CONDITION OF COLIFORM ORGANISMS INFLUENCING RECOVERY OF SUBCULTURES ON SELECTIVE MEDIA[1]

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Abstract

The effects of stress of coliform bacteria resulting from exposure to heat, radiation, or sodium chloride on behavior of the progeny were studied. After exposure to stress and subsequent growth on plating media, colonies were picked by random design and grown in nutrient broth for further comparison of their ability to form colonies on plate count and violet red bile agar. After 6 hr in nutrient broth, average counts on violet red bile agar were less than half those obtained with plate count agar. Sensitivity to the selective medium was lost by repeated transfer and growth in nutrient broth or by repeated picking from the selective medium and subculture of colonies.

Cultures with moderate sensitivity to violet red bile agar were obtained from raw sewage through picking of colonies from plate count agar. Attempts to obtain a stable sensitive strain through selective enrichment were unsuccessful. A laboratory strain of *Escherichia coli*, with extreme sensitivity to violet red bile agar, however, was used to determine that tolerance was acquired by stepwise adaptation to a selective medium. Occurrence of sensitive coliform cells in nature indicates their potential importance in tests for indicator organisms of public health significance.

Coliform bacteria associated with food handling systems are of interest as indicators of organisms of public health significance. Bacteria in the microenvironment of food handling equipment are commonly subjected to stress and thus become more sensitive to their growth environment *(2, 4, 6-10, 12)*. Some are apparently "injured." Injured cells are sensitive to surface active agents in selective media normally useful for enumerating coliform bacteria *(4, 11)*.

It has been tacitly assumed that stressed cells on subsequent recovery and growth gave rise to normal cells. Simple methods for proving this assumption, however, were not available. Purposes of research reported here were to examine the hypothesis that stress treatments influence subsequent cultures and to study conditions required for the progeny to regain normal resistance to selective components of media.

Methods

Cultures

Enterobacter aerogenes and one strain of *Escherichia coli* were from the departmental culture collection. A strain of *E. coli* with extreme sensitivity to selective media for coliform determinations was obtained from the Department of Microbiology, University of Nebraska, Lincoln. The cultures were propagated in nutrient broth (NB; Difco) at 32 C for 18-24 hr and held at 3-5 C for storage.

Media

The medium which served as a standard for comparison was plate count agar (PCA; BBL or Difco). The selective medium was violet red bile agar (VRBA; BBL or Difco). Brilliant green lactose bile broth (BGLBB; Difco) was used to determine gas production and "presumptive evidence" of coliform organisms *(1)*. Minimal agar (MA) consisted of $NH_4H_2PO_4$, 0.3%; K_2HPO_4, 0.2%; iron as $FeSO_4 \cdot 7H_2O$, 0.5 ppm; $MgSO_4 \cdot 7H_2O$, 0.05%; glucose, 0.3%; agar, 1.5%. The pH was adjusted to 7.0 by the addition of 5N KOH. Solutions of glucose and $MgSO_4$ were autoclaved separately and added to the medium before plating.

Plating and enumeration

Plating and counting procedures were those recommended by the American Public Health Association *(1)*. The difference between the PCA count and the VRBA count was attributed to injured or to sensitive cells. When colonies were picked for further study it was by random design from countable plates. They were then grown for 6 hr in NB at 32 C and stored at 2 C until plate counts were made—the elapsed time never exceeded 24 hr.

Procdure for stress of cells

To obtain heat stressed cells, cultures were heated without agitation at 60 or 65 C according to a previously described method *(4)*. Obtaining stress by exposure to 5% NaCl has also been described *(4)*. Radiation stress was by exposure to cobalt-60 as described by Tiwari and Maxcy *(13)*. Each process was repeated at least three times after adjustment of conditions to obtain approximately 95-99% kill of the original culture.

Enrichment of sensitive cultures

Procedures for selective enrichment were based on the work of Lederberg and Zinder *(3)*. Growing cells are sensitive to penicillin. After destruction of penicillin by penicillinase, previously inactive cells can be made to grow.

Adaptation of a sensitive culture

A sensitive strain of *E. coli* was grown in progressive, challenging concentrations of quaternary ammonium compound in NB according to a procedure described by Maxcy et al. *(5)* to obtain a resistant strain.

Results

Injury and effect on subsequent generations

To determine if subcultures remained sensitive to selective media, colonies were picked from PCA and subcultured in NB. Presumably, if subcultures re-

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TABLE 1. THE EFFECT OF VARIOUS FORMS OF INJURY ON THE SENSITIVITY OF SUBCULTURES TO VIOLET RED BILE AGAR

Method of injury	Organism	Number of isolates observed	Per cent injured cells		
			Mean	Range	Standard deviation
Heat	*E. coli*	78	58	16-99	5
Heat	*E. aerogenes*	68	34	0-70	—
Radiation	*E. coli*	78	69	0-98	25
NaCl	*E. coli*	72	57	15-97	21

tained sensitivity, injury was to a genetically transmissible trait. Three systems of cell stress were used to study the response of *E. coli*. Results were expressed in terms of per cent of injured cells, which was the difference between the PCA and VRBA counts divided by the PCA count with the quotient multiplied by 100. From a study of 78 subcultures of heat injured *E. coli*, for example, the mean per cent of cells recovered was 58, thus indicating 42% of the cells had altered characteristics compared to the parent culture. A summary of the results with subcultures is in Table 1. The mean per cent of injured cells for the various treatments and bacteria was 34-69. These results are in agreement with results obtained with the parent culture as previously reported *(4)*. Thus the sensitive characteristic persisted through a subculture indicating the phenomenon was genetically related. Results with *E. aerogenes* were similar to those with *E. coli* as judged by data pertaining to heat stress.

Isolates showing the greatest sensitivity to VRBA were studied further in an attempt to obtain strains with a high degree of sensitivity. From the most sensitive 6-hr culture, an 18-hr subculture in NB was prepared and subjected to stress. Platings were made and 30 colonies were picked for 6-hr subcultures in NB. Comparative platings of the individual subcultures were then made on PCA and VRBA. The results indicated that the mean per cent of injured cells was similar to the original culture before the stress treatment. Thus it was not possible to obtain a further increased proportion of sensitive cells.

Stressed cells were treated with various concentrations of penicillin and for various times after which penicillinase was added to inactivate the penicillin. This system to destroy growing cells did not provide the hoped for increase in proportion of sensitive cells in the population.

To obtain data on the relative stability of sensitivity in cultures after stress treatments, subcultures were made in NB. The most sensitive cultures reverted to the normal resistance of the original stock culture within 6 daily transfers.

To determine if there was a difference in the proportion of sensitive cells from colonies growing on VRBA and on PCA, isolates were obtained from VRBA plates made to evaluate stress treatments. Subculture of these isolates for 6 hr in NB followed by plating on VRBA and PCA gave results that showed the cells had the same sensitivity toward the selective medium as when colonies taken from PCA were similarly subcultured and plated.

Cultures treated to obtain stressed cells developed various colony sizes when grown on VRBA. There was no apparent difference between small and large colonies, however, in comparative sensitivity to VRBA.

With this system of observation it was apparent that the type of injury was similar irrespective of the method of stress. Perhaps the apparent similarity resulted from the arbitrary adjustment of the stress treatment to get a 95-99% kill of the test culture.

Occurrence of VRBA-sensitive strains in nature

Samples of mixed raw sewage, representing effluent from most of the city of Lincoln, Nebraska (population approximately 150,000), were plated on PCA. Ten colonies from each of three countable plates were picked by random design into NB and into BGLBB. Those colonies producing gas in BGLBB were considered coliform organisms and observed further by using the inoculum that had been put into NB. Comparative counts from the NB were made with duplicate plates using PCA and VRBA. When the VRBA count was less than 50% of the PCA count and a repetition of the plating also indicated less than 50%, the isolate was arbitrarily considered sensitive to VRBA. From 31 samples of sewage, 102 coliforms were isolated and 6 of these were sensitive to VRBA.

Comparisons of VRBA and PCA counts on the sewage samples indicated that 9.9% of the total population was coliforms. Isolates from PCA counts were obtained by random design, and results showed 14% of the total population to be coliforms as judged by their ability to produce gas in BGLBB. A comparison of these methods indicated only 71% of the coliforms were enumerated by the VRBA method.

Gain and loss of VRBA sensitivity

A particularly sensitive strain of *E. coli* was used to determine conditions contributing to development of resistance to VRBA. A typical count for an 18-hr culture from NB was 3.2×10^8 on PCA and 6.0×10^2 on VRBA. After subculturing seven times on

VRBA, a typical count from an 18-hr culture of NB was 3.1×10^8 on PCA and 3.0×10^7 on VRBA. The adaptation process was stepwise. Counts on MA were not significantly different from those on PCA.

When the strain of *E. coli* particularly sensitive to VRBA was subcultured for 10-12 days by serial transfer in BGLBB, there was a marked loss in sensitivity to VRBA. A similar reduction in sensitivity was attained through stepwise adaptation of the sensitive parent culture to 28 mg of quaternary ammonium compound per liter of NB. Altered sensitivity to VRBA was maintained even after 5 subcultures in NB without quaternary ammonium compound.

DISCUSSION

Cell stress and altered recovery on various media is well recognized. While the mechanism is not understood, it most often has been attributed to changes in nutritional requirements, because richer media commonly yielded more cells. Auxotrophic mutants, however, have not been found. Furthermore, in the work reported here attempts to increase the proportion of sensitive cells in a culture were unsuccessful.

The effect of stress persisted through subculture, therefore, indicating a genetic relation. Conditions for isolating these strains with sensitivity as a stable factor, however, remained obscure. Since the sensitive strain from the Department of Microbiology acted as a mutant, it may be projected from our data that the sensitivity is genetically related and not nutritionally dependent. Classical methods for isolating auxotrophic cultures, as used by Postgate and Hunter (8) and Russell and Harris (9), therefore, would not be applicable. Since the sensitive culture acquired tolerance for VRBA through subculture on VRBA, growth in BGLBB, and growth in the presence of a quaternary ammonium compound, sensitivity apparently is related to surface activity.

Sensitive strains occur in nature therefore emphasizing the problem of cell stress and recovery on selective media. Sewage should not be considered an extremely adverse environment; a greater proportion of sensitive cells, therefore, might be expected from other more adverse environments. There are numerous conditions in the food industry where stress is common, and cells would remain in an adverse environment. The problem of stress and recovery of stressed cells on selective media, therefore, continues to be of interest and of public health significance.

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