Stability of Bacterial Mutants in Saline

A. M. KROPINSKI

Department of Microbiology and Immunology, Queen's University, Kingston, Ontario, Canada

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By storage in 1% NaCl, genetically characterized strains of Escherichia coli, Salmonella typhimurium, and Pseudomonas aeruginosa were stable for more than 1 year at 4°C. This method of preservation was more effective than maintenance of the strains in stab culture.

Numerous techniques for the long-term preservation of bacterial cultures have been devised. These include freezing of bacterial suspensions in the presence (8) or absence (13) of glycerol; storage in liquid nitrogen; and preservation on silica gel (7) in stab culture, on sealed agar slants (1), or by lyophilization (2, 9). In addition, fungi, yeasts, and some bacterial strains have been successfully maintained in sterile distilled water (5, 11).

We have experienced difficulties in maintaining Pseudomonas aeruginosa strains and certain mutants of Escherichia coli in stab culture at 4°C, and Antheunisse (2) observed a poor degree of recovery of P. aeruginosa strains from the lyophilized state.

Chance (3) described a method for the preservation of prototrophic bacterial cultures in 1% NaCl (saline). Using this procedure, he kept E. coli, Serratia marcescens, Bacillus subtilis, Staphylococcus aureus, and P. aeruginosa strains viable for approximately 4 years at room temperature. In this paper I describe the application of this technique to the maintenance of genetically characterized strains of E. coli, Salmonella typhimurium, and P. aeruginosa and compare the stability of the strains in saline and in stab culture.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains together with their sources and genotypes are given in Table 1.

Media. The complex media used were tryptic soy broth (TSB; Difco), nutrient broth (Difco), L broth (10), and MacConkey agar base (Difco) supplemented with 1% (wt/vol) sugars. The minimal medium was that of Davis and Mingioli (4), whereas the nutrient broth stock stab agar was that described by Miller (12).

Growth of microorganisms and preparation of stored cultures. All cultures were grown overnight at 37°C in 50 ml of TSB at 150 rpm in a water bath shaker (New Brunswick Scientific Co., model G-86). The broth cultures were inoculated by using a straight needle into stock stab agar (7 ml) in screw-capped tubes (13 by 125 mm). The agar containing the pur and pyr auxotrophs was overlayed with 0.1 ml of adenine (5 mg/ml) and uracil (5 mg/ml), respectively. After overnight incubation at 37°C, the stab cultures were also stored at 4°C.

The cultures were then harvested by centrifugation for 10 min at 6,000 x g, and the cell pellets were suspended in 250 ml of cold 1% saline. Six-milliliter samples were distributed to sterile screw-capped tubes (13 by 125 mm) and stored at 4°C.

Plate counts. The viability of the cells in saline was determined by diluting samples in TSB and plating duplicate 0.1-ml samples by the spread plate technique on TSB + 1.5% agar (TSBA) plates. After incubation at 37°C for 24 to 48 h, the colonies were counted.

The viability of the cells in the stab media was assayed by streaking out loopfuls on TSBA. When no growth occurred on these TSBA plates, 1 ml of TSB was added to the stab culture, and after overnight incubation another loopful was plated out.

RESULTS AND DISCUSSION

Rates of decrease in viability of the bacteria stored in saline at 4°C are shown in Fig. 1. None of the populations showed typical exponential death kinetics, but rather a series of death phases with different rates of decline. E. coli AB1157 performed typically (Fig. 1A). The initial degree of auxotrophy did not influence the stability of the cultures in saline. Indeed, one of the most stable mutants, E. coli CGSC4442, carries 15 known markers. Viability of this strain of E. coli and that of P. aeruginosa PAO177 proved the most stable of the cultures incubated in saline, declining by 2.9 and 2.6 logs over the study period. The numbers of viable cells of the other strains decreased 3.9 to 4.8 logs. It is of interest to note that G. Greer (personal communication) has maintained a dilute suspension of P. aeruginosa KC-IIIR in saline at room temperature for almost 2 years. In spite of the long storage time in saline, colony
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Table 1. Genetic characteristics of the bacterial cultures used

| Organism                  | Genotype                                      | Source       |
|---------------------------|-----------------------------------------------|--------------|
| *Escherichia coli* K-12 E5014 | F' lac⁺ proA⁺, B⁺/Δ (lac pro)                 | B. Magasanik |
| *E. coli* K-12 AB1157     | F⁻ argE his leu proA thy thr ara galK lacY mtl xyl str tax λ⁻ sup | CGSC*        |
| *E. coli* K-12 CGSC4518   | F⁻ argE his leu proA pyrE thy thr ara galK lacY mtl xyl str λ⁻ | CGSC         |
| *E. coli* K-12 CGSC4442   | F⁻ argH his leu pro purE thy thr gal lac mtl xyl str ton chlA λ⁺ λ⁻ | CGSC         |
| *Salmonella typhimurium* LT2 SA571 | F⁻ hisF iloA metA pyrE trpE gal strA | K. E. Sanderson |
| *Pseudomonas aeruginosa* PA0177 | FF⁻ arg⁻ ilv⁻ 202 met⁻ 28 str⁻ | B. W. Holloway |

* CGSC, Coli Genetic Stock Center.

Development on TSBA exhibited normal morphology and growth rate.

After more than 400 days of storage of the cells in saline, the viability of the saline suspensions was tested by directly plating 0.2-ml samples from five vials of each organism on TSBA. In addition, the contents of five stock vials were streaked out on TSBA. To enhance prospects for recovery of organisms present in low numbers in the stabs, 1 ml of TSB was added to each vial. These were incubated overnight and then tested for viable microorganisms. The organisms were, in general, more stable in the saline than in stock stab medium (Table 2). These results are difficult to compare since there were initially probably more cells in the saline than in the stock stabs. Auxotrophs such as *P. aeruginosa* PA0177, *S. typhimurium* LT2 SA571, and *E. coli* AB1157 were less stable in the stab medium than were the other organisms tested.

The effect of plating samples on TSBA, nutrient agar, MacConkey lactose agar, and L agar plates was examined for possible differences in recoveries. With the exception of *E. coli* CGSC4442 and 4518, higher counts were obtained with L agar than with TSBA. Viable counts for population spread on nutrient agar varied considerably, whereas the counts with MacConkey lactose agar were significantly lower with all organisms. *S. typhimurium* yielded $2.22 \times 10^8$ colony-forming units per ml when plated on TSBA but only 50 colony-forming units per ml on MacConkey lactose plates. This represented the most significant difference in recovery between the media. The reason for this phenomena is unknown but should be noted when salmonellae are isolated from natural water by selective techniques.

Twenty-five colonies of each auxotroph derived from plating out samples from saline were patched onto TSBA and replica plated onto...
TABLE 2. Comparison of the stability of bacterial auxotrophs in saline and in stock stab medium (SSM) after 410 days of storage at 4°C

| Culture      | No. of stocks tested | Viable cells recovered from: | Saline | −TSBa | +TSBa |
|--------------|----------------------|------------------------------|--------|-------|-------|
| E. coli      |                      |                              |        |       |       |
| E5014        | 5                    | 5                            | 3      | 4     |       |
| AB1157       | 5                    | 5                            | 3      | 5     |       |
| CGSC4518     | 5                    | 5                            | 1      | 1     |       |
| CGSC4442     | 5                    | 5                            | 1      | NT    |       |
| S. typhimurium | 5                   | 5                            | 1      | 1     |       |
| SA571        | 5                    | 5                            | 4      | 1     |       |
| P. aeruginosa| 5                    | 5                            | 1      | 1     |       |
| PAO177       | 5                    | 5                            | 1      | 1     |       |

* Samples were tested for viability before and after the addition of approximately 1 ml of TSB and overnight incubation at 37°C.
* NT, Not tested (positive without added TSB).

both appropriately supplemented minimal agar plates and MacConkey sugar agar plates to assay the retention of auxotrophic and sugar fermentative characteristics (see Table 1) by the strains. Survival of the storage period in saline did not eliminate the markers. In addition, the F' lac+ element in E. coli E5014 was stable.

Storage of bacterial mutants in saline appears advantageous and is now used exclusively for this purpose in our laboratory. It may be useful as well in the maintenance of type cultures for antibiotic and pyocin typing. The latter is currently under study. Whether this method is an improvement over the storage of bacteria, particularly P. aeruginosa strains (5, 6) in distilled water is not known, but storage in saline is clearly preferable to maintaining cultures in stabs. As originally suggested (3), this convenient and inexpensive method permits long-term preservation of auxotrophic bacterial cultures.

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