INDUCTION AND MECHANISMS OF ARSENITE RESISTANCE
IN PSEUDOMONAS PSEUDOMALLEI

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ABSTRACT

ARIMA, KEI (University of Tokyo, Tokyo, Japan), and MICHIKO BEPPU. Induction and mechanisms of arsenite resistance in Pseudomonas pseudomallei. J. Bacteriol. 88:143-150. 1964.—Pseudomonas pseudomallei strain 54, able to grow in the presence of 2 × 10^{-3} \text{M} arsenite, was isolated from soil. After a short lag period, it grew at a normal growth rate. In the organisms grown with 10^{-3} \text{M} arsenite, oxidation of \( \alpha \)-ketoglutarate and other substrates proceeded in the presence of the same concentration of the drug. The concentration of arsenite which was half-inhibitory to \( \alpha \)-ketoglutarate oxidation was 1.5 × 10^{-2} \text{M} in the sensitive bacteria and 3.3 × 10^{-2} \text{M} in the resistant ones. Cells capable of oxidizing \( \alpha \)-ketoglutarate in the presence of arsenite were induced rapidly by contact with arsenite in growing cultures; when the drug was removed from the cultures, resistance was maintained for about two generations and then gradually disappeared. From the data presented, it was concluded that resistance in this organism is a physiological change and not a hereditary one. Further studies were carried out to investigate the arsenite-resistance mechanisms. \( \alpha \)-Ketoglutarate dehydrogenase activity in the cell-free extracts of the resistant bacteria was sensitive to arsenite. An increase in the contents of this enzyme and sulfhydryl compounds, involving lipoic acid, was not observed in the resistant bacteria. The possibility of detoxication of arsenite was ruled out. Treatment of the resistant cells with cetyl-trimethylammonium bromide made them susceptible to 2 × 10^{-3} \text{M} arsenite, although untreated cells were resistant to the same concentration of the drug. These data suggest the decreased permeability to arsenite of the resistant bacteria as a main mechanism of resistance.

It is a well-known concept that microorganisms have extraordinary adaptability to changes of environmental conditions; an important example of such ability is the phenomenon of drug resistance. Despite their great theoretical interest and clinical importance, the biological mechanisms underlying the development of bacterial resistance to various drugs are at present imperfectly understood. To study drug resistance in microorganisms, it may be of some advantage to use strains resistant to enzyme inhibitors, since their structures are more or less simple and their inhibiting mechanisms have been established. For the purpose of investigating drug resistance, we isolated from soil one strain which was highly resistant to arsenite. Arsenite is a well-known specific inhibitor to the dithiol enzyme, i.e., pyruvate and \( \alpha \)-ketoglutarate dehydrogenases. The organism was named Pseudomonas pseudomallei strain 54 and was examined for its mechanism of resistance to arsenite.

Resistance to various drugs in living microorganisms can take place both at a populational and individual level. For instance, inheritable acquisition of resistance to various antibiotics occurs through mutation and selection (Newcombe and Hawirko, 1949), whereas physiological adaptation is also found in the drug resistance of various microorganisms (Shint and Kelly, 1962; Ashida and Nakamura, 1959).

In the present report, evidence is presented for physiological acquisition of arsenite resistance in P. pseudomallei 54. Data are also given which suggest the decreased permeation of arsenite into the cell as a main mechanism of resistance in this organism. A succeeding report will elucidate this point further.

MATERIALS AND METHODS

Organisms and media. Arsenite-resistant P. pseudomallei 54 isolated from soil was used throughout this study. Bouillon-peptone broth was used for cultures of bacteria.

Procedure for isolation of drug-resistant microorganisms. After inoculation of a drop of soil suspensions into medium containing 2 × 10^{-2} \text{M} sodium arsenite, stationary cultivation was car...
ried out overnight at 30 C. When marked prolifera-
tion of microorganisms was observed, the cul-
tivated media were spread, after appropriate
dilution, on an agar plate of medium containing
the same concentration of the drug. The micro-
organisms thus isolated were stocked on agar slants
containing the drug.

Conditions and measurements of growth. The
organisms were grown overnight aerobically at
30 C in bouillon-peptone broth, and samples of
this culture were inoculated in the same medium
with or without arsenite. Cultivation was under-
taken in L-type test tubes tilting slowly at 30 C.
Turbidity was measured photometrically with a
Kotaki nephelometer. To prepare cell-free ex-
tracts, large-scale cultivation was undertaken in
500-ml Sakaguchi flasks by reciprocal shaking.

Preparation of cell-free extracts. Organisms
grown in the presence or absence of 10^{-2} M ar-
senite were harvested in the middle of exponential
growth. The washed cells were suspended in
phosphate buffer (0.2 M, pH 7.4) and were dis-
rupted by treatment in a sonic oscillator (Toyo
Riko, 10 kc) for 5 min. After removal of cell debris
by centrifugation at 10,000 X g for 10 min, the
sonic extracts were used as crude cell-free pre-
parations.

Manometric measurements. Oxidation of various
substrates by P. pseudomallei 54 was measured by
Warburg manometric techniques. The cells were
harvested in the middle of the logarithmic growth
phase and suspended in phosphate buffer (0.2 M,
pH 7.4). Warburg vessels contained appropriate
amounts of cell suspension, 100 μmoles of phos-
phate buffer (pH 7.4), and 20 μmoles of the
substrates (total volume, 2.0 ml; gas phase, air;
temperature, 30 C).

Assay of α-ketoglutarate dehydrogenase in the
cell-free preparations. Reduction of nicotinamide
adenine dinucleotide (NAD) was measured spec-
trophotometrically at 340 μm with a Hitachi
EPU-2 spectrophotometer; the cell (light path, 1
cm) contained 1 μmole of NAD, 0.1 mg of co-
enzyme A (CoA), 0.2 μmole of coenzyme A; 0.3
μmole of MgCl2, 10 μmole of L-cysteine, 5 μmole
of α-ketoglutarate, 80 μmole of phosphate buffer
(pH 7.4), and 0.2 ml of crude cell-free extract.
Arsenite was added if necessary. The final volume
was adjusted to 3 ml. The reaction was started
by the final addition of α-ketoglutarate. One unit
of the enzyme was defined as that amount which
caused an initial rate of increase in optical density
at 340 μν of 0.001 per min under the conditions
described above.

Analytical methods. Total content of lipoic acid
in the cells was estimated by bioassay with Strep-
tococcus faecalis after the hydrolysis of the cells
according to the method of Wagner et al. (1956)
or Stockstad et al. (1960).

Total free sulfhydryl compounds were esti-
mated by the method of Saville (1958) after de-
proteinization of each material.

Estimation of arsenite was carried out by io-
dometry (Feigel, 1954). Protein concentration
was measured by the micro-Kjeldahl method.

Biochemical reagents. NAD, CoA, and cocar-
boxylase were purchased from Mann Research
Laboratories, Inc., New York, N.Y.

RESULTS

Isolation of drug-resistant microorganisms from
soil. Seven strains of bacteria able to grow on the
medium containing 2 X 10^{-2} M arsenite could be
isolated from soil. For further study, we chose one
bacterial strain, 54, which could grow on medium
containing 10^{-2} M arsenite. Strain 54 can oxidize
α-ketoglutarate rapidly. It was identified as be-
longing to P. pseudomallei according to Bergey's
Manual of Determinative Bacteriology. Taxonomic
descriptions were as follows.

Agar colonies: Circular, convex, smooth.
Agar slant: Growth moderate, butyrous,
smooth.

Nutrient broth: Turbid.
Gelatin stab: Liquefaction.
Litmus milk: Slightly acidic.
Acid formed from glucose.
Starch not hydrolyzed.
H2S not produced.
Citrate not used as a sole carbon source.
Oxidase activity test by Nadi-reaction: Negati-
ve.

Indole production: Negative.
Nitrite not produced from nitrate.

Effect of arsenite and arsenate on growth. The in-
hibitory effect of serially increased concentrations
of arsenite on growth of P. pseudomallei 54 is il-
ustrated in Fig. 1. A progressive increase in the
lag period was observed with increasing con-
centrations of arsenite, but the exponential growth
rate and final amount of growth equalled that of
the uninhibited control. Complete inhibition occurred at a concentration of $3 \times 10^{-2}$ M arsenite.

To determine whether cultures that had resumed growth now became resistant to arsenite, cells were prepared for inoculation as described above and inoculated into fresh medium containing $10^{-2}$ M arsenite. Figure 2 shows that the prolonged lag typical of adapting cultures was eliminated or shortened by preliminary growth in the presence of arsenite. This shows that the cells acquired a resistant character during growth with arsenite.

The growth of bacteria subjected to preliminary growth with $10^{-2}$ M arsenite was also resistant to $3 \times 10^{-2}$ M arsenate, although this concentration of arsenate was sufficient to depress the growth rate of the unadapted organisms (Fig. 3A). Conversely, organisms grown in the presence of $10^{-2}$ M arsenate were able to grow without any inhibitory effect when they were transferred to the medium containing $10^{-2}$ M arsenite (Fig. 3B).

Effect of arsenite and arsenate on oxidation of $\alpha$-ketoglutarate. Oxidation of the various substrates by the cells which had grown in the presence of $10^{-3}$ M arsenite was found to be resistant to the same concentration of arsenite (Table 1). In the case of $\alpha$-ketoglutarate oxidation, which may be a key reaction inhibited by arsenite, oxygen uptake became completely resistant toward arsenite, and a slight activation was sometimes observed. Thus, oxidation activity of $\alpha$-ketoglutarate in the presence of arsenite might be used as a measure of arsenite resistance.

Inhibition of $\alpha$-ketoglutarate oxidation was
FIG. 3. Mutual cross-resistance to arsenite and arsenate inhibitions observed with bacterial growth. (A) Effect of $3 \times 10^{-3} \text{ M}$ arsenate on growth of unadapted bacteria (▲) and adapted bacteria after preliminary growth with $10^{-2} \text{ M}$ arsenate (○). Culture without arsenate is the control (●). (B) Effect of $10^{-2} \text{ M}$ arsenite on growth of the unadapted bacteria (▲) and adapted ones after preliminary growth with $10^{-2} \text{ M}$ arsenate (○). Culture without arsenite is the control (●).

TABLE 1. Effect of $10^{-2} \text{ M}$ arsenite on oxidation of various substrates by sensitive and resistant cells

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sensitive cells</th>
<th>Resistant cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$Q_{O_2}$</td>
<td>$Q_{O_2}$ with arsenite</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>62.5</td>
<td>8</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>210</td>
<td>22</td>
</tr>
<tr>
<td>Succinate</td>
<td>250</td>
<td>81</td>
</tr>
<tr>
<td>Malate</td>
<td>282</td>
<td>35</td>
</tr>
</tbody>
</table>

* Expressed as μmoles of oxygen consumed per mg of cell N per 30 min at 30 C.
† Stimulation.

measured at various concentrations of arsenite with the organisms which were grown in the absence or presence of $10^{-2} \text{ M}$ arsenite. When the inhibition ratio was plotted against the concentration of arsenite, the inhibition curves illustrated in Fig. 4 were obtained. The concentration of arsenite required for 50% inhibition was about $1.6 \times 10^{-3} \text{ M}$ in the sensitive cells and about $3.3 \times 10^{-2} \text{ M}$ in the resistant cells grown in the presence of $10^{-2} \text{ M}$ arsenite; i.e., the latter was about 20 times higher than the former. Not only a great difference in the half-inhibitory concentrations, but a considerable change in the shape of these curves, is observed with resistant cells.

Cross-resistance to arsenite of the arsenate-grown organisms was also observed with oxidative activity of α-ketoglutarate. Oxidation of α-ketoglutarate in cells grown in the presence of $10^{-2}$ or $10^{-3} \text{ M}$ arsenate became resistant to arsenite at a concentration of $10^{-2} \text{ M}$ (Table 2).

Table 2. Effect of $10^{-3} \text{ M}$ arsenite on oxidation of α-ketoglutarate by the cells grown in the absence or presence of arsenate

<table>
<thead>
<tr>
<th>Concentration of arsenate added during growth</th>
<th>$Q_{O_2}$</th>
<th>$Q_{O_2}$ with arsenite</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M$</td>
<td>0</td>
<td>286</td>
<td>12</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>272</td>
<td>228</td>
<td>17.5</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>266</td>
<td>227</td>
<td>14.5</td>
</tr>
</tbody>
</table>

* Expressed as μmoles of oxygen consumed per mg of cell N per 30 min at 30 C.
arsenite. When cells were placed in medium containing \(10^{-2} \text{ M} \) arsenite at 30 C with shaking, a rapid rise of arsenite resistance with \(\alpha\)-ketoglutarate oxidation occurred prior to initiation of growth (Fig. 5A). During this time, only a slight increase of turbidity was observed. Full resistance was reached within about 2 or 3 hr. Acquisition of resistance to arsenite did not occur in the arsenite-containing incubation medium which did not support bacterial growth.

When the resistant cells growing in the presence of \(10^{-2} \text{ M} \) arsenite were resuspended in new medium without arsenite, the resistant character was maintained during about two generations (about 2 hr) and then gradually disappeared (Fig. 5B).

Mechanism of arsenite resistance in *Pseudomonas*. The results described above demonstrated that resistance to arsenite in *Pseudomonas* was acquired through the process of physiological adaptation. There are many possibilities concerning the direct mechanism responsible for this resistant character. Formation of alternative metabolic pathways insensitive to drugs, increased destruction of drugs (Pollock, 1957), increase in amounts of enzyme inhibited by drugs (Mizushima and Arima, 1960), increase in a content of a cofactor that antagonizes drugs (Harington, 1959), and decreased permeability of the cell to drugs (Isaki and Arima, 1963) have been postulated to be responsible for drug resistance. These possible mechanisms were examined in the experiments described below.

\(\alpha\)-Ketoglutarate dehydrogenase activity in cell-free preparations. Activities of the \(\alpha\)-ketoglutarate dehydrogenase systems extracted from both sensitive and resistant cells required CoA for their activities and were both inhibited almost equally by arsenite (Table 3). Also, a significant difference in their specific activity was not observed. These activities were 135 units per mg of protein N in the extracts of the sensitive cells and 111 in those of the resistant ones.

Contents of lipoic acid and sulphydryl compounds in the cells. The organisms grown with or without \(10^{-2} \text{ M} \) arsenite were harvested, and appropriate amounts of the cells were hydrolyzed to determine contents of lipoic acid and sulphydryl compounds. Although considerable diversity of the values was observed, depending on the method used to prepare the cell hydrolysates, a significant increase in the contents of these compounds in the resistant cells could not be observed (Table 4).

Detoxication of arsenite. As reported by Mandel and Mayersak (1962), detoxication of arsenite by oxidation is one of the mechanisms of arsenite resistance. However, oxidation of arsenite could not be observed in the *Pseudomonas* cells by chemical estimation of arsenite.
Effect of cetyltrimethyl ammonium bromide (CTAB) on the resistance to arsenite. There has been considerable evidence suggesting that treatment of microorganisms with cationic detergents (for instance, CTAB) destroys the permeability barrier of the cells to various metabolites (Bruemmer and Wilson, 1961; Sato and Arima, in press). If this is true and arsenite resistance is caused by decreased permeation of arsenite into the cells, then the treatment may decrease the degree of arsenite resistance of Pseudomonas cells. This is the case, as is illustrated in Fig. 6. Organisms grown with $10^{-2}\, \text{M}$ arsenite were resuspended in distilled water, and CTAB was added to give a final concentration of 100 $\mu\text{g/ml}$. After incubation for 10 to 20 min at 30 $\degree\text{C}$ with gentle shaking, the cells were recentrifuged and subjected to manometry without washing. This treatment itself did not impair severely the oxidative activity of $\alpha$-ketoglutarate of the cell. Oxidation of $\alpha$-ketoglutarate by the untreated organisms was not inhibited at all by arsenite at $2 \times 10^{-2}\, \text{M}$. However, remarkable inhibition was observed when the same concentration of arsenite was added to the organisms after treatment with CTAB.

**DISCUSSION**

The process by which microorganisms acquire resistance against various drugs may fall largely in two different groups in nature: one involves a hereditary change in resistance to an inhibitor, and the other involves physiological changes which result in more or less temporary acquisition of resistance. It can be concluded that the arsenite resistance in the Pseudomonas cells described in this paper is a physiological phenomenon induced by contact with the drug. The
reasons are summarized as follows. (i) A very short lag time was observed with growth in the presence of arsenite. It is too short to be explained by the mutation theory (Fig. 1). (ii) Very rapid acquisition of resistance was observed with oxidation of $\alpha$-ketoglutarate (Fig. 5A). (iii) Rapid disappearance of resistance was observed when the resistant organisms were transferred to medium containing no arsenite (Fig. 5B). (iv) Induction of almost full resistance was observed with an extremely low concentration of arsenite, which was not observed with the untreated organisms. These results were attributed to destruction of the permeability barrier of the cells for succinate and antimycin A, respectively. The data presented in Fig. 6 indicate decreased permeation of arsenite in the resistant cells. Treatment with CTAB might facilitate penetration of arsenite into the cells and cause it to have an inhibitory effect even on the resistant cells.

Inhibition sites of arsenate ($\text{As}_2\text{O}_4^-$) are known to be distinct from those of arsenite ($\text{As}_2\text{O}_3^-$). However, mutual cross-resistance to arsenite and arsenate shown by the arsenate- and arsenite-grown cells indicates some common mechanisms in their resistance. Although the occurrence of interconversion between these two compounds by oxidation-reduction has been reported in the various bacteria (Turner, 1954; Turner and Legge, 1954), decrease of arsenite in cultures owing to oxidation to arsenate was not observed with this organism.

The intermediary formation of the dithiol form of the lipoic acid coenzyme during the turnover of the keto acid dehydrogenases renders these enzymes particularly susceptible to inhibition by arsenite. As shown in Table 3, the properties of $\alpha$-ketoglutarate dehydrogenase in resistant cells were not altered.

An arsenite-resistance mechanism was reported by Harington (1959) with blue tick. He observed a remarkable increase of the contents of sulfhydryl compounds in an arsenic-resistant strain of blue tick larvae, and concluded that higher levels of sulfhydryl compounds conferred upon them a greater power to detoxicate given amounts of arsenicals. This mechanism was negated by the results shown in Table 4.

Detoxication of arsenite by oxidation to arsenate was reported by Mandel and Mayersak (1962) with Bacillus cereus. Though we could not prove the oxidation of arsenite by Pseudomonas, there were some possibilities that slow reaction occurred in these organisms (Turner, 1954). However, it seems unlikely that the large amounts of arsenite ($10^{-4} \text{M}$) are detoxicated by the slow oxidation. The fact that the organisms grown in the presence of arsenite acquired a resistant nature excluded detoxication as the mechanisms of resistance in this organism.

The effect of treatment with CTAB was recently confirmed with various microorganisms. Bruemmer et al. (1961) reported that treatment of Azotobacter with CTAB unmasked its succinodioxidase activity. Sato et al. (in press) observed that a similar treatment caused an appearance of inhibitory effect of antimycin A on B. subtilis, which was not observed with the untreated organisms. These results were attributed to destruction of the permeability barrier of the cells for succinate and antimycin A, respectively. The data presented in Fig. 6 indicate decreased permeation of arsenite in the resistant cells. Treatment with CTAB might facilitate penetration of arsenite into the cells and cause it to have an inhibitory effect even on the resistant cells.

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