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Effect of Coliform and *Proteus* Bacteria on Growth of *Staphylococcus aureus*[1]

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ABSTRACT

DIGIACINTO, J. V. (University of Wisconsin, Madison), AND W. C. FRAZIER. Effect of coliform and *Proteus* bacteria on growth of *Staphylococcus aureus*. Appl. Microbiol. **14**:124–129. 1966.—Cultures of coliform and *Proteus* bacteria, mostly from foods, were tested for their effect on growth of *Staphylococcus aureus* in Trypticase Soy Broth. Inhibition of the staphylococcus by these competitors increased with increasing proportions of inhibiting (effector) bacteria in the inoculum and decreasing incubation temperatures (37 to 10 C). Time required for 2×10^4 staphylococci to increase to 5×10^6 cells per milliliter, the minimal number assumed to be necessary for food poisoning, varied with the species of effector, the original ratio of effector bacteria to staphylococci in the medium, and the incubation temperature. When the original ratio was 100:1, the staphylococci did not reach 5×10^6 cells per milliliter at 10, 15, 22, or 30 C (with one exception), when growing with cultures representing six species of coliform bacteria and two of *Proteus*. When the ratio was 1:1, all effectors either greatly delayed the staphylococcus or prevented it from reaching hazardous numbers at 15 C, six of the eight caused a delay of 2 to 3 hr at 22 C, and only *Escherichia coli* delayed the coccus at 30 C. All effectors were ineffective at 22 and 30 C when original numbers of effectors and staphylococci were in the ratio 1:100. Greatest overall inhibition was by *E. coli*, *E. freundii*, and *Proteus vulgaris*, and these species were more effective than the others at 22 and 30 C. *Aerobacter cloacae* and *Paracolobactrum aerogenoides* were more effective at 15 C. In general, results were similar with different strains of a species. Except for *Aerobacter aerogenes*, *Klebsiella* sp., and *P. aerogenoides*, which apparently did not compete for nutrients, inhibition of the staphylococcus was by a combination of antibiotic substances and competition for nutrients.

Previous work (8) had indicated that spot-plate tests for inhibition or stimulation of *Staphylococcus aureus* by other bacteria were not always reliable. Organisms that were inhibitory by the spot-plate method usually were so by growth in liquid medium, but cultures that were stimulatory or without effect on spot plates often showed inhibition of the staphylococcus in liquid medium. For this reason, tests are being made with representative cultures of different groups of food bacteria. The present work is concerned with the effect of various coliform bacteria and a few *Proteus* cultures on growth of *S. aureus*.

Inhibition of *S. aureus* by coliform bacteria has been reported by Neufeld and Kuhn (12), Wynne (17), Bowling and Wynne (3), Wynne and Norman (18), Blackford, Parr, and Robbins (2), Cook et al. (4), Higgenbottom (9), Dack and Lippitz (6), Oberhofer and Frazier (13), Troller and Frazier (15), Graves and Frazier (8), and others. Inhibition by *Proteus* cultures has been reported by Dolman (7), Graves and Frazier (8), and Troller and Frazier (16).

MATERIALS AND METHODS

To 26 cultures of coliforms and one each of *P. vulgaris* and *P. morganii*, selected from those used by Graves and Frazier (8), were added stock cultures of *Escherichia coli*, *Aerobacter aerogenes*, *A. cloacae*, and *Proteus vulgaris*. The *S. aureus* cultures were strain 196E, an enterotoxigenic strain from G. M. Dack, and strain W-1, a nonenterotoxigenic mastitis strain from J. B. Wilson. All 34 effector cultures were tested for their effect on *S. aureus* 196E when grown together on Trypticase Soy Agar spot plates at 15, 22, 30, and 42 C. Also, each effector culture was grown

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with *S. aureus* 196E in Trypticase Soy Broth to determine its effect on the growth of the staphylococcus. In the preparation of inocula, both the staphylococcus and the effector organisms were grown for three successive transfers in Trypticase Soy Broth at 30 C, the first two transfers being incubated 18 hr and the third transfer 12 hr. In all experiments, the inoculum of staphylococci was about 2×10^4 cells per milliliter, and inocula of effectors were 2×10^2, 2×10^4, or 2×10^6 cells per milliliter.

Counts of *S. aureus* during the period of growth were made by means of Mannitol Salt Agar spread plates, with incubation for 48 hr at 30 C. All dilutions were in sterile 0.1% peptone solution.

RESULTS

Spot plates. Of the 11 *Escherichia* cultures tested (4 of *E. coli*, 1 of *E. intermedia*, and 6 of *E. freundii*), all but four strains of *E. freundii* inhibited *S. aureus* at 30 C. Three of the inhibitory cultures also inhibited at 15 C, one at 22 C, and none at 42 C. Six of the 10 *Aerobacter* (*A. cloacae* and *A. aerogenes*) cultures and one of the three *Klebsiella* cultures (not identified) were weakly inhibitory toward *S. aureus* at 30 C. Otherwise, the *Klebsiella* strains did not inhibit at any of the four temperatures. Two of the *A. cloacae* cultures inhibited at 15, 22, and 30 C. An *A. aerogenes* culture and one of *A. cloacae* were stimulatory. Only three of seven *Paracolobactrum aerogenoides* cultures were inhibitory, and then at both 22 and 30 C. All three *Proteus* cultures were inhibitory.

Preliminary tests in broth. All 34 effector cultures inhibited *S. aureus* in Trypticase Soy Broth at 25 C, when the ratio of effector bacteria to staphylococci in the inocula was 1:1 and counts were made after 8 and 24 hr. In general, strains of *E. coli*, *E. freundii*, and *P. vulgaris* were more effective than strains of the other species. All strains of *E. freundii*, five of nine strains of *A. cloacae*, the single strain *A. aerogenes*, and all seven strains of *P. aerogenoides* prevented the staphylococcus from reaching 5×10^6 cells per milliliter in 8 hr.

Effect of variation in incubation temperature and size of inoculum. Eight of the 34 effector cultures were selected as representative of species or genus. These cultures were tested for the effect of incubation at 15, 22, and 30 C, for the effect of ratios of numbers of effector bacteria to numbers of staphylococci in the inoculum, 2×10^4 cocci being added, and for numbers of effectors required to make ratios of effectors to cocci of 1:100, 1:1, and 100:1. Special attention was paid to the time required to reach 5×10^6 staphylococci per milliliter. This figure approximates the minimal number of *S. aureus* organisms assumed to be necessary for appreciable enterotoxin production. Minimal numbers quoted by various workers include: 100,000 to 1 million staphylococci per gram given by Dolman (7), 500,000 suggested by Allison (1), eight to hundreds of millions according to Peterson (14), hundreds of millions reported by Dack (5), and some millions estimated by Hobbs (10). Mossel (11) estimates the minimal toxic dose as 10^7 staphylococci. Low numbers in toxic food, such as 100,000 to 500,000 per gram, may not represent maximal numbers attained according to Dolman (7), for he has demonstrated that numbers may be reduced from millions to these lower levels by competing bacteria during holding of the food. Most counts on foods soon after incrimination in poisoning are in tens to hundreds of millions of staphylococci per gram; but numbers will vary, of course, with amounts and types of enterotoxin produced by different strains of *S. aureus*.

Table 1 indicates numbers of hours required for the original 2×10^4 staphylococci per milliliter, growing with each of the eight effectors, to reach 5×10^6 cells per milliliter at 15, 22, and 30 C, with ratios of effectors to staphylococci in the inocula of 1:100, 1:1, and 100:1. Growth was followed into the maximal stationary phase. Comparison with the time required by *S. aureus* alone to reach 5×10^6 cells per milliliter at 15, 22, and 30 C, namely, 32, 12, and 6 hr, respectively, shows that: (i) when the original ratio of effectors to staphylococci was 100:1, the staphylococcus was held below the hazardous number at all three temperatures, except with *A. cloacae* at 30 C, when the delay was for 92 hr; (ii) at 15 C, when the ratio was 1:1, *E. freundii*, *A. cloacae*, *P. aerogenoides*, and *P. vulgaris* kept the count below 5×10^6, and the other effectors delayed the attainment of these numbers for from 10 to 34 hr; (iii) a ratio of 1:1 at 22 C resulted in no delay in reaching hazardous numbers with *P. aerogenoides* and *P. morganii*, and a delay of 2 to 3 hr with the other effectors; (iv) at 30 C and with a ratio of 1:1, only *E. coli* appreciably delayed the staphylococcus; (v) at 15 C with a ratio of 1:100, only *E. freundii* and *P. aerogenoides* did not delay growth of the staphylococcus, and the other effectors caused delays of from 5 to 13 hr; (vi) with an original ratio of 1:100 at both 22 and 30 C, all of the effectors were ineffective in delaying increase of the staphylococci to 5×10^6 cells per milliliter.

At 10 C (not shown in table) with a 1:1 inoculum, it took 190 hr in the presence of *E. coli* and *Klebsiella* for the staphylococcus to increase to 5×10^6 cells per milliliter, a count that was not

TABLE 1. *Effect of coliform and Proteus bacteria on growth of Staphylococcus aureus 196E in Trypticase Soy Broth*

Organism	Incubation temp° C	Ratio* E:S	Hr to reach 5 × 10^6 cocci/ml	Maximal no.† of cocci/ml × 10^6	R_I
Escherichia coli	15	1:100	45	79	.32
		1:1	66	7.9	.49
		100:1	—‡	0.7	.76
	22	1:100	12.7	200	.15
		1:1	15.3	22	.35
		100:1	—	1.1	.65
	30	1:100	6.3	380	.15
		1:1	8.2	44	.36
		100:1	—	2.4	.61
E. freundii	15	1:100	32	50	.33
		1:1	—	4.5	.54
		100:1	—	0.6	.76
	22	1:100	13	160	.17
		1:1	14.7	8.8	.38
		100:1	—	0.5	.69
	30	1:100	6	700	.09
		1:1	6.3	92	.26
		100:1	—	3.7	.57
Aerobacter aerogenes	15	1:100	37	168	.21
		1:1	47	8.2	.49
		100:1	—	0.4	.74
	22	1:100	12	460	.08
		1:1	14.3	18	.34
		100:1	—	0.9	.62
	30	1:100	6	1,200	.05
		1:1	6	85	.21
		100:1	—	1.7	.52
A. cloacae	15	1:100	38	46	.32
		1:1	—	3.7	.56
		100:1	—	0.2	.83
	22	1:100	13.7	640	.10
		1:1	13.7	63	.24
		100:1	—	1.9	.56
	30	1:100	6	880	.08
		1:1	6	190	.19
		100:1	98	10	.47
Klebsiella sp.	15	1:100	37	140	.22
		1:1	45	9.9	.47
		100:1	—	0.6	.73
	22	1:100	12	347	.11
		1:1	14	18	.35
		100:1	—	0.9	.62
	30	1:100	6	1,200	.06
		1:1	6	80	.22
		100:1	—	2.3	.58
Paracolobactrum aerogenoides	15	1:100	32	46	.30
		1:1	—	4.6	.55
		100:1	—	0.5	.81
	22	1:100	12	540	.07
		1:1	12	82	.25
		100:1	—	1.8	.52
	30	1:100	6	1,300	.05
		1:1	6	88	.21
		100:1	—	3.5	.52

TABLE 1—*Continued*

Organism	Incubation temp	Ratio* E:S	Hr to reach 5 × 10^6 cocci/ml	Maximal no.† of cocci/ml × 10^6	R_I
	C				
Proteus vulgaris	15	1:100	42	27	.37
		1:1	—	2.3	.59
		100:1	—	0.2	.88
	22	1:100	12	470	.07
		1:1	15	17	.34
		100.1	—	0.6	.70
	30	1:100	6	630	.09
		1:1	6	120	.23
		100:1	—	3.5	.56
P. morganii	15	1:100	42	36	.31
		1:1	42	6.9	.46
		100:1	—	0.5	.74
	22	1:100	12	370	.08
		1:1	12	35	.25
		100:1	—	1.1	.54
	30	1:100	6	1,500	.05
		1:1	6	105	.21
		100:1	—	2.6	.52
S. aureus alone	15		32	4,000	
	22		12	1,760	
	30		6	4,000	

* Ratio E:S = no. of effector bacteria–no. of staphylococci in inoculum; 2 × 10^4 staphylococci per milliliter always added.

† Maximal number of staphylococci after 111 hr at 15 C; 34 hr at 22 C; and 17 hr at 30 C.

‡ Dash indicates 5 × 10^6 staphylococci per milliliter not attained.

reached in that period by any of the other six cultures. Inhibition was poor at 37 C.

Table 1 also indicates the numbers of staphylococci in their maximal stationary phase. At all temperatures and with all ratios of effectors to staphylococci, the numbers of the latter were held down to a count considerably below that of *S. aureus* growing alone, and again total numbers decreased with incubation temperature and percentages of staphylococci in the inocula.

The R_I value in Table 1, calculated as described by Troller and Frazier (15), is the area under the growth curve of the staphylococcus when grown with the test or effector organism (*AE*) as measured by a planimeter, divided by the area under the growth curve of the staphylococcus alone (*AC*), subtracted from 1 to give a value that varies directly with the amount of repression of growth. The formula is: $R_I = 1 - AE/AC$. The effector prevented the staphylococcus from reaching 5 × 10^6 cells per milliliter when the R_I value was 0.52 or greater. Averages of R_I values indicated that *E. coli*, *E. freundii*, and *P. vulgaris* gave the greatest overall inhibition, and that these species were more effective than the others at 22 and 30 C. On the other hand, *A. cloacae* and *P. aerogenoides* had their greatest effect at 15 C.

The time of the start of inhibition of the staphylococcus differed with the inhibiting organism, temperature, and original ratio of effectors to staphylococci. Inhibition by *E. coli* began soon and continued throughout growth. With most effectors, however, except with the 100:1 ratio and at 30 C, little inhibition took place until the staphylococci were well into their exponential phase.

Differences in the effectiveness of strains of species are shown in Table 2. For the most part, there were not great differences in results with different strains. One exception was *E. coli*, where the Gratia strain, usually considered atypical, gave the high counts; otherwise, the range was 6.6 × 10^6 to 7.3 × 10^6 cells per milliliter at 8 hr, and 24 × 10^6 to 56 × 10^6 at 24 hr. The other exception was *A. cloacae*, of which three strains were poorer inhibitors than the other six.

Tests with strain W-1 of *S. aureus* gave results similar to those with strain 196E.

Causes of inhibition. Sterile, Millipore-filtered filtrate of each of the effector cultures representing the eight species was prepared from an 18-hr,

TABLE 2. *Ranges in numbers of Staphylococcus aureus organisms growing in Trypticase Soy Broth with different strains of species of effector bacteria after 8 and 24 hr at 25 C**

Species	No. of strains	No. of staphylococci × 10^6	
		8 hr	24 hr
Escherichia coli	4	6.6–10.7	24–168
E. freundii	6	0.95–1.45	2–5.4
Aerobacter cloacae	9	1.9–8.9	10–34
Paracolobactrum aerogenoides	7	1–2.2	1.8–4.6
S. aureus alone	1	13.5	1,250

* Inoculum ratio was 1:1.

37 C culture in Trypticase Soy Broth. The filtrate was incorporated in various proportions in sterile broth, which was inoculated with *S. aureus* and incubated at 25 C. All filtrates inhibited the staphylococcus, and effectiveness increased with concentration of added filtrate. However, even when the filtrate made up as much as 10% of the culture medium, repression of the staphylococcus was inconsiderable, and more than 40% was required for marked inhibition. Apparently, action of antibiotic substances produced by an effector would be effective only after considerable growth, i.e., well along in the growth curve of the competing staphylococcus.

Evidence that competition for nutrients also was a factor in the repression of *S. aureus* was the resumption of active growth of that organism, in the presence of five of the eight effector organisms, after growth had almost ceased, when sterile broth concentrate was added. *S. aureus* alone was not affected by addition of the broth concentrate. The three cultures that showed no apparent competition for nutrients were *A. aerogenes*, *Klebsiella*, and *P. aerogenoides*.

Colicins and acid production apparently were not involved in the inhibition.

DISCUSSION

Coliform bacteria are considered undesirable in some foods because they may indicate fecal contamination from human sources. Most raw foods contain them, however, for they are part of the natural flora of green plants and of the surface and intestinal flora of animals. If conditions permit growth, they will collaborate with other food bacteria in repressing the growth of staphylococci. The staphylococci usually will be considerably outnumbered by competitors, making repression the more successful. Aiding in this repression is the fact that usually the food will be held at below usual room temperatures, where the inhibitory effect of the competitors will be enhanced. Similar considerations hold for the *Proteus* bacteria as competitors. The effect of other competitors, e.g., the lactic acid bacteria and the non-gasforming, gram-negative rods, will be reported elsewhere.

Adequate precooking of foods will kill the staphylococci, but also will destroy all or most competing bacteria, leaving a staphylococcus that recontaminates the food opportunity for unrestrained growth. This recontamination, however, may also add competitors for the staphylococcus, including coliform bacteria.

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