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Survival of plasmid-containing strains of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* in phenylmercuric nitrate and thiomersal*

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Strains of *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* harbouring plasmids that confer mercury resistance grew in nutrient broth containing concentrations of phenylmercuric nitrate (PMN) that were inhibitory to isogenic plasmid-less strains. Decimal reduction times of *P. aeruginosa* and *S. aureus* in aqueous PMN solution were also increased by the presence of plasmids. The viable count of a plasmid-containing *P. aeruginosa* strain in Davis and Mingioli's minimal medium (DM) containing 10 μg ml^{-1} PMN fell by approximately 99% after 5 h. The count then remained constant for two weeks, when growth recommenced. This pattern of death followed by growth was also observed with the *P. aeruginosa* strain in DM + 10 μg ml^{-1} thiomersal.

Plasmid-mediated mercury resistance occurs in many bacterial species, including *Staphylococcus aureus* (Richmond & John, 1964), *Escherichia coli* and *Salmonella typhimurium* (Smith, 1967), *Pseudomonas aeruginosa* (Loutit, 1970) and *Klebsiella pneumoniae* (Nakahara, Ishikawa & others, 1977). Although the genetics (Smith, 1967; Novick, 1969; Loutit, 1970) and biochemistry (Komura & Izaki, 1971; Summers & Lewis, 1973) of mercury-resistance plasmids have been studied extensively, there is little information available about their effect on bacterial survival in simple aqueous systems preserved with mercury. The viabilities of plasmid-containing (P$^+$) and isogenic plasmid-less (P$^-$) strains of *E. coli, P. aeruginosa* and *S. aureus* in complex media, in water and in minimal media containing phenylmercuric nitrate (PMN) or thiomersal have therefore been determined to see if the presence of such plasmids significantly improves bacterial survival.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli strain J5–3 *pro met* (R471–1), *Pseudomonas aeruginosa* strain PAO2 *ser* (FP2) and *Staphylococcus aureus* strain PS81 were used together with their isogenic P$^-$ derivatives. R plasmid R471–1, which is a deletion mutant of R471 (Hedges, Rodriguez-Lemoine & Datta, 1975), confers ampicillin and mercury resistance; the sex plasmid FP2 codes for mercury resistance (Loutit, 1970), and *S. aureus* strain PS81 (Bynoe, Elder & Comtois, 1956) harbours a plasmid conferring both penicillin and mercury resistance.

Media

Nutrient broth was Oxoid No. 2 (code CM67) and nutrient agar was Oxoid blood agar base (code CM55). The minimal medium used was that of Davis & Mingioli (DM) (1950), with 0·25% D-glucose as carbon source.

Spectrophotometric measurement of growth

Organisms were grown over-night in nutrient broth at 37° without shaking. 0·2 ml of these stationary phase cultures were added to 9·8 ml of nutrient broth in 1 inch diameter Bausch and Lomb spectrophotometer test tubes. These were incubated at 37° in a water bath with shaking at 120 throws min^{-1}. The absorbance of the suspension at 540 nm was measured in a Bausch and Lomb Spectronic 20 spectrophotometer at 30 min intervals. PMN was added to these growing cultures after 60 min to give final concentrations of 1 or 10 μg ml^{-1}. Overnight cultures exposed to 100 μg ml^{-1} PMN in nutrient broth were inoculated directly into this concentration at time zero.

Viability in water or in minimal medium + PMN or thiomersal

0·2 ml of unwashed overnight cultures grown at 37° in nutrient broth and subsequently cooled to 25° were added to 19·8 ml aqueous PMN solution in a 250 ml conical flask at 25°. The viability of

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the suspension was determined by diluting samples in nutrient broth and plating on nutrient agar + 0·1% thioglycollic acid. Organisms exposed to PMN or thiomersal in DM medium were treated similarly. *E. coli* strain J5–3 *pro met* was inoculated into DM medium containing PMN and supplemented with 40 μg ml⁻¹ L-proline and 50 μg ml⁻¹ DL-methionine. DM medium for *P. aeruginosa* strain PAO2 *ser* was supplemented with 40 μg ml⁻¹ L-serine. *S. aureus* strain PS81 was inoculated into unsupplemented DM medium.

RESULTS

Growth in complex media

Resistance levels were initially determined by streaking P^+ and P^- strains on nutrient agar containing 1, 10 or 100 μg ml⁻¹ PMN. The highest PMN concentration permitting growth of *S. aureus* strain PS81 was 1 μg ml⁻¹. *E. coli* strain J5–3 (R471–1) grew on 10 μg ml⁻¹ PMN and *P. aeruginosa* strain PAO2 (FP2) was not inhibited by 100 μg ml⁻¹ PMN. No growth of the respective isogenic P^- derivatives occurred under these conditions. Resistance levels were confirmed by spectrophotometric measurement of cultures growing in nutrient

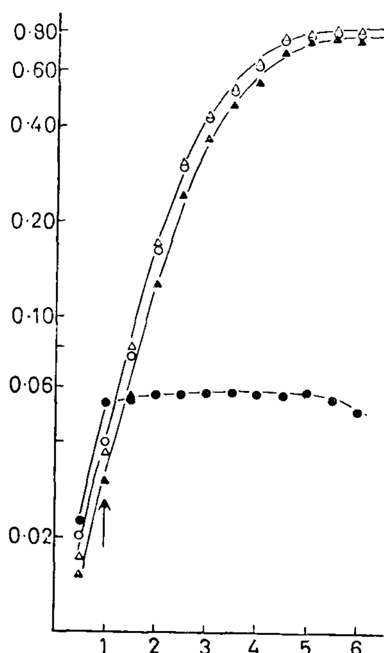

FIG. 1. Resistance of P^+ *S. aureus* strain to 1 μg ml⁻¹ PMN in nutrient broth. ○ and △ = control cultures of P^- and P^+ strains respectively in absence of PMN. ● and ▲ = P^- and P^+ strains respectively in presence of PMN added after 1 h (indicated by arrow). Ordinate: Absorbance at 540 nm. Abscissa: Time (h).

broth. PMN at 1 μg ml⁻¹ immediately inhibited growth of the P^- *S. aureus* strain PS81–Hg^s, whereas the growth rate of the P^+ strain PS81–Hg^r in nutrient broth + 1 μg ml⁻¹ was the same as a control culture growing in drug-free broth (Fig. 1). Addition of PMN at 10 μg ml⁻¹ inhibited growth of *E. coli* strain J5–3 (R471–1) for 1 h, after which time the culture commenced growing at the control, uninhibited rate (Fig. 2). No growth of the isogenic

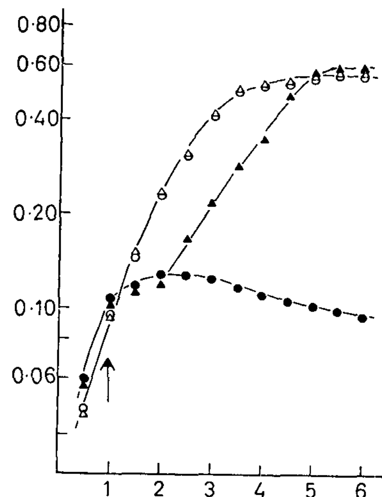

FIG. 2. Resistance of P^+ *E. coli* strain to 10 μg ml⁻¹ PMN in nutrient broth. Symbols and axes as in Fig. 1.

P^- *E. coli* strain occurred in 10 μg ml⁻¹ PMN (Fig. 2). The presence of 100 μg ml⁻¹ PMN in nutrient broth had no effect on the growth rate of *P. aeruginosa* strain PAO2 (FP2) (Fig. 3), whereas the P^- mercury-sensitive strain was completely inhibited under the same conditions (Fig. 3).

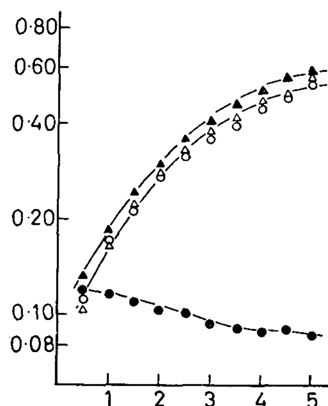

FIG. 3. Resistance of P^+ *P. aeruginosa* strain to 100 μg ml⁻¹ PMN in nutrient broth. Symbols and axes as in Fig. 1. PMN was present at time zero.

Death in simple aqueous systems

The presence of plasmid R471–1 had little effect on the survival of *E. coli* strain J5–3 in aqueous $10\,\mu g\,ml^{-1}$ PMN solution (Fig. 4). The survival level of the P^+ strain was three- and tenfold higher than the P^- strain after 2 and 3 h respectively, but there was little difference in viable count after 4 or 5 h exposure. No viable organisms of either strain were recoverable after 24 h. However, the presence of mercury-resistance plasmids in both the *P. aeruginosa* and *S. aureus* strains significantly increased survival in aqueous $10\,\mu g\,ml^{-1}$ PMN solution (Fig. 5): the decimal reduction time (D value) of the *S. aureus* strain was increased from 140 to 300 min and that of the *P. aeruginosa* strain from 70 to 115 min by the presence of the plasmids (Fig. 5). A comparison of the results in Figs 4 and 5 with the minimum inhibitory concentration values obtained on nutrient agar or in nutrient broth show that whereas *S. aureus* was the organism most sensitive to PMN in complex media, it was the most resistant of the three organisms when tested in aqueous PMN solution.

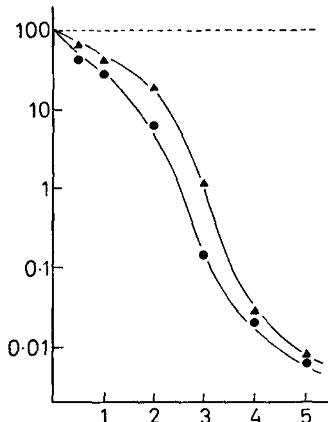

FIG. 4. Survival of P^+ (▲) and P^- (●) *E. coli* strains in aqueous $10\,\mu g\,ml^{-1}$ PMN solution. Dashed line is survival of P^+ and P^- strains in water. Ordinate: % survivors. Abscissa: Time (h).

Survival in minimal media

The survival of *E. coli* strain J5–3 *pro met* in DM medium + $10\,\mu g\,ml^{-1}$ PMN and supplemented with proline and methionine was similar to that in aqueous $10\,\mu g\,ml^{-1}$ PMN solution (Fig. 4); the presence of R plasmid R471–1 made little difference and no viable organisms were detected after 24 h. The *S. aureus* strains, which are highly resistant to PMN in water (Fig. 5), were even more resistant in DM basal medium. There were 70 and 57% survivors of the Hg^r and Hg^s strains respectively after 5 h in DM, compared with 23 and 4·1% after the same time in $10\,\mu g\,ml^{-1}$ PMN in water. 33% of the Hg^r strain were still viable after 24 h in DM basal medium + $10\,\mu g\,ml^{-1}$ PMN, compared with 1·1% of the Hg^s *S. aureus* strain. Thus, although *S. aureus* will not grow in unsupplemented DM medium, its survival is significantly increased in this phosphate-buffered system compared with that in water (Fig. 5). The P^- *P. aeruginosa* strain

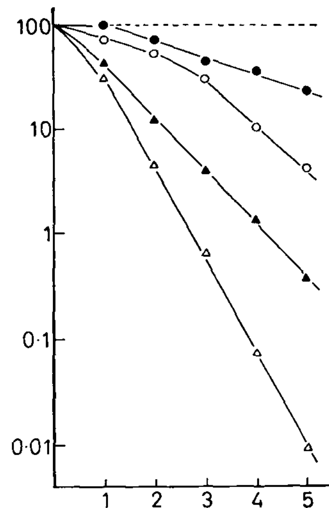

FIG. 5. Survival of P^+ (▲) and P^- (△) *P. aeruginosa* strains and P^+ (●) and P^- (○) *S. aureus* strains in aqueous $10\,\mu g\,ml^{-1}$ PMN solution. Dashed line as in Fig. 4. Ordinate: % survivors. Abscissa: Time (h).

had a D value of approximately 60 min in DM + $10\,\mu g\,ml^{-1}$ PMN and no viable organisms were recoverable after 24 h or on repeated sampling during the next 5 weeks (Fig. 6). However, the viability of *P. aeruginosa* strain PAO2 (FP2) decreased from 6×10^7 to $1 \times 10^5\,ml^{-1}$ after 5 h, but after 24 h there was no further fall in viability. The count remained nearly constant for 2 weeks, when viability increased to reach 2×10^8 and $4 \times 10^8\,ml^{-1}$ after 3 and 5 weeks respectively (Fig. 6). A similar pattern of death followed by growth was observed with the P^+ *P. aeruginosa* strain in DM + $10\,\mu g\,ml^{-1}$ thiomersal (Fig. 7), but in this case the count rose to $2 \times 10^8\,ml^{-1}$ after only 96 h. No viable cells of the isogenic P^- strain were recoverable after 48 h under the same conditions (Fig. 7).

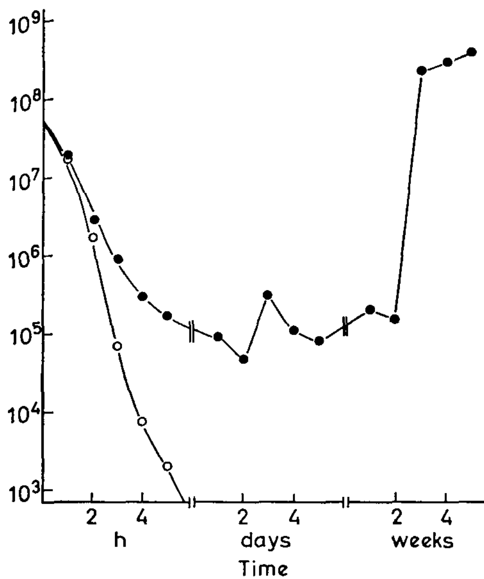

FIG. 6. Survival of P^+ (●) and P^- (○) *P. aeruginosa* strains in DM minimal medium + 10 μg ml^{-1} PMN. Ordinate: Viable count ml^{-1}.

DISCUSSION

Tonomura, Maeda & others (1968) showed that a mercury-resistant strain of Pseudomonas vapourized mercury from phenylmercuric acetate. The mechanism of resistance to mercuric chloride in strains of *E. coli* carrying plasmids has also been shown to be due to the conversion of Hg^{2+} to volatile metallic mercury, which is eliminated from the culture medium as mercury vapour (Komura & Izaki, 1971; Summers & Silver, 1972). Further work by Summers & Lewis (1973) demonstrated that strains of *E. coli*, *S. aureus* and *P. aeruginosa*, which all carried genes determining mercury resistance on naturally occurring plasmids, also converted mercuric chloride to a volatile form of mercury. Thus the ability to volatilize mercury seems to be the general mechanism of resistance determined by plasmid-borne genes.

Active removal of metallic mercury would account for the regrowth, after death, of the *P. aeruginosa* strain PAO2 (FP2) in DM medium + PMN (Fig. 6) or thiomersal (Fig. 7). The plasmid enables sufficient of the cells to survive and actively detoxicate the mercuric compound until its concentration is below the minimum inhibitory level. The surviving cells can then grow in the nutrient DM medium. Although *E. coli* strain J5–3 will grow in drug-free DM medium containing its auxotrophic requirements (proline and methionine), no regrowth of the P^+ *E. coli* strain was observed in such DM medium + PMN during a 5 week sampling period. This difference in sensitivities of the two Gram-negative rods is probably a reflection of both the intrinsically higher mercury resistance of the Pseudomonas compared with the *E. coli* strain (Figs 2 and 3) and also the ability of the free-living Pseudomonad to survive better than the enteric Escherichia species in a simple aqueous system. On this basis it is possible to predict that plasmid-containing strains of other water-borne genera, such as Klebsiella

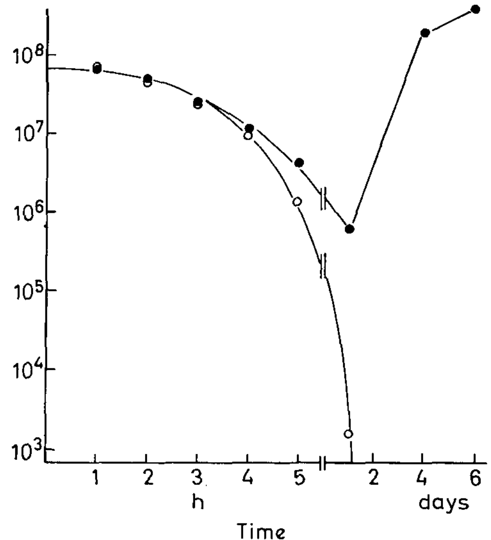

FIG. 7. Survival of P^+ (●) and P^- (○) *P. aeruginosa* strains in DM minimal medium + 10 μg ml^{-1} thiomersal. Ordinate: Viable count ml^{-1}.

and Alcaligenes, might also be highly resistant to mercuric preservatives in minimally nutrient medium. It is also relevant that once the concentration of mercury in a system has been reduced, for example by a P^+ Pseudomonas strain, it may lose its inhibitory capacity for other species.

Plasmids, therefore, increase the mercury resistance levels of *E. coli*, *P. aeruginosa* and *S. aureus*. Minimum inhibitory concentrations in complex media are increased, death rates are decreased in aqueous systems, and perhaps most alarmingly, in the case of *P. aeruginosa* in minimally nutrient medium, death may be followed by growth. Since mercury-resistance is often linked to antibiotic resistance determinants on the same plasmid, pathogenic organisms that survive in systems preserved with mercury, may also be resistant to chemotherapy.

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