *Y. enterocolitica* serotype 0:8, previously isolated from a human, was selected for these studies. This strain (E663) was autoagglutination positive (6) and was previously found to be infective for HeLa cells (3). Other gram-negative bacteria used in this study were isolated from foods by our laboratory and were identified by their characteristic biochemical profiles.

Stock cultures were preserved in glycerol-peptone at −20°C. Subcultures were prepared in Trypticase soy broth (BBL Microbiology Systems) incubated at room temperature for 2 to 3 days. Dilutions were prepared in 0.85% saline for inoculation of test media. Plate counts completed to determine the size of inocula and used to calculate multiplicities (i.e., the ratio of antagonist to *Y. enterocolitica*) were made with Trypticase soy agar (BBL) containing 0.6% yeast extract adjusted to pH 7.4 (TSY).

Antagonism across a dialysis membrane. Fourteen strains of gram-negative bacteria were screened for antagonistic activity against *Y. enterocolitica* in a system which separated the organisms by a cellulose dialysis membrane (Spectrapor 6; molecular weight cut off, 50,000). The membrane sac was attached to the end of a piece of glass tubing inserted through the metal cap on a 250-ml flask. The end of the sac was sealed with a clamp, and the upper end of the glass tube was sealed with a plastic cap. Approximately 10 to 12 ml of Trypticase soy broth was placed inside the sac, and 190 ml was placed in the flask outside of the sac. The prepared unit was sterilized by autoclaving. The potential antagonistic gram-negative organism was introduced into the outside medium at ca. 3×10^6/ml, and *Y. enterocolitica* was introduced into the sac at ca. 6×10^2/ml, thus providing densities of ca. 5,000:1. A control unit inoculated with *Y. enterocolitica* only was included in each experiment. The test units

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TABLE 1. Growth of *Y. enterocolitica* in the presence of other gram-negative bacteria separated by a dialysis membrane[a]

Gram-negative organism	Log no. of *Y. enterocolitica* cells per ml at:	
	24 h	48 h
None	8.848	10.205
Pseudomonas fluorescens	9.686	9.895
Providencia alcalifaciens	7.929	ND[b]
Pseudomonas putida	8.387	ND
Enterobacter agglomerans	6.320	7.633
Klebsiella pneumoniae	6.223	7.449
None	9.792	9.748
Enterobacter agglomerans	6.062	5.796
Citrobacter freundii	5.312	4.653
Citrobacter amalonaticus	5.021	4.690
Providencia rettgeri	6.371	5.942
Achromobacter spp. (biotype 1)	9.097	ND
Acinetobacter calcoaceticus	9.255	9.978
Pseudomonas putida	9.279	8.884
Citrobacter diversus	5.690	5.033
Providencia stuartii	7.380	7.892

[a] Bacteria were incubated statically at 32°C in Trypticase soy broth.
[b] ND, No data.

were incubated statically at 32°C, and plate counts were completed on *Y. enterocolitica* after 24 and 48 h by removing a portion of the broth from the inside of the dialysis sac through the tube protruding from the flask cap.

New enrichment broth. A series of experiments was undertaken to formulate a new enrichment broth that would hopefully reduce the antagonism of *Klebsiella pneumoniae*, selected for study from the group of organisms that had demonstrated antagonism across the dialysis membrane, towards *Y. enterocolitica* at 32°C. The basal medium consisted of phosphate (0.05 M)-buffered salts (NaCl, 0.1%; KCl, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.001%; $CaCl_2$, 0.001%) supplemented with Special Peptone (Oxoid Ltd.) and yeast extract (Difco Laboratories). Various modifications and conditions were examined, including addition of sodium chloride, bile, pyruvate, reducing agents (cystine, thioglycollate), and activated charcoal; adjustment of pH and the degree of aeration provided; spiking the medium at intervals during incubation with peptone-yeast extract; and stepwise transfers during incubation into fresh medium. The final selected medium (PEM) consisted of phosphate-buffered salts with 1.0% peptone and 2.0% yeast extract adjusted to pH 8.3. The medium was prepared by mixing two separate solutions sterilized by autoclaving: (i) Na_2HPO_4 plus NaCl plus KCl and (ii) Special Peptone plus yeast extract (final concentrations, 1.0 and 2.0%, respectively). To the cooled mixture were added filter-sterilized stock solutions of $MgSO_4 \cdot 7H_2O$ and $CaCl_2$. The final pH of the medium was 8.3.

Broth cultures were prepared in Erlenmeyer flasks and incubated either in a water bath shaker or on a rotary shaker (Psychrotherm, New Brunswick Scientific Co.). A very slow shake, sufficient only to keep the cells suspended uniformly, was used during most experiments. This was increased only when vigorous aeration to eliminate available oxygen as a variable was desired. All mixed cultures were inoculated with ca. 10^4 cells of the gram-negative antagonist and 10^2 cells of *Y. enterocolitica* per ml of medium to give a multiplicity of ca. 100:1.

Agar media for counts on mixed cultures. *Y. enterocolitica* was quantitatively measured in the presence of *K. pneumoniae* by using a selective medium consisting of TSY agar with 15 μg of sodium nitrofurantoin (Norwich-Eaton) per ml. In experiments with mixed cultures containing *Y. enterocolitica* and *Citrobacter freundii*, TSY with 15 μg of carbenicillin per ml was used for counting *Y. enterocolitica*, and TSY with 40 μg of sodium nitrofurantoin per ml was used for counting *C. freundii*. The media for selective counting of *Y. enterocolitica* and *Pseudomonas fluorescens* in mixed cultures were CIN (Yersinia Selective Agar, Difco) and TSY with 40 μg of sodium nitrofurantoin per ml, respectively. These media were developed by titrating various antimicrobial agents for their ability to completely inhibit the antagonistic organism with no decrease in the quantitative recovery of *Y. enterocolitica*. When *C. freundii* and *P. fluorescens* were included in the same experiment, counts for the control culture of *Y. enterocolitica* were completed with both selective media (i.e., CIN and TSY with 15 μg of carbenicillin per ml), and the reported count was taken as the arithmetic average.

RESULTS

Antagonism across a dialysis membrane. The growth of *Y. enterocolitica* in Trypticase soy broth under static conditions at 32°C in the presence of other bacteria separated by a dialysis membrane was restricted when the second organism was a fermenter from the family *Enterobacteriaceae* (Table 1). Species of *Citrobacter* were the most inhibitory. Organisms with an oxidative form of metabolism were far less inhibitory.

Effect of multiplicity. *K. pneumoniae* and *Y. enterocolitica* were grown together in PEM broth at 32°C under low-aeration conditions at three different multiplicities (Fig. 1).

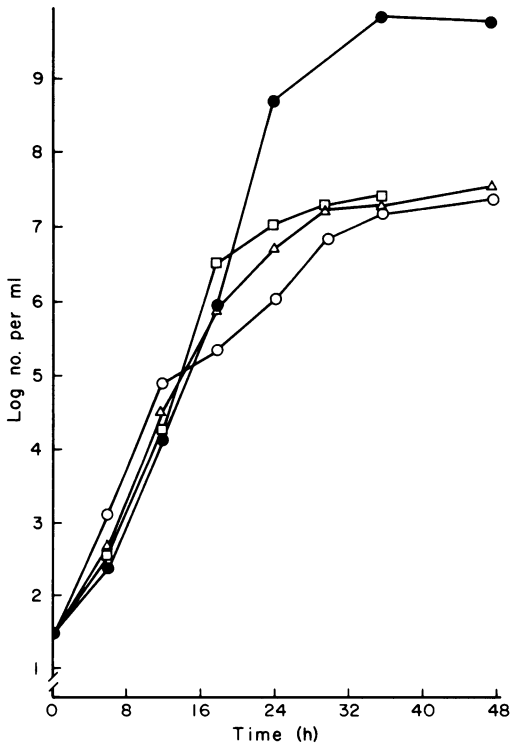

FIG. 1. Growth of *Y. enterocolitica* alone (●) and in the presence of *K. pneumoniae* at various multiplications: 2×10^4:1 (○), 2×10^2:1 (△), and 2×10^0:1 (□).

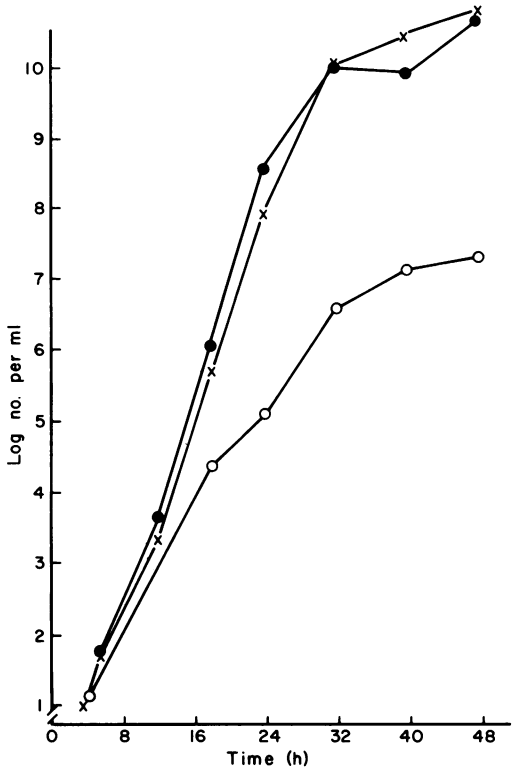

FIG. 2. Growth of *Y. enterocolitica* alone (●) and in the presence of *K. pneumoniae* (○) in fresh broth and alone in peptone-yeast extract-supplemented, pH-adjusted, spent broth from a *K. pneumoniae* culture (x).

The growth of *Y. enterocolitica* in mixed cultures was not different initially from the control; however, the point at which the growth rate began to decline varied with multiplicity. The final population established in all inhibited cultures was approximately the same.

Growth in supplemented, pH-adjusted, spent broth. *K. pneumoniae* was grown in PEM broth at 32°C for 24 h and

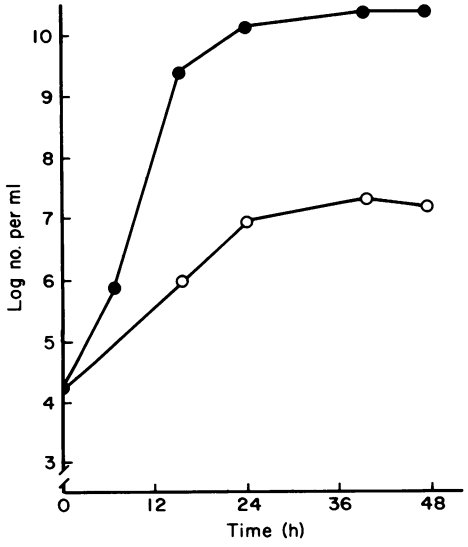

FIG. 3. Growth of *Y. enterocolitica* alone (●) and in the presence of *K. pneumoniae* suspended in fresh broth at stationary-phase density (○) under conditions of high aeration.

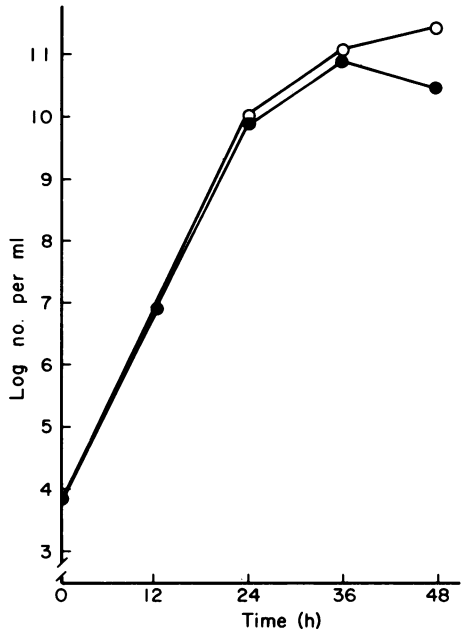

FIG. 4. Growth of *Y. enterocolitica* alone (●) and in the presence of *K. pneumoniae* cells killed by boiling and then suspended in fresh broth at stationary-phase density (○) under conditions of high aeration.

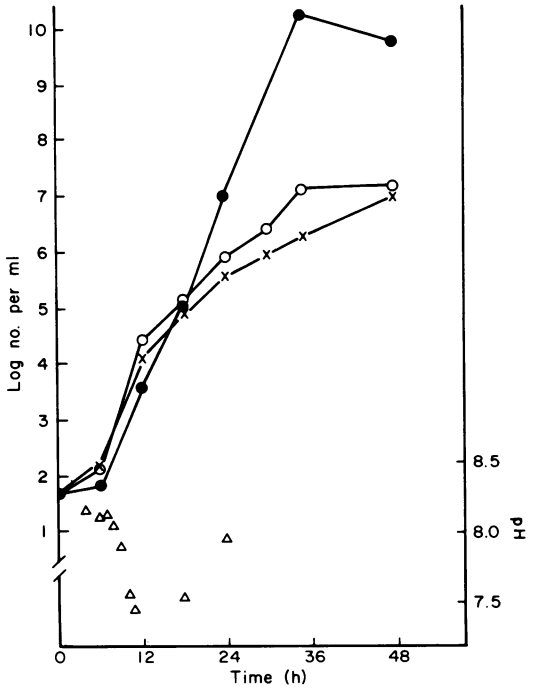

FIG. 5. Growth of *Y. enterocolitica* alone (●) and in the presence of *K. pneumoniae* with (×) and without (○) pH adjustment and peptone-yeast extract supplementation during the first 24 h of incubation under high aeration. The pH (△) is shown for the unadjusted mixed culture (○). The pH for *Y. enterocolitica* alone (●) varied between 8.1 and 8.2, and for the adjusted mixed culture (×), it varied between 8.1 and 8.3.

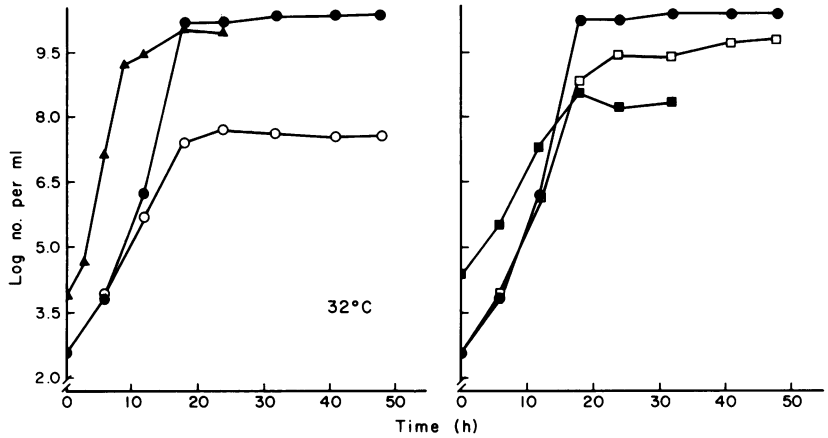

FIG. 6. Growth of *Y. enterocolitica* alone (●) and in the presence of *C. freundii* (○) and *P. fluorescens* (□), and growth of C. freundii (▲) and *P. fluorescens* (■) in the presence of *Y. enterocolitica* at 32°C.

then removed by centrifugation. The spent broth was filter sterilized and supplemented with 1.0% Special Peptone and yeast extract, the pH was raised from 7.58 to 8.30, and the medium was filter sterilized again. *Y. enterocolitica* introduced alone into this supplemented, pH-adjusted, spent broth grew as well as it did in fresh broth (Fig. 2). In mixed culture in fresh broth, the inhibition of *Y. enterocolitica* was similar to that observed in the previous multiplicity experiment. The final media pH values for *Y. enterocolitica* alone in fresh and spent broth were 7.99 and 8.18, respectively, and 8.48 in the mixed culture after 48 h of incubation.

Effect of viability and crowding. Two cultures of *K. pneumoniae* were grown to stationary phase, recovered by centrifugation, and then suspended in fresh broth. In one case the cells were first killed by boiling. *Y. enterocolitica* was introduced into the fresh media containing live and dead cells of *K. pneumoniae* at stationary-phase density, and the mixed cultures were grown under vigorous aeration. The inhibitory effect of the live cells on the growth of *Y. enterocolitica* was immediate (Fig. 3), whereas the killed cells at an equal density had no effect (Fig. 4).

Antagonism with supplementation and pH adjustment. An experiment was undertaken to eliminate three potential influences on the inhibition of *Y. enterocolitica* by *K. pneumoniae* in mixed cultures: (i) depletion of an essential nutrient(s), (ii) unfavorable change in pH, and (iii) reduction in available oxygen. The pH was monitored during the first 24 h of growth in all three cultures. In the control culture with *Y. enterocolitica* alone, it varied between 8.1 and 8.2. In the unadjusted mixed culture, it dropped quickly to ca. 7.4 by 12 h and then rose to 8.0 by 24 h of incubation. In the second mixed culture, the pH was adjusted at each observation time back to 8.3, and it stayed between 8.1 and 8.3. At each observation time, 1.0 ml of an autoclaved solution of 4.0% yeast extract–2.0% peptone was added to the mixed culture. Incubation of all cultures was at 32°C under conditions providing vigorous aeration. Growth of *Y. enterocolitica* in the supplemented, pH-adjusted, mixed culture was not improved (Fig. 5).

Effect of temperature on antagonism. Mixed cultures of *Y. enterocolitica* with either *C. freundii* or *P. fluorescens* were incubated at three different temperatures, 32, 25, and 15°C, and the growth of each organism was followed. A control culture of *Y. enterocolitica* alone was included at each experimental temperature. The results showed that the inhibition of *Y. enterocolitica* occurred when the antagonistic organism first reached the stationary phase (Fig. 6, 7, and 8). The antagonism in terms of a reduction in the maximum

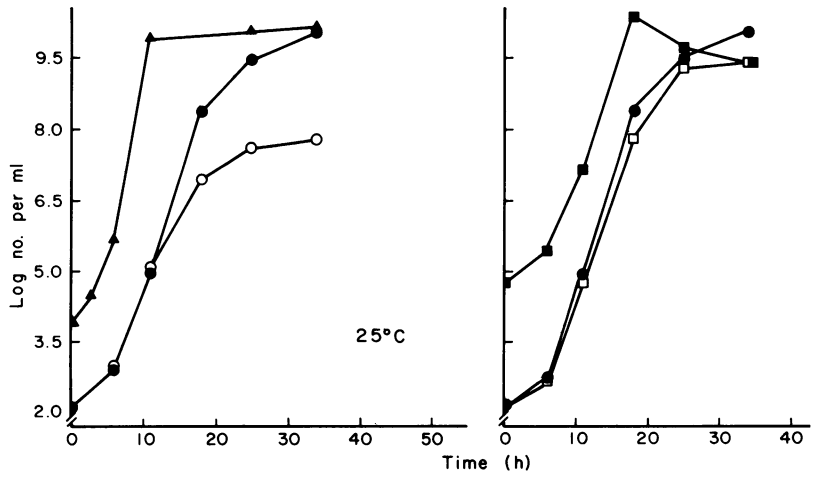

FIG. 7. Growth of *Y. enterocolitica* alone (●) and in the presence of *C. freundii* (○) and *P. fluorescens* (□), and growth of *C. freundii* (▲) and *P. fluorescens* (■) in the presence of *Y. enterocolitica* at 25°C.

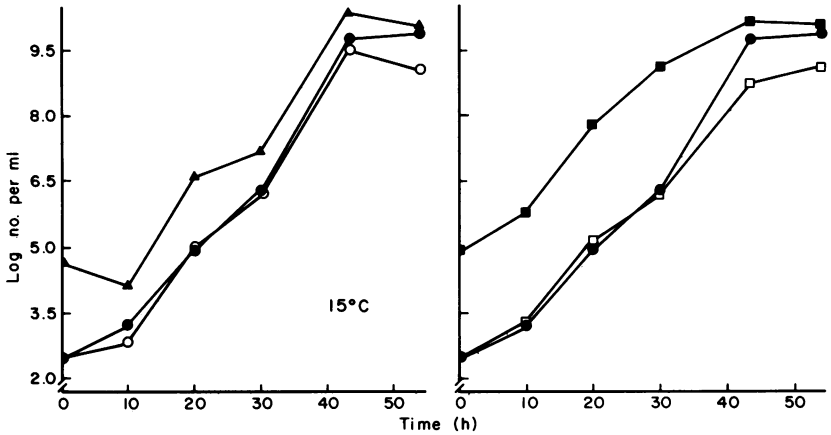

FIG. 8. Growth of *Y. enterocolitica* alone (●) and in the presence of *C. freundii* (○) and *P. fluorescens* (□), and growth of *C. freundii* (▲) and *P. fluorescens* (■) in the presence of *Y. enterocolitica* at 15°C.

population compared with the control culture of *Y. enterocolitica* alone was greater with *C. freundii* than with *P. fluorescens* at both 32 and 25°C. This difference could be related to the faster growth of *C. freundii* at these temperatures. At 15°C the time it took for both *C. freundii* and *P. fluorescens* to reach the stationary phase was approximately the same, and the effect on the growth of *Y. enterocolitica* was essentially identical. At this lower temperature the growth rates of both organisms in the mixed cultures were nearly identical, in contrast to the differences observed at higher incubation temperatures.

DISCUSSION

These experiments demonstrated that the antagonistic effect of certain gram-negative bacteria toward the growth of *Y. enterocolitica* in mixed cultures at higher temperatures is not the result of nutrient depletion, reduction in oxygen tension, the production of any toxic metabolite, or an unfavorable change in pH. The data show that growth of *Y. enterocolitica* was halted when the second organism reached the stationary phase, which supports the theory regarding the necessity of a certain critical space for continued cell multiplication. It appears that this space requirement is "biological" (or "metabolic") and not merely physical, since live but not dead cells of *K. pneumoniae* inhibited the multiplication of *Y. enterocolitica* when introduced into fresh media at stationary-phase cell densities.

The beneficial effect of "cold" enrichment for isolation of *Y. enterocolitica* appears to result from changing the growth rates in a way that allows *Y. enterocolitica* to come closer to attaining its potential maximum population. It is not that the growth of other gram-negative bacteria ceases at lower temperatures, but only that the growth rates are more equalized. Under these conditions, *Y. enterocolitica* can attain a higher cell density before the effects of metabolic crowding by other organisms are exerted. This is not to suggest that under other circumstances factors such as nutrient depletion, oxygen supply, toxic metabolites, and pH may in various combinations exert inhibitory effects, but only that when these influences are eliminated, the only one remaining is metabolic crowding.

The hope of developing an enrichment medium for *Y. enterocolitica* that could be used at higher temperatures and thereby reduce the time required for isolation was dashed by this study. We did, however, develop a new medium that was an improvement over Trypticase soy broth and presents a potential alternative for preenrichment. The results from the temperature studies suggest that it is not necessary to go as low as 4°C, which is commonly used for cold enrichment of *Y. enterocolitica* from mixed cultures (9, 11, 14). Our data show that antagonisms are essentially eliminated at 15°C, a temperature that would serve equally as well for enrichment while decreasing the time period substantially. The common use of 4°C for enrichment of *Y. enterocolitica* has more likely been encouraged by the availability of refrigerators at this temperature rather than on the basis of any physiological benefit for improving isolation.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Abdel-Bar, N. M., and N. D. Harris.** 1984. Inhibitory effect of *Lactobacillus bulgaricus* on psychrotrophic bacteria in associative cultures and in refrigerated foods. J. Food Prot. **47**:61–64.
2. **Bail, O.** 1929. Ergebnisse experimenteller Populationsforschung. Zitatsf. Immun. Forsch. **60**:1–22.
3. **Devenish, J. A., and D. A. Schiemann.** 1981. HeLa cell infection by *Yersinia enterocolitica*: evidence for lack of intracellular multiplication and development of a new procedure for quantitative expression of infectivity. Infect. Immun. **32**:48–55.
4. **Gilliland, S. E., and M. L. Speck.** 1977. Antagonistic action of *Lactobacillus acidophilus* toward intestinal and foodborne pathogens in associative cultures. J. Food Prot. **40**:820–823.
5. **Ingraham, J. L., O. Maaloe, and F. C. Neidhardt.** 1983. Growth of the bacterial cell. Sinauer Associates, Inc., Sunderland, Mass.
6. **Laird, W. J., and D. C. Cavanaugh.** 1980. Correlation of autoagglutination and virulence of *Yersiniae*. J. Clin. Microbiol. **11**:430–432.
7. **Lammana, C., M. F. Mallette, and L. Zimmerman.** 1973. Basic bacteriology: its biological and chemical background, 4th ed. The Williams & Wilkins Co., Baltimore.
8. **Monod, J.** 1949. The growth of bacterial cultures. Annu. Rev. Microbiol. **3**:371–394.
9. **Pai, C. H., S. Sorger, L. Lafleur, L. Lackman, and M. I. Marks.** 1979. Efficacy of cold enrichment techniques for recovery of *Yersinia enterocolitica* from human stools. J. Clin. Microbiol. **9**:712–715.
10. **Pritchard, R. H., and D. W. Tempest.** 1982. Growth: cells and population, p. 99–123. *In* J. Mandelstam, K. McQuillen, and I. Dawes (ed.), Biochemistry of bacterial growth, 3rd ed. Blackwell Scientific Publications, Oxford.

11. **Schiemann, D. A.** 1982. Development of a two-step enrichment procedure for recovery of *Yersinia enterocolitica* from food. Appl. Environ. Microbiol. **43**:14–27.
12. **Schiemann, D. A.** 1983. Comparison of enrichment and plating media for recovery of virulent strains of *Yersinia enterocolitica* from inoculated beef stew. J. Food Prot. **46**:957–964.
13. **Speck, M. L.** 1972. Control of food-borne pathogens by starter cultures. J. Dairy Sci. **55**:1019–1022.
14. **van Pee, W., and J. Stragier.** 1979. Evaluation of some cold enrichment and isolation media for the recovery of *Yersinia enterocolitica*. Antonie van Leeuwenhoek J. Microbiol. Serol. **45**:465–477.