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Mercury and Cadmium Resistances Mediated by the Penicillinase Plasmid in *Staphylococcus aureus*

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Resistance of *Staphylococcus aureus* mediated by the penicillinase (Pc-ase) plasmid to divalent metal ions of Hg and Cd was found to be controlled by different mechanisms. The Hg resistance of the Pc-ase plasmid-carrying organisms is based upon a process of changing the ion incorporated in the cell into a somewhat innocuous form. This process is independent of temperature and seems to be controlled by an inducible enzyme. The killing effect of Hg salts was not influenced by the coexistence of other di- or monovalent ions such as $MgCl_2$, $CaCl_2$, $MnCl_2$, and NaCl. No vaporization of Hg, which explains the resistance mechanism such as that proposed by Komura et al. for R factor-mediated Hg resistance in enterobacilli, was found in the case of Hg resistance in staphylococci. On the other hand, the resistance to Cd ion is mediated by some protective mechanism to retain the ion outside the cell. Pc-sensitive organisms not carrying the Pc-ase plasmid incorporate Cd ions into the cells, whereas the Pc-ase plasmid-carrying organisms do not. The incorporation of this ion is temperature dependent and does not take place at 4 C. When incubated with this ion at 4 C, Pc-sensitive organisms as well as Pc-resistant organisms are also able to show a resistance. The addition of $CaCl_2$ could eliminate the killing effect of $CdCl_2$ with a dose-effective response.

It has been known since the report by Novick et al. that the penicillinase (Pc-ase) plasmid in *Staphylococcus aureus* also carries genes determining resistance to several heavy metal ions (13, 14) as well as those to erythromycin and other antibiotics (12). The authors have been especially interested in the resistances to these metal ions, not only from the point of view of microbial genetics but also from the medical aspects. Most of these metal salts have recently been listed as established or possible causes of environmental pollution. For example, in Japan organic mercury salts are known to cause the "Minamata disease" (6, 19, 23), and cadmium salts are found to be responsible for the "Itai-Itai disease" (4, 22, 24).

Plasmid-mediated resistances to these heavy metals have also been observed in entric bacilli, especially in R factor-carrying organisms (16), and have recently been studied by several workers (7, 8, 17, 18, 20, 21). Nevertheless, the mechanisms of resistance to heavy metal ions in staphylococci have not been studied as much as those in enteric bacilli or the mechanisms of staphylococcal penicillin resistance.

The present paper is a preliminary report of studies on the mechanisms of resistance to mercury and cadmium salts mediated by Pc-ase plasmid in *S. aureus*.

We have been able to show that these two resistances were based upon quite different mechanisms through comparative studies on the Pc-ase plasmid-carrying and the -noncarrying organisms of the same strain. Characteristics of these resistances as well as differences found between them will be described.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* 248βH is one of the stock cultures in our department and has been employed in previous studies (9, 10). It produces a beta hemolysin, a coagulase, a fibrinolysin, and a deoxyribonuclease, but no alpha hemolysin, and ferments mannitol. It carries Pc-ase plasmid and is resistant to penicillin but not to erythromycin. This Pc-ase plasmid also has genes determining resistances to mercury, cadmium, arsenic, lead, and zinc ions, as well as the gene controlling production of Pc-ase. This strain will hereafter be abbreviated Pc^r.

A plasmid-negative segregant of 248βH, Pc^s, was obtained from this Pc^r strain after treatment of the organism with ethidium bromide. An overnight culture of Pc^r strain in nutrient broth containing 0.5 μg of ethidium bromide (Boots Pure Drug Co., Ltd., England) per ml was washed three times with broth and

then plated on nutrient agar plates containing 0.3% soluble starch and 0.5 U of staphcillin (Banyu Pharm. Co., Ltd., Tokyo) per ml. Iodine-potassium iodide solution containing 20,000 U of penicillin per ml was poured onto the colonies produced on the plates, according to an iodometric procedure (2, 3), for examination of the Pc-ase production. Colonies producing Pc-ase were surrounded by colorless, clear halos on a dark-blue background, but Pc-ase-deficient colonies were not. Isolates from these latter colonies were examined for their resistance to heavy metal ions and for phage patterns. Strains which lost all features mediated by the Pc-ase plasmid and retained all other characteristics possessed by the original Pc^r strain were established as the Pc^s segregant strain of 248βH.

These Pc^r and Pc^s strains were exclusively employed in this study.

Chemicals. $HgCl_2$, $CdCl_2$, $MgCl_2$, $MnCl_2$, and NaCl were all dissolved in distilled water, sterilized by means of a membrane filter (Millipore Corp., pore size 0.45 μm), and added to a previously sterilized culture medium in designated concentrations.

Culture. All experiments were conducted with cells from the late exponential or the early stationary phase of cultures grown aerobically in nutrient broth (10 g of Lender meat extract, 10 g of Eiken peptone, and 2 g of NaCl per liter) at 37 C with shaking. Numbers of living cells were indicated as colony-forming units (CFU).

Test. Screening tests for heavy metal resistance were carried out with blank 4-mm diameter disks as in the sensitivity tablet method (Eiken Co., Ltd.). Disks were impregnated with 0.02 ml of a metal salt solution of a desired concentration, dried, and then placed on the agar plates that had been seeded previously with overnight cultures of the strain to be tested. Pc-ase activity was estimated by the iodometric assay procedure.

Measurement of inorganic salts. Quantitative measurements of inorganic salts were done by atomic absorption spectrophotometry. An equal volume of concentrated HCl solution was added to the whole bacterial culture or the culture medium containing the inorganic salt to be tested. Ampoules containing the mixture were sealed under vacuum and heated at 100 C for 2 h to completely hydrolyze the bacterial cells. Culture filtrates or culture media were also treated in the same manner. Concentrations of inorganic salts contained in these treated samples were measured with a Hitachi 207 atomic absorption spectrophotometer.

RESULTS

Susceptibilities of Pc^s and Pc^r strains of 248βH to $HgCl_2$ and $CdCl_2$. Bacterial suspensions of Pc^s and Pc^r (about 10^5 organisms per ml) were inoculated into broths containing various concentrations of $HgCl_2$ or $CdCl_2$. The CFUs obtained after overnight culture at 37 C were estimated. Results with $HgCl_2$-broth and $CdCl_2$-broth are indicated in Fig. 1 and 2, respectively. The maximal concentration of $HgCl_2$ under which Pc^r strain was able to grow was 20 μg/ml, 10 times as much as that for Pc^s

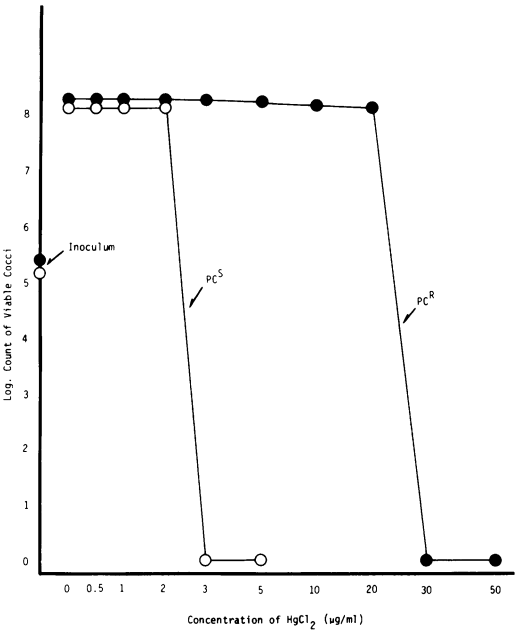

FIG. 1. *Resistance of Pc^r and Pc^s strains of 248βH to $HgCl_2$. Symbols: O, log counts of viable organisms of strain Pc^s (S. aureus 248βH) when cultured overnight at 37 C in broths containing various concentrations of $HgCl_2$; ●, Pc^r strain cultured in the same media. In both cases the initial inocula were adjusted to a level of about 10^5 organisms per ml.*

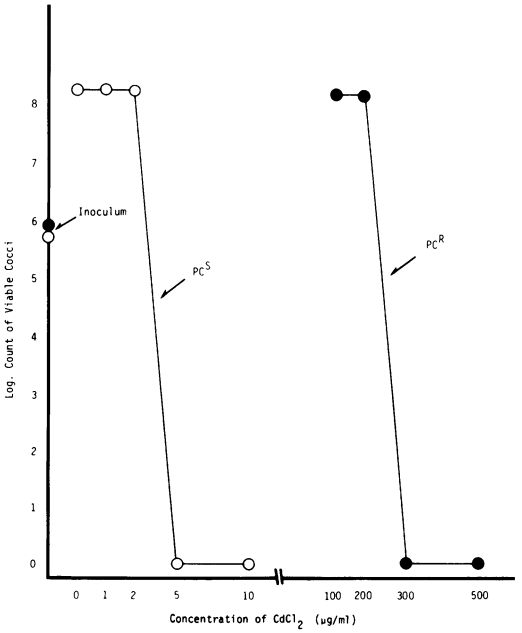

FIG. 2. *Resistance of Pc^r and Pc^s strains (S. aureus 248βH) to $CdCl_2$. The same symbols are used as in Fig. 1. The resistance of Pc^r strain to $CdCl_2$ is shown to be 100 times that of Pc^s strain.*

strain, whereas the maximal concentration of $CdCl_2$ for the growth of Pc^r strain was 200 μg/ml, corresponding to 100 times that for the Pc^s strain.

Growth curves of Pc^s and Pc^r strains in $HgCl_2$-broth and $CdCl_2$-broth. The Pc^r strain was cultured at 37 C in broths containing the respective half concentrations of the maximal doses of these heavy metal salts allowed for the growth of this strain. CFUs were followed up at various intervals (Fig. 3). In $CdCl_2$-broth, the Pc^r strain was able to grow with almost the same curve of CFU as that obtained in the control broth culture. However in $HgCl_2$-broth, the CFU of this strain showed a rapid and steep decrease down to about 10^{-5} of the initial inoculum during the first several hours, and then turned and gradually increased to the same level as that in the control broth culture after 30 h. It formed a characteristic V-shaped pattern, different from the growth curve of this strain in $CdCl_2$-broth. It was shown that such a V-shaped pattern of CFU curve did not result from a selected growth of resistant mutants, since the same pattern was observed when the grown cells were washed and again inoculated into the same $HgCl_2$-broth.

Influence of temperature on $HgCl_2$ and $CdCl_2$ resistance. Pc^r and Pc^s, respectively, were inoculated into broths containing 10 μg of $HgCl_2$ or 100 μg of $CdCl_2$ per ml, and incubated at 37 and 4 C. Figure 4 indicates the time courses of CFU change in $HgCl_2$-broth. The marked difference of resistance between Pc^r and Pc^s cells is manifested only at the 37 C incubation, and the typical V-shaped pattern can be

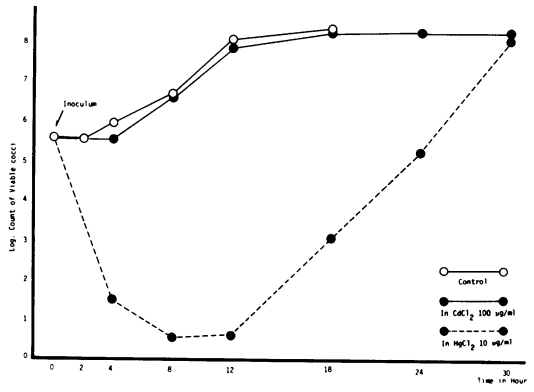

FIG. 3. *Growth curve of Pc^r strain in the usual broth, $CdCl_2$ (100 μg/ml) broth, and $HgCl_2$ (10 μg/ml) broth. The concentrations of the heavy metal salts correspond to the respective half doses of the maximal concentration in which Pc^r cells are able to grow. A characteristic V-shaped pattern can be seen in the growth curve of this strain in $HgCl_2$-broth, but not in the cure for $CdCl_2$-broth.*

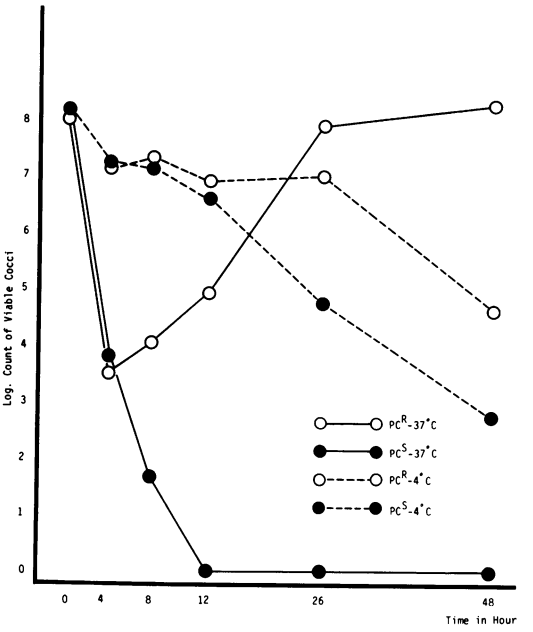

FIG. 4. *Fates of Pc^s and Pc^r cells incubated in $HgCl_2$ (10 μg/ml) broth at 37 and 4 C. The difference in resistance to $HgCl_2$ between Pc^r and Pc^s is only observed at 37 C. Under the condition of 4 C both Pc^s and Pc^r cells are killed, the latter with a more gradual pace.*

seen in the CFUs of the former cells but not in those of the latter cells. However, in the incubation at 4 C a gradual decrease of CFU is observed in Pc^r strain as well as Pc^s strain, although the former seems to be slightly more resistant than the latter. It should be noticed that $HgCl_2$ could kill both strains of Pc^s and Pc^r under this cold temperature.

On the other hand, in the case of $CdCl_2$-broth (Fig. 5), a marked difference of resistance between Pc^s and Pc^r is also indicated in the incubation at 37 C but not at 4 C. In addition, under the condition of 4 C, the CFUs of Pc^s cells did not decrease and formed a plateau line similar to that for the Pc^r cells.

These experimental results indicate a temperature dependency in the resistance of Pc^r to $HgCl_2$ and in the susceptibility of Pc^s to $CdCl_2$.

Changes of concentrations of $HgCl_2$ and $CdCl_2$ in the culture medium during bacterial growth. In the first place, the possibility of Hg vaporization from the whole culture system, which has recently been proposed by some workers as a major factor of mercury resistance in R factor-bearing enterobacilli or in plasmid-bearing staphylococci, was examined. Pc^r strain was cultured in broth containing a sublethal dose of $HgCl_2$ at 37 C. Total quantities of $HgCl_2$

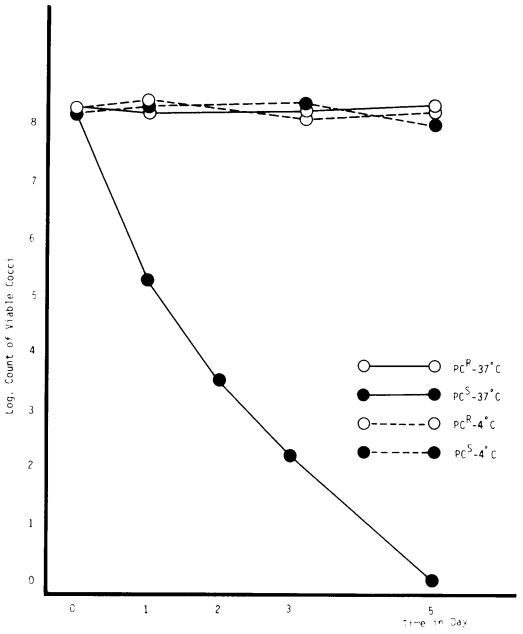

FIG. 5. *Fates of Pc^s and Pc^r cells incubated in $CdCl_2$ (100 μg/ml) broth at 37 and 4 C. The decrease of CFU is only observed in Pc^s cells incubated in this medium at 37 C, and the killing effect of $CdCl_2$ on Pc^s cells did not manifest itself at 4 C.*

contained in the initial culture medium and in the whole cultures of Pc^r after various intervals were measured by atomic absorption spectrophotometry as described above. No significant changes or differences were detected in the quantities of $HgCl_2$ in the culture medium or in cultures before and after incubation with Pc^r cells.

The distribution of Hg and Cd ions in the culture medium inoculated with Pc^r or Pc^s organisms was examined at various time intervals. Viable organisms of Pc^r and Pc^s with about 10^8 CFUs per ml were inoculated into the broths containing $HgCl_2$ (0.5 μg/ml) and $CdCl_2$ (10 μg/ml), and incubated at 37 C for 24 h. Quantities of $HgCl_2$ and $CdCl_2$ in the supernatants from these cultures and the CFUs of both strains were measured at various intervals.

The CFU curves of these two strains formed almost parallel plateau lines, but the quantities of $HgCl_2$ in the respective supernatants fell rapidly during the first 4 h and then gradually decreased to below 10% of the initial value after 24 h (Fig. 6).

On the other hand, a decrease in the concentration of $CdCl_2$ was observed in the culture supernatant of strain Pc^s, but not in that of strain Pc^r (Fig. 7).

The results indicated in Fig. 7 suggest that Pc^r cells would possess a certain kind of capability for resistance to $CdCl_2$, mediated by a specific recognition mechanism for this harmful metal ion, whereas Pc^s cells would incorporate this ion instead of some other essential ion because of the lack of this recognition mechanism.

To examine this possibility, we added several divalent cations to the broths containing 50 μg of $CdCl_2$ and 10 μg of $HgCl_2$ per ml, respectively, and examined their influences on the killing of Pc^s organisms by Cd or Hg ions. No significant influences were observed upon the addition of $MnCl_2$ and $MgCl_2$, but a certain dose-responsive competitive influence was observed with the addition of $CaCl_2$ (Fig. 8). On the other hand, the killing effect of $HgCl_2$ was not influenced by any of these ions (Fig. 9).

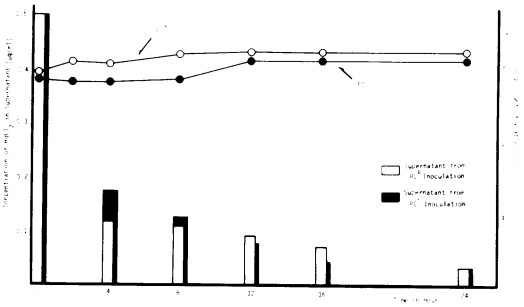

FIG. 6. *Changes of $HgCl_2$ concentration in the culture supernatant and colony-forming units in the whole culture when Pc^s and Pc^r cells are cultured in $HgCl_2$-broth at 37 C.*

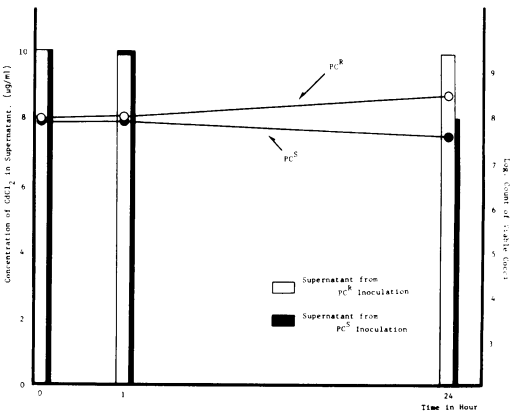

FIG. 7. *Changes of $CdCl_2$ concentration in the culture supernatant and colony-forming units in the whole culture when Pc^s and Pc^r cells are cultured in $CdCl_2$-broth at 37 C.*

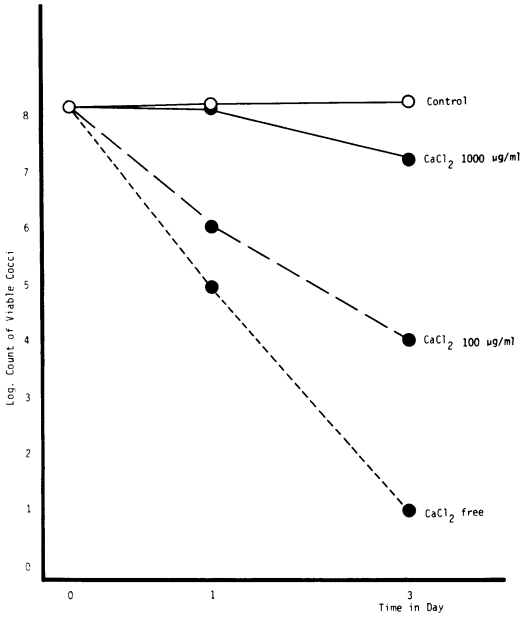

FIG. 8. *Protective effect of $CaCl_2$ on the death of Pc^s strain caused by $CdCl_2$ (50 µg/ml). Symbols: O, CFUs recovered from the culture of Pc^s strain in the usual broth; ●——●, those in the $CdCl_2$ (50 µg/ml) broth added with $CaCl_2$ (1,000 µg/ml); ●— —●, those in the $CdCl_2$ broth added with $CaCl_2$ (100 µg/ml); and ●- - -●, those in the $CaCl_2$ free broth.*

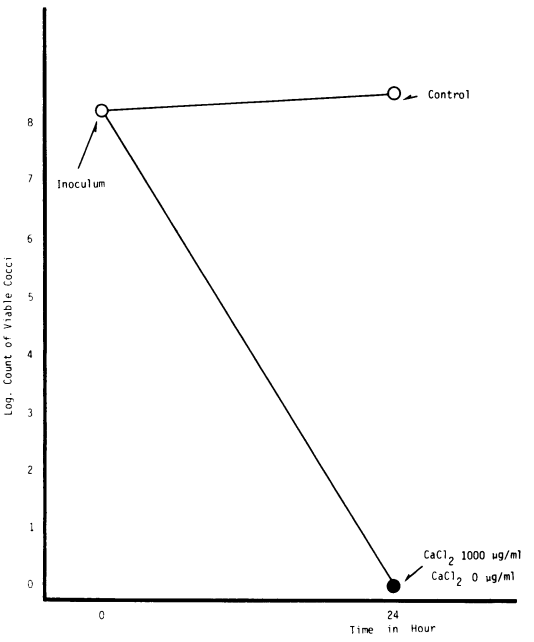

FIG. 9. *Effect of $CaCl_2$ on the death of strain Pc^s caused by $HgCl_2$ (10 µg/ml). Symbols: O, CFU (in log count) of strain Pc^s cultured in the usual broth; ●, those cultured in $HgCl_2$ (10 µg/ml) broth free from and added with $CaCl_2$ (1,000 µg/ml). No protecting effect of $CaCl_2$ was observed on the death of strain Pc^s cultured in $HgCl_2$ broth.*

DISCUSSION

Nothing is yet known about the origin or raison d'être of Pc-ase plasmids. Nevertheless, it is of interest that many genes which determine resistances to environmental substances that could be noxious for this organism, at least under the existing environmental conditions, are also located on the Pc-ase plasmids.

In general, the possible resistance mechanisms of microorganisms to environmental poisons could be classified as follows: (i) the detoxication of noxious substances by extracellular enzymes outside the bacterial cells, e.g., penicillin-resistant staphylococci can produce penicillinase and discharge it outside the cells; (ii) the inhibition of transfer of toxic substances into the bacterial cells by a conformational change in the cell membranes resulting in their impermeabilities for the respective toxic substances (the mechanism of Cd resistance of Pc^r should be classified in this category); (iii) the detoxication of noxious substances introduced into the bacterial cells by some intercellular mechanism which somehow changes them into innocuous forms (the mechanism of resistance of Pc^r to Hg ions would be considered to belong to this category, although it is yet unknown into what forms the Hg ions will finally be changed); (iv) removal of the invading toxic substances outside the bacterial cells. The R factor in enterobacilli also has been reported by Smith (16) to carry the gene determining mercury resistance. Komura et al. (7, 8) and Summers et al. (17, 18) proposed the vaporization of $HgCl_2$ by the R factor-carrying enterobacilli as the essential mechanism for their resistance to this divalent mercury ion. On the other hand, Tomura et al. (20, 21) reported the existence of a metabolic system in pseudomonads for changing $HgCl_2$ into metallic mercury. Such a vaporization system could be considered as one example in the fourth category of bacterial protection.

As far as the results obtained in the present study are taken into consideration, the killing effects of $CdCl_2$ on staphylococci seem to be much different from those of $HgCl_2$, and the resistance mechanisms of staphylococci to the cadmium ions differ from those to the mercury ions.

Hg ions are rapidly transferred into bacterial cells, and more than 90% of the ions are

removed from the culture supernatant after 24 h when the broth containing $HgCl_2$ inoculated with Pc^s or Pc^r cells is incubated at 37 C (Fig. 6). Similar transfer of these ions into cells was also observed in the incubation of the same medium at 4 C, although with much more gradual pace. On the other hand, the resistance of Pc^r to $HgCl_2$ can only be manifested at 37 C (Fig. 4).

Thus, the resistance mechanisms of Pc^r to $HgCl_2$ are considered to work at 37 C after the mercury salts are incorporated into the bacterial cells. This is in marked contrast to the results with $CdCl_2$. Cadmium ions were transferred only into Pc^s cells, but not Pc^r cells, under the 37 C conditions, whereas neither Pc^s nor Pc^r cells incorporated the ions under the 4 C conditions. The $CdCl_2$ killing effects of Pc^s cells are considered to be unable to manifest at 4 C due to the incapability of this metallic salt of penetrating the resting staphylococcal cells. It was also confirmed that dead cells of strains Pc^r or Pc^s heated at 121 C for 15 min could not remove this metallic salt from the medium when incubated at 37 C.

It is of much interest that $CaCl_2$ could prevent the killing effects of $CdCl_2$ in proportion to the concentration of the added salt. Moreover, this competitive action of $CaCl_2$ was not observed at 4 C. Perhaps Pc^s cells would be unable to distinguish between a harmful Cd ion and a physiological Ca ion, whereas some mechanism of differential recognition of the harmful Cd ion would be present in Pc^r cells.

Evidence that Cd ions do not penetrate the cells of Pc^r was also supported by the results of the following additional experiment. The supernatant of the culture of Pc^r in $CdCl_2$ (2 μg/ml) broth was heated and again inoculated with Pc^s cells in order to determine the concentration of the remaining $CdCl_2$. The results indicated that the concentration of $CdCl_2$ initially added to the medium did not decrease below 2 μg/ml, the minimal inhibitory concentration of the salt to this strain, since the Pc^s cells inoculated in this supernatant medium were not able to grow.

Taking all this evidence into consideration, there are two possible explanations for the protection mechanisms in Pc^r cells to the penetration of Cd ions. (i) Some conformational changes of the cytoplasmic membrane are mediated by Pc-ase plasmid, and consequently the cells become impermeable to Cd ions. (ii) Some differential recognition mechanisms are provided by the Pc-ase plasmid in Pc^r so that the cells can prevent the penetration of Cd ions, by distinguishing these harmful ions from Ca ions, one of the vital ions for most microorganisms. Specific binding proteins for the Cd and Ca ions are known, for example, in rat testis (15) and chicken intestinal mucosa (25). It should also be recalled that, with respect to animal cells, Cd ions are known to be effective as uncouplers of the oxidative phosphorylation in mitochondria (5, 11). Since animal mitochondrial membranes and bacterial cytoplasmic membranes are similar, it may be possible that Cd ions transferred into staphylococcal cells would also uncouple the oxidative phosphorylation in their cytoplasmic membranes, resulting a gradual death of these microorganisms.

The V-shaped pattern of the CFU curve observed in Pc^r strain which was cultured in $HgCl_2$-broth seems to suggest that the resistance mechanisms of this strain to $HgCl_2$ would be inducible ones. This is also supported by the additional experiments since the Pc^r cells which are preincubated with low concentrations of $HgCl_2$ were prevented from the early death observed in Pc^r cells directly inoculated into broth containing the salt.

Additional experiments should be conducted for the examination of these possibilities as well as for the elucidation of the essential nature of cadmium and mercury resistance mechanisms of staphylococci.

ACKNOWLEDGMENT

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