

Repression of *Staphylococcus aureus* by Food Bacteria

I. Effect of Environmental Factors on Inhibition¹

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ABSTRACT

TROLLER, JOHN A. (University of Wisconsin, Madison) AND W. C. FRAZIER. Repression of *Staphylococcus aureus* by food bacteria. I. Effect of environmental factors on inhibition. *Appl. Microbiol.* **11**:11-14. 1963.—The effects of environmental factors on the inhibition of an enterotoxin-producing strain of *Staphylococcus aureus* by food bacteria were investigated. Type of medium and temperature of incubation were important factors in determining the amount of inhibition. The pH range of maximal inhibition was found to be 7.4 to 6.2. Availability of oxygen was not a factor. As the ratios of inhibitor to staphylococcus were increased from 1:1 to 10:1 and 100:1, the amount of inhibition was markedly increased. Inhibition occurred in custard, where it increased with increasing ratios of effector to staphylococcus. The repression of the staphylococcus in all media usually was sufficient to be of practical significance.

Growth of enterotoxigenic staphylococci in foods is affected by competitive growth of other microorganisms. The present work deals with the influence of various food bacteria, found to be inhibitory toward *Staphylococcus aureus*, on the growth of an enterotoxigenic staphylococcus under different environmental conditions and with different proportions of inocula.

Peterson, Black, and Gunderson (1962*a, b, c, d*) described the repression of growth of staphylococci by mixed populations of bacteria found naturally in foods. The suppression was enhanced as the proportion of staphylococci was decreased, as temperatures were increased toward room temperature, and as the pH was decreased below 6.0 or increased above 8.0; but the staphylococci were favored by salt concentrations above 3.5% and by added sucrose. By the time large populations of staphylococci had developed, the numbers of saprophytes had usually become great enough to render the food organoleptically unacceptable. Other examples of the suppression

of staphylococci by growth of saprophytes were reported by Straka and Combs (1952) for creamed chicken, Miller (1955) for ground pork, and Newman (1943) for milk. Oberhofer and Frazier (1961) found that the temperature of incubation of mixed cultures of *Escherichia coli* and *S. aureus* markedly influenced the extent of inhibition of staphylococci.

MATERIALS AND METHODS

Cultures employed. *S. aureus* 196, an enterotoxigenic strain obtained from G.M. Dack, was used as a test organism after trials had indicated that the several enterotoxigenic strains available reacted similarly. The inhibitory food bacteria were *Bacillus cereus*, *Proteus vulgaris*, *Serratia marcescens*, *Escherichia coli* H-52, and *Aerobacter aerogenes* from the stock cultures of the Department of Bacteriology, *Pseudomonas* sp. CS-1 isolated from meat, and *Achromobacter* sp. isolated from milk.

Culture media. Culture media used were nutrient broth, a semisynthetic medium (SYN), and SYN plus 4 g/liter of yeast extract (Difco). The composition of SYN is as follows (g/liter): acid-hydrolyzed vitamin-free casein, 10.0; tryptophan, 0.1; cystine, 0.1; thiaminehydrochloride, 0.001; nicotinic acid, 0.001; MgSO₄, 0.2; FeSO₄·7H₂O, 0.2; sodium acetate, 2.0; K₂HPO₄, 2.0; and distilled deionized water (pH 7.0). Also, 400 ml of egg custard, prepared according to the formula of Angelotti et al. (1959), were autoclaved at 10 psi for 20 min in a stainless-steel Waring Blendor cup (Waring Products Corp., New York, N.Y.) to facilitate mixing prior to withdrawal of a sample.

Unless otherwise noted, inocula of equal numbers of staphylococcus and effector organisms were employed. Growth curves of staphylococci were derived from plate counts of mixed and control cultures on mannitol salt agar.

Determination of R_I. Growth curves illustrating the results are too numerous for presentation. Therefore, results were condensed and expressed as relative inhibition (R_I), which is the area under the growth curve of the staphylococcus alone (AC) as measured by a planimeter, divided by the area under the growth curve of the staphylococcus when grown with a test or effector organism (AE), subtracted from 1 to give a value that varies directly with the amount of repression of growth. The formula would be: $R_I = 1 - AE/AC$.

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RESULTS

Examination of the 105 growth curves of staphylococci growing alone and with an effector organism showed that the time when inhibition of the coccus first became evident, the numbers attained by that time, and the total maximal numbers varied with the temperature of incubation, the

kind of culture medium and its pH, the effector organism tested, and the initial ratio of effector to staphylococcus. In most instances, the greater part of the inhibition of the staphylococcus took place after 8 or 10 hr. This is illustrated in Fig. 1, where results at 20 C for equal initial inocula of staphylococcus and effector bacteria are sum-

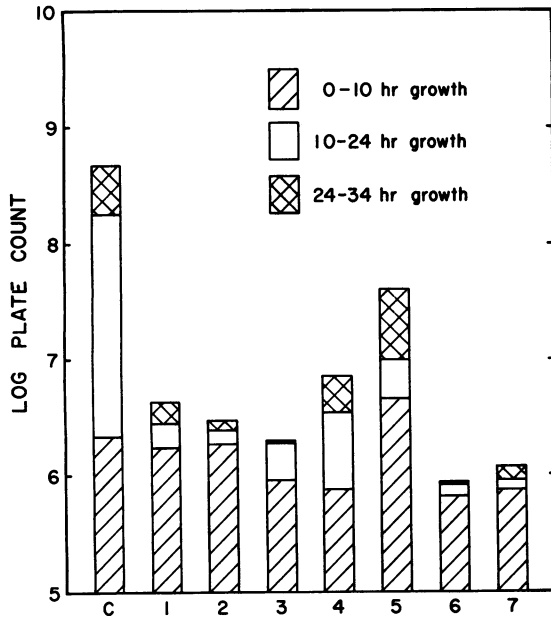


FIG. 1. Amount of growth of *Staphylococcus aureus* 196 in nutrient broth at 20 C in pure culture (C), and in mixed culture with *Bacillus cereus* (1), *Aerobacter aerogenes* (2), *Serratia marcescens* (3), *Achromobacter sp.* (4), *Proteus vulgaris* (5), *Escherichia coli H-52* (6), and *Pseudomonas sp. CS-1* (7).

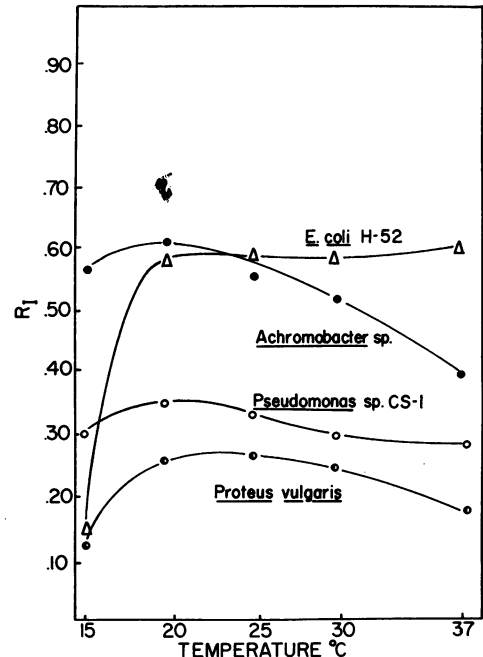


FIG. 3. Inhibition of *Staphylococcus aureus* 196 at 15, 20, 25, 30, and 37 C in nutrient broth by *Proteus vulgaris*, *Escherichia coli H-52*, *Achromobacter sp.*, and *Pseudomonas sp. CS-1*, expressed as relative inhibition (R_1).

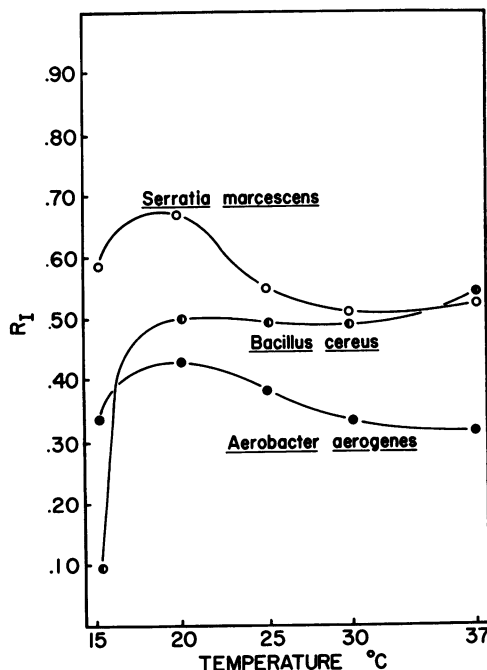


FIG. 2. Inhibition of *Staphylococcus aureus* 196 at 15, 20, 25, 30, and 37 C in nutrient broth by *Bacillus cereus*, *Aerobacter aerogenes*, and *Serratia marcescens*, expressed as relative inhibition (R_1).

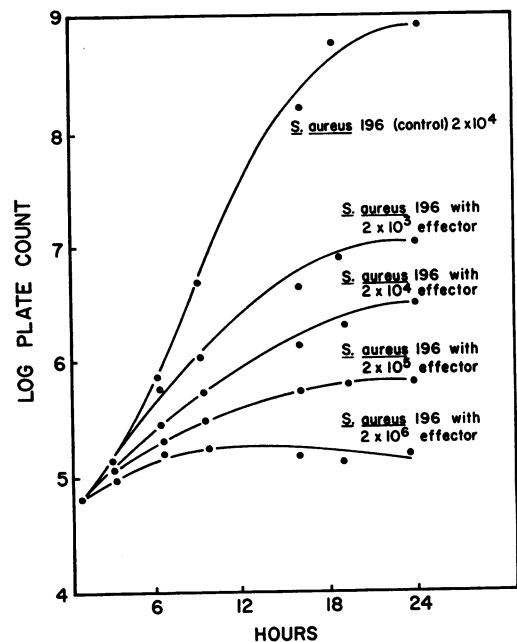


FIG. 4. Growth curves of *Staphylococcus aureus* 196, alone and in mixed culture with *Escherichia coli H-52*, in SYN medium at 30 C with a fixed original inoculum of staphylococcus and with various initial numbers of effector organisms.

TABLE 1. Comparison of the effects of shaking and incubation in shallow layers on plate counts of *Staphylococcus aureus* 196 and of effector bacteria

Organism	No. per ml after 24 hr of incubation at 30 C in SYN medium				
	Unshaken flask*		Shaken flask†		Roux bottle‡
	<i>S. aureus</i> 196	Effector	<i>S. aureus</i> 196	Effector	<i>S. aureus</i> 196
<i>S. aureus</i> 196 control	3.3×10^8		6.4×10^8		5.2×10^8
<i>Achromobacter</i> sp.	4.2×10^6	2.1×10^8	5.7×10^8	7.3×10^8	9.2×10^6
<i>Proteus vulgaris</i>	4.7×10^6	3.2×10^8	2.5×10^8	1.7×10^9	8.9×10^6
<i>Escherichia coli</i> H-52	1.3×10^6	6.9×10^8	4.8×10^4	2.2×10^9	4.4×10^6

* Oxidation-reduction potential = + 230 mv.

† Oxidation-reduction potential = + 270 mv.

‡ Oxidation-reduction potential = + 255 mv.

marized. The figure also illustrates the considerably lowered population of staphylococci after 24 and 34 hr of competition with other bacteria at 20 C. At 37 C, the staphylococcus attained 5 to 8×10^8 organisms per ml, but when grown with effector bacteria reached only 2.5 to 6×10^6 cocci.

Temperature and kind of medium. The temperature of incubation for maximal inhibition of *S. aureus* 196 varies with the kind of effector organism as well as with the culture medium. The maximal inhibition occurred mostly at 20 to 25 C, especially when the medium was either nutrient broth or SYN supplemented with yeast extract. However, in a poorer medium than either of the other two (SYN), the inhibition seemed to be maximal at 15 C. In both SYN with yeast extract and nutrient broth, there was a definite decline in the R_T value when incubation was at 15 C. Figures 2 and 3 show the influence of incubation temperature on the R_T values in nutrient broth. It was also noted that, at higher incubation temperatures, the inhibition occurred earlier than at lower incubation temperatures.

Effect of pH. The greatest amount of inhibition occurred in the pH range 7.4 to 6.2, regardless of the effector organism used. At lower initial pH values of the medium, the inhibition decreased until, at and below pH 5.4, the inhibitory activity had virtually disappeared.

TABLE 2. Plate counts of *Staphylococcus aureus* 196 with inoculum (2×10^4 per ml) growing in custard alone (at 30 C) or with an inoculum of effector bacteria (2×10^4 or 2×10^6 per ml)

Organism	No.* per ml with 2×10^4 effector organisms per ml			No.* per ml with 2×10^6 effector organisms per ml		
	6 hr	10 hr	24 hr	6 hr	10 hr	24 hr
<i>S. aureus</i> 196 (alone)	29	2,300	6,100	810	6,200	8,400
<i>Proteus vulgaris</i>	15	500	200	63	17	21
<i>Escherichia coli</i> H-52	15	360	480	15	20	31
<i>Achromobacter</i> sp.	6.5	52	120	10	23	22
<i>Pseudomonas</i> sp. CS-1	28	130	200	5	8.2	9
<i>Aerobacter aerogenes</i>	22	520	460	15	16	16
<i>Bacillus cereus</i>	84	250	610	23	27	27
<i>Serratia marcescens</i>	38	290	520	62	71	29

* All figures $\times 10^6$.

Effect of initial proportions of S. aureus 196 and effector organisms. Figure 4 shows a typical example of the effects of ratios of numbers of effector organisms to numbers of *S. aureus* 196 growing in SYN medium at 30 C. As the ratio of effector organism to staphylococcus was increased, there was a marked increase in the inhibitory effect. However, even when the staphylococci outnumbered the effector organisms by a ratio of 10:1, the staphylococcus was found to be inhibited by at least one logarithmic cycle after 24 hr. This effect of increasing inhibition of staphylococci with increasing ratios of effector to staphylococcus was exhibited by all of the inhibitor bacteria and to about the same degree. With *B. cereus*, with a ratio of 100 effectors to 1 staphylococcus, there was an actual lowering of the numbers below those in the original inoculum.

Effect of shaking. The inhibitory activity of three of the seven inhibiting organisms, *Achromobacter* sp., *P. vulgaris*, and *E. coli* H-52, was greatly enhanced by incubation for 24 hr on a shaker, but the inhibitory activity of the remaining four effector organisms was not affected by the shaking process. Table 1 compares numbers of effector organisms and staphylococci in mixed culture, and the staphylococci in pure culture, when shaken, static, and in a Roux bottle. All cultures had essentially equal oxidation-reduction readings after incubation. The restoration of the normal amount of inhibition in the thin broth layer indicates that the increased inhibition occurring with the three organisms was caused most probably by the mechanical action of the shaking alone.

Inhibition of S. aureus 196 in custard. *S. aureus* 196 was inhibited (Table 2) when effector and staphylococcus were inoculated into sterile custard in equal numbers in the same manner as into the other culture media. When the initial ratio of effector to staphylococcus was 2×10^6 to 2×10^4 per ml (ratio 100:1), the inhibition was even greater, the numbers of staphylococcus reaching less than 3×10^6 organisms per ml in 10 hr (except for *S. marcescens*). The appearance of the custard remained unchanged after growth of the mixed cultures, except that, when *B. cereus* was employed as the effector organism, the custard was liquefied by starch-hydrolyzing enzymes secreted by the organism. There also appeared to be little

off-odor production, with the exception of custard in which *P. vulgaris* was used as the effector organism, where there was a rather objectionable sulfurlike odor.

DISCUSSION

Inhibition of *S. aureus* 196 by food organisms was markedly influenced by environmental conditions. Both the growth medium and temperature of incubation were factors in the amount of inhibition occurring. If, as has been reported (Frazier, 1958), enterotoxin production is best between 21 and 36 C, it is fortunate that maximal inhibition by the food bacteria tested has been found to occur in approximately the same range. Of practical significance is the fact that maximal inhibition usually was at about 20 to 25 C in a good medium. Foods implicated in food-poisoning outbreaks usually have had a history of storage from some time at or above room temperature, allowing growth of the staphylococcus. The time that growth diminished in rate or stopped depended on the incubation temperature, the repression occurring sooner at higher temperatures. The time of cessation of growth also depended on the effector bacterium and the culture medium.

It is important that *S. aureus* was inhibited to an increasing extent as it was outnumbered more and more by the inhibitory bacteria, since the staphylococcus probably is usually in the minority in foods. The inoculation of equal and fairly large numbers of staphylococci and effector organisms certainly was in favor of the staphylococcus, for when the inoculum of effector bacteria was increased to 10 to 100 times that of the staphylococcus, the inhibition of the staphylococcus was greatly increased. The time for retardation of growth was shortened as the numbers of effector bacteria were increased, and effective levels of the inhibitor were obtained earlier. The availability of oxygen apparently had little influence on inhibition, indicating that a competing bacterium might be as inhibitory toward the staphylococcus in deeper layers of a food as at the surface.

The results indicate that the ten million or more

staphylococci, considered by some (Peterson, 1962) to be necessary for production of significant amounts of enterotoxin, were seldom attained at different temperatures and in different media, even when equal inocula of staphylococcus and effector organism were added. The causes of inhibition of the staphylococcus by the effector bacteria will be reported elsewhere.

LITERATURE CITED

- ANGELOTTI, R., E. WILSON, M. J. FOTER, AND K. H. LEWIS. 1959. Time-temperature effects on salmonellae and staphylococci in foods. I. Behavior in broth cultures and refrigerated foods. Technical Report F59-2. U.S. Department of Health, Education, and Welfare, Cincinnati.
- FRAZIER, W. C. 1958. Food microbiology. McGraw-Hill Book Co., Inc., New York.
- MILLER, W. A. 1955. Effect of freezing ground pork and subsequent storing above 32°F upon the bacterial flora. Food Technol. **9**:332-334.
- NEWMAN, R. W. 1943. The problem of staphylococci in a milk supply. Bull. Dep. Agr., Calif. **32**:42-45.
- OBERHOFER, T. R., AND W. C. FRAZIER. 1961. Competition of *Staphylococcus aureus* with other organisms. J. Milk Food Technol. **24**:172-175.
- PETERSON, A. C. 1962. An ecological study of frozen foods. Proc. Low Temp. Microbiol. Symp. (Campbell Soup Co.), p. 157-192.
- PETERSON, A. C., J. J. BLACK, AND M. F. GUNDERSON. 1962a. Staphylococci in competition. I. Growth of naturally occurring mixed populations in precooked frozen foods during defrost. Appl. Microbiol. **10**:16-22.
- PETERSON, A. C., J. J. BLACK, AND M. F. GUNDERSON. 1962b. Staphylococci in competition. II. Effect of total numbers and proportion of staphylococci in mixed cultures on growth in artificial culture medium. Appl. Microbiol. **10**:23-30.
- PETERSON, A. C., J. J. BLACK, AND M. F. GUNDERSON. 1962c. Staphylococci in competition. III. Influence of pH and salt on staphylococcal growth in mixed populations. Bacteriol. Proc., p. 33.
- PETERSON, A. C., J. J. BLACK, AND M. F. GUNDERSON. 1962d. Staphylococci in competition. IV. Effect of starch and kind and concentration of sugar on staphylococcal growth in mixed populations. Bacteriol. Proc., p. 33.
- STRAKA, R. P., AND F. M. COMBS. 1952. Survival and multiplication of *Micrococcus pyogenes* var. *aureus* in creamed chicken under various holding, storage and defrosting conditions. Food Res. **17**:448-455.