Note

Comparison of Characteristics of Hexavalent Chromium-tolerant Bacterium, *Pseudomonas ambigua* G-1, and Its Hexavalent Chromium-sensitive Mutant

Hiroyuki HORITSU, Satoshi FUTO, Kazuhiro OZAWA and Keiichi KAWAI

Department of Agricultural Chemistry, Faculty of Agriculture, Gifu University, Yanagido, Gifu 501-11, Japan

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In recent years, a few studies have been made on the isolation of Cr^{6+}-tolerant bacteria.[1~3] The Cr^{6+}-tolerant mechanism of these bacteria has not been sufficiently clarified.

In a previous work,[4] we isolated a Cr^{6+}-tolerant bacterium from activated sludge and identified it as *Pseudomonas ambigua* G-1. To elucidate the Cr^{6+}-tolerant mechanism of *P. ambigua* G-1, we attempted the isolation of Cr^{6+}-sensitive mutant(s) from the parent.

This paper reports that Cr^{6+}-sensitive mutants were derived from tolerant *P. ambigua* G-1 and some characteristics of a Cr^{6+}-sensitive mutant were compared with those of the parent G-1.

P. ambigua G-1 was previously marked as a streptomycin-resistant and methionine threonine-double auxotroph by treatment with 2,000 μg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) at 37°C for 2 hr. Cr^{6+}-sensitive mutants were derived from this marked *P. ambigua* G-1. The cells washed with 50 mM Tris-maleate buffer (pH 6.0) were treated with MNNG under the same conditions used for obtaining the auxotrophs. Then, the treated cells were spread on nutrient agar plates. After overnight incubation at 37°C, the colonies appearing were transferred to Cr^{6+} (2,000 ppm) containing nutrient agar plates by the replica plating method. The transferred colonies were incubated overnight at 37°C. Colonies which grew on a nutrient agar plate but not on a Cr^{6+} containing nutrient agar plate were picked up from master plates as Cr^{6+}-sensitive mutants. The origin of these mutants was confirmed by determining the drug resistance and amino acid requirement. Thus, we obtained seven Cr^{6+}-sensitive mutants. Tolerance concentrations for these sensitive mutants of some heavy metals were determined by the gradient concentration method as described previously.[5] The parent G-1 was able to grow on nutrient agar containing up to 4,000 ppm Cr^{6+} or 250 ppm Cd^{2+} or 200 ppm $Cu.^{2+}$ On the other hand, the sensitive mutants did not grow on the nutrient agar containing these heavy metals, except that two Cr^{6+}-sensitive mutants, strains S-1 and S-3, could grow on nutrient agar containing up to 200 ppm and 50 ppm Cu^{2+}, respectively. It is of interest that strain S-1 was as tolerant to Cu^{2+} as the parent G-1 was. This result suggests that the tolerant mechanism for Cr^{6+} was different from that for $Cu.^{2+}$

Next, we compared Cr^{6+} uptake by cells between parent

TABLE I. COMPARISON OF HEXAVALENT-CHROMIUM UPTAKE BETWEEN THE PARENT G-1 AND MUTANT S-1

The cells of the parent G-1 and mutant strain S-1 cultivated aerobically in nutrient broth for 18 hr at 30°C (stationary phase) were harvested, washed with 50 mM Tris-HCl (pH 7.0), and then suspended in the same buffer containing 200 ppm Cr^{6+}. After incubation for 3 hr at 30°C, the cells were collected and sonicated. The sonicates were centrifuged at 15,000 × *g* for 15 min to obtain the soluble and insoluble fractions. The chromium contents were determined with a Hitachi Atomic Absorption Spectrophotometer Type 170-40.

Fraction	Parent G-1	Mutant S-1
	Total chromium (mg/100 mg dry cells)	
Cells	0.061	0.356
Soluble	0.056	0.307
Insoluble	0.005	0.049

TABLE II. COMPARISON OF FATTY ACID COMPOSITION

The cells of the parent G-1 and mutant strain S-1 cultivated as in Table I, were harvested and washed with deionized water. The total lipid of the cells was extracted according to the method of Bligh and Dyer.[6] The lipid obtained was subsequently transesterified with BF_3 in methanol (14%, v/v).[7] Fatty acid methylesters were analyzed with a Shimadzu GC-4C Gas Chromatograph equipped with a glass column packed with 10% SILAR 10C on Uniport B (60～80 mesh).

Component	Parent G-1 (%)	Mutant S-1 (%)
12:1	5.1	2.7
12:3	2.2	1.0
14:0	3.1	0.9
14:1	17.5	6.5
15:1	2.1	0.8
16:0	32.6	15.7
16:1	10.2	17.5
Unknown	7.2	0.8
16:2	6.8	1.1
18:0	11.0	7.3
18:1	2.2	45.7

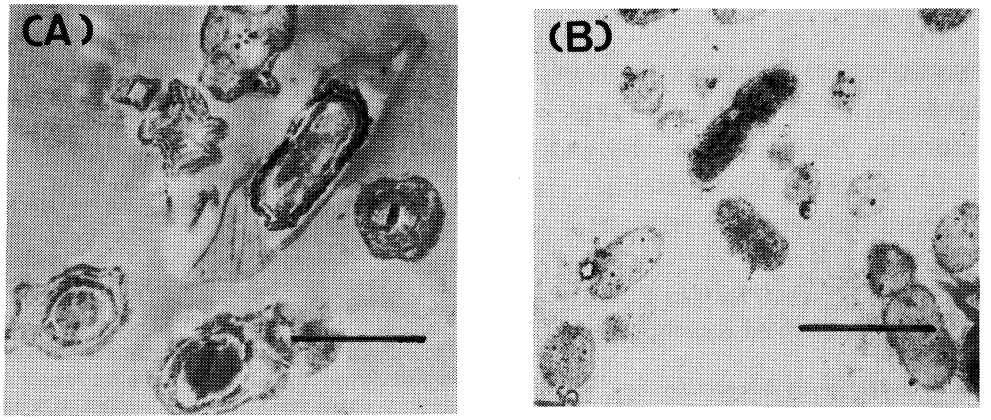

FIG. 1. Electron Micrographs of the Parent G-1 and Mutant S-1.

The cells grown aerobically in nutrient broth for 18 hr at 30°C (stationary phase) were washed with 0.2 M phosphate buffer (pH 7.2). The growth of mutant strain S-1 was essentially the same as that of the parent G-1 under the cultural conditions. Preparation of the samples for transmission electroscopy was performed as described previously.[5] The sliced samples were observed with a JEMT-7 (JEOL) transmission microscope. Bar, 1 μm. (A) parent G-1; (B) mutant strain S-1.

G-1 and mutant strain S-1. As Table I shows, Cr^{6+} uptake of the mutant strain into cells, and the soluble and insoluble fractions were 5.8-times, 5.5-times and 9.8-times, respectively, as much as those of the parent. Thus, the Cr^{6+}-sensitive mutant strain took up much more Cr^{6+} into the cells than the parent, suggesting that the membrane barrier for the permeation of Cr^{6+} may be injured in the sensitive mutant strain. As shown in Table II, there is a considerable difference in the fatty acid composition between the parent G-1 and sensitive mutant strain S-1. In the parent, the major constituent was a saturated C 16 acid, but in the mutant strain, a monounsaturated C 18 acid. Furthermore, observation by transmission electron microscopy of these two strains showed that there were extraordinary differences between them (Fig. 1). The parent was found to be surrounded by a thick envelope, while in mutant strain S-1, the thick envelope was lost. These results suggest that the differences in membrane structure and the surface structure of cells may be related to the Cr^{6+}-tolerance mechanism.

Recently, we found the existence of a Cr^{6+}-reducing enzyme in the cell-free extract of parent G-1.[8] In Cr^{6+}-sensitive mutant strain S-1, the specific activity of the Cr^{6+}-reducing enzyme was found to be decreased to about one-fourth of that of the parent G-1. The decrease in the enzyme activity may also cause the sensitivity to Cr^{6+} in Cr^{6+}-sensitive mutant strain S-1. The Cr^{6+}-reducing enzyme of parent G-1 will be reported elsewhere.

REFERENCES

1) K. Shimada, Abstracts of Papers, the Annual Meeting of Agricultural Chemical Society of Japan, Kyoto, April 1976, p. 58; *ibid.*, Yokohama, April 1977, p. 188; *ibid.*, Nagoya, April 1978, p. 282.
2) K. Koiwai and Y. Nayama, Abstracts of Papers, the Annual Meeting of the Agricultural Chemical Society of Japan, Nagoya, April 1978, p. 281.
3) A. O. Summers and G. A. Jacoby, *Antimicrob. Agents Chemother.*, **13**, 637 (1978).
4) H. Horitsu, H. Nishida, H. Kato and M. Tomoyeda, *Agric. Biol. Chem.*, **42**, 2037 (1978).
5) H. Horitsu and T. Ito, *Agric. Biol. Chem.*, **44**, 2317 (1980).
6) E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.*, **37**, 911 (1959).
7) W. R. Morrison and L. M. Smith, *J. Lipid Res.*, **5**, 600 (1964).
8) H. Horitsu, S. Futo, S. Ogai and K. Kawai, Abstracts of Papers, the Annual Meeting of the Agricultural Chemical Society of Japan, Kyoto, March 1981, p. 84.