faster than the acetylcholinesterase is inactivated. Thus, the lethal dose increased by a factor of at least 280 due to the presence of the phosphotriesterase. This pesticide resistance, however, was short-lived since all caterpillars exposed to recombinant or wild type baculovirus died after approximately six days. In the moth stage, the LD_{50} was found to be 1.2 ± 0.3 µg of paraoxon.

The results presented above demonstrate that reduced toxicity to paraoxon and perhaps other pesticides can be induced in insects by expression of an enzyme that is known to efficiently hydrolyze these molecules to non-toxic products. In this manner, a symbiotic relationship is created between the host insect and the baculovirus. Similarly, the use of a nonpathogenic bacterium as a symbiont vector may be feasible for the incorporation of the phosphotriesterase into other insects.

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2 Foster,T. J., Biol Rev. *47* (1983) 361.
3 Lewis, V. E., Donarski, W. J., Wild, J. R., and Raushel, F. M., Biochemistry *27* (1988) 1591.
4 Donarski, W. J., Dumas, D. P., Heitmeyer, D. P., Lewis, V. E., and Raushel, F. M., Biochemistry *28* (1989) 4650.
5 Dumas, D. P., Caldwell, S. R., Wild, J. R., and Raushel, F. M., J. biol. Chem. *264* (1989) 19659.
6 McDaniel, C. S., Harper, L. L., and Wild, J. R., J. Bact. *170* (1988) 2306.
7 Serdar, C. M., and Gibson, D. T., Bio/Technology *3* (1985) 567.
8 Ohkawa, H., in: Insecticide Mode of Action, p. 163. Ed. J. R. Coats. Academic Press, New York, 1982.
9 Summers, M. D., and Smith, G. E., Tex. Agric. Exp. Stn Bull. No. 1555 (1987).
10 Luckow, V. A., and Summers, M. D., Bio/Technology *6* (1988) *47*.
11 Luckow, V. A., and Summers, M. D., Virology *170* (1989). 31.
12 Harper, L. L., Raushel, F. M., Luckow, V. A., Summers, M. D., and Wild, J. R., (1990) submitted for publication.
13 Finney, D. J., J. Pharmac. *104* (1952) 440.
14 Busvine, J. R., A Critical Review of Techniques for Testing Insecticides, 2nd edn, p. 267. Dorset Press, Dorchester, England 1971.
15 Ashford, J. R., and Smith, C. S., Biometrics *21* (1965) 181.

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Chromate reduction in *Streptomyces*

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Summary. Streptomyces species 3M grew in peptone yeast extract medium with 1000 µg/ml $K_2Cr_2O_7$. Incubation of the chromate with different cell fractions in the presence of NADH and NADPH resulted in a decrease of Cr^{6+} in the reaction mixture. The level of Cr^{6+} was reduced by 82.7% by a particulate cell fraction obtained by centrifugation at 105,000 × g for 1 h, in the presence of NADH. The reducing enzyme was associated with this cell fraction. The enzyme was constitutive and reduced Cr^{6+} to Cr^{3+}.
Key words. Streptomyces; chromate tolerance; reducing enzyme; reduction of Cr^{6+} to Cr^{3+}.

Chromium compounds are toxic, but there are microorganisms which grow in the presence of chromium [2–5]. Various mechanisms have been suggested to be involved in chromium resistance and reduction. Aerobic heterotrophic bacteria accumulated chromium in the extracellular polymer material of the cell; this binding of chromium to the extracellular material was a mechanism of chromium tolerance [6]. Reduction, changing the valency of chromium from the toxic Cr^{6+} to the relatively less toxic Cr^{3+}, was reported in rat liver microsomes [7]. The Cr^{6+} tolerant *Pseudomonas ambigua* G-1 exhibited a membrane barrier against the penetration of Cr^{6+} into the cells as a mechanism of Cr^{6+} tolerance [8]. Later it was shown that Cr^{6+} reduction in *P. ambigua* G-1 was due to a reducing enzyme [9], and enzymatic reduction of chromate to Cr^{3+} was reported in *P. fluorescens* LB 300 [10]. There are no reports so far on the reduction of Cr^{6+} by *Streptomyces* species.

Materials and methods

A *Streptomyces* sp. isolated from soil where chrome-tanned leather had been dumped grew in peptone yeast extract [3] (PYE) broth in the presence of 375 µg/ml $K_2Cr_2O_7$. The organism was adapted in the laboratory to tolerate 1000 µg/ml $K_2Cr_2O_7$. The parent strain was designated 3W and the adapted strain 3M.

Streptomyces sp. 3M was grown in PYE broth containing 100 µg/ml $K_2Cr_2O_7$. After 72 h incubation at 28 °C the cells were spun down, and the supernatant was treated with *s*-diphenylcarbazide [11] and assayed for Cr^{6+} at 540 nm. Cr^{6+} in the supernatant was also estimated after incubation with glucose and sodium azide. Uninoculated medium containing $K_2Cr_2O_7$ was assayed for 1) Cr^{6+} with *s*-diphenylcarbazide, 2) total chromium with an atomic absorption spectrometer (Varian, AA575 series) using an oxidizing air acetylene flame at 425.4 nm and a 0.1 spectral band pass. $Cr(OH)SO_4$ was used as a stan-

dard. A strain of *S. griseorubiginosus* (NRRL B-12384) sensitive to 60 µg/ml $K_2Cr_2O_7$ was grown in PYE medium containing 25 µg/ml $K_2Cr_2O_7$ and the supernatant assayed for Cr^{6+}.

For cell fractionation 3M was grown in PYE broth containing 30% sucrose and 10 mM $MgCl_2$ for 3 days on a rotary shaker. 100 µg/ml $K_2Cr_2O_7$ was added for induction, and incubation prolonged for 6 h. Cells were harvested by filtering, washed thrice with 0.05 M Tris HCl buffer (pH 7.4), weighed, frozen for 1–1.5 h with neutral alumina, thawed, crushed and suspended in buffer. This was then centrifuged at 9000 × g for 15 min to obtain the supernatant as a cell-free extract (fraction I), which was centrifuged at 15,000 × g for 20 min. The sediment was fraction II and the supernatant fraction III. Fraction II was suspended in the buffer while fraction III was ultracentrifuged at 105,000 × g for 1 h. The pellet obtained was fraction IV and the supernatant fraction V. Fraction IV was homogenized and suspended in 1–2 ml buffer. All centrifugations were made at 4 °C.

The protein contents of the cell fractions were estimated[12] using bovine serum albumin as standard. Enzyme activity was determined by the method of Gruber and Jennette[7]. 1 ml Tris buffer (pH 7.4) containing 0.4 mM $K_2Cr_2O_7$, 1.6 mM NADH/NADPH and cell fractions with a constant protein content of 0.323 mg was incubated at 37 °C for 6 h with constant shaking. Chloroform-isoamylalcohol mixture (24:1) was added, and the mixture then shaken and centrifuged at 3600 × g for 15 min. The upper aqueous layer was carefully removed and the concentrations of chromate and NADH/NADPH were determined spectrophotometrically at 400 and 340 nm. Tris HCl buffer containing cell fractions with no chromate and NADH/NADPH was used as control. Absorbances were read with the buffer containing $K_2Cr_2O_7$ and NADH/NADPH (without cell fractions) to demonstrate that no significant amount of chromate was lost from the reaction mixture during the experiment.

Fractionation studies were also carried out with *S. griseorubiginosus* and enzyme activities were determined after incubation with 25 µg/ml $K_2Cr_2O_7$.

An electron paramagnetic resonance (EPR) spectrum was obtained with fraction IV on a Varian spectrometer after incubation with $K_2Cr_2O_7$ in the presence of NADH for 15 min. The reaction mixture with chromate and NADH but without fraction IV was used as the control. $CrCl_3$ was run for comparison. The values of g were calculated relative to DPPH as reference for which g = 2.0036[13]. Spectrophotometric estimations of Cr^{6+} at 400 nm and Cr^{3+} at 595 nm were also made with the reaction mixture.

For determining the constitutive or inducible nature of the reducing enzyme, 3W and 3M were grown separately in 2 batches in the absence of $K_2Cr_2O_7$. After 3 days, one batch of each strain was induced with 100 µg/ml $K_2Cr_2O_7$ while the other batch was grown without $K_2Cr_2O_7$. After an additional 6-h incubation, induced and uninduced cells were fractionated and fraction IV was incubated at 37 °C for 6 h in Tris HCl buffer with $K_2Cr_2O_7$ and NADH. Cr^{6+} was estimated at 400 nm.

Results and discussion

This is the first report on the reduction of C^{6+} to Cr^{3+} by a streptomycete. Incubation of *Streptomyces* sp. 3M in PYE broth in the presence of $K_2Cr_2O_7$ resulted in a decrease of 25–30% of Cr^{6+} in the culture filtrate. A decrease of 50% was observed in *S. griseorubiginosus*. PYE medium was developed with a low organic content to reduce binding of chromium to the medium constituents[3]. We also noted that Cr^{6+} added to the uninoculated PYE medium did not decrease. No loss of total chromium was observed in the uninoculated medium during the experiment. Addition of 0.5% glucose decreased Cr^{6+} in 3M; after 72-h incubation 97% of Cr^{6+} was lost from the culture filtrate. This implies that glucose acts as an electron donor in the uptake and reduction of Cr^{6+} by 3M. Resting cells of *Pseudomonas fluorescens* LB 300 reduced chromate only if glucose or another suitable electron donor was present in the medium[10]. But in *P. ambigua* G-1, glucose was not required for the reduction of chromate[9]. Sodium azide, when added at different concentrations to the incubation mixture, did not effect the loss of Cr^{6+} from the culture filtrate of 3M.

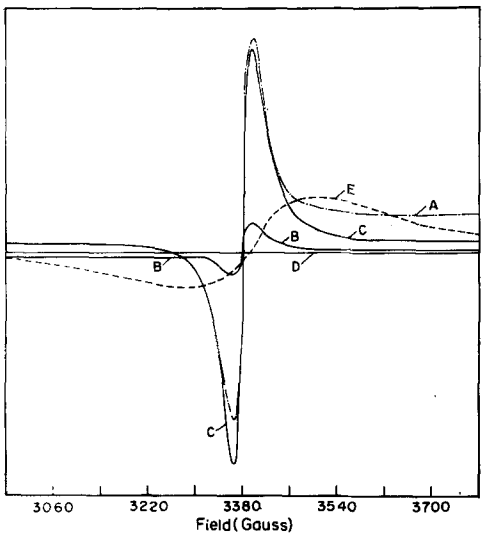
Reduction of chromate (from $K_2Cr_2O_7$ which is in the chromate form at pH 7.4) was observed with cell fractions I and III of 3M in the presence of NADH and NADPH. Cr^{6+} reduction in rat liver microsomes required NADPH[7] and in *P. ambigua* G-1 required NADH[9], but in *Streptomyces* 3M, NADH was a better cosubstrate than NADPH (table 1). The sedimented fraction IV from the 105,000 × g centrifugation reduced 82.7% of Cr^{6+} in the presence of NADH.

A clear concept of the cellular organelles in *Streptomyces* is yet to be reported. Fraction IV of *Streptomyces* 3M cells was particulate and probably contained cytoplasmic membranes[14]. The soluble fraction V reduced chromate to a lesser extent. This is in contrast to the situation in *P. ambigua* G-1, in which the soluble fraction of 105,000 × g

Table 1. Decrease of hexavalent chromium when incubated with cell fractions of *Streptomyces* sp. 3M

Fractions	Decrease of Cr^{6+} (%) in the presence of	
	NADH	NADPH
I	58.2	30.0
II	36.5	29.5
III	60.4	38.7
IV	82.7	80.7
V	6.6	1.9

Reaction mixture: 1 ml of 0.05 M Tris HCl buffer (pH 7.4) containing cell fraction protein 0.323 mg/ml, CrO_4^{2-} 0.4 mM; NADH/NADPH 1.6 mM. Shake incubation at 37 °C for 6 h. Cell fractions obtained by centrifugation: I, supernatant of 9000 × g for 15 min; II, sediment of 15000 × g for 20 min; III, supernatant of 15000 × g for 20 min; IV, sediment from III at 105,000 × g for 1 h; V, supernatant of 105,000 × g for 1 h. Results are the average of 3 experiments.



EPR spectra were run on a Varian spectrometer at 100-kHz modulation frequency, 0.8 × 10 G modulation amplitude, 5 mW microwave power, 9.43-GHz microwave frequency and 2.5×10^3 gain. Spectra *A*, resulting from incubation of chromate (0.4 mM) with cell-fraction IV of *Streptomyces* sp. 3M (1 mg/ml) and NADH 1.6 mM in 0.05 Tris. HCl buffer pH 7.4 at 22 °C for 15 min, *B*, 'A' without fraction IV, *C*, $CrCl_3$, *D*, $K_2Cr_2O_7$ *E*, fraction IV.

Table 2. Enzymatic reduction of hexavalent chromium by *Streptomyces* spp. 3W and 3M

Strains	Cr^{6+} reduced (%)	Activity (units)*	Specific activity (unit/mg protein)
3W	20.2	1.7	0.02
3W-I	23.7	2.0	0.02
3M	46.1	3.8	0.04
3M-I	61.4	5.2	0.06

Reaction mixture: 1 ml 0.05 M Tris HCl buffer (pH 7.4) containing cell fraction IV, protein content 0.293 mg/ml; CrO_4^{2-} 0.4 mM; NADH/NADPH 1.6 mM. Incubated with shaking at 37 °C for 6 h. *unit = amount of enzyme that reduced 1 µmole Cr^{6+}/min.

centrifugation reduced Cr^{6+}. *S. griseorubiginosus* cells could take up more chromate from the culture broth than *Streptomyces* 3M, but only 87% of the chromate added to *S. griseorubiginosus* cell fraction IV was reduced, whereas 100% was reduced in *Streptomyces* 3M. Apparently chromate uptake, resistance and reduction are not related in *Streptomyces* species. No relation between chromate sensitivity and reduction could be found in *Pseudomonas* spp. either [10].

Since the decrease of Cr^{6+} was maximal with fraction IV in the presence of NADH, further experiments were carried out with fraction IV and NADH. The EPR spectrum of the reaction mixture showed a signal with $g = 1.99$ and $\Delta H = 32$. These values were similar to the g and ΔH values obtained with the trivalent form of chromium $CrCl_3$ (fig.). When analysed spectrophotometrically the reaction mixture showed a decrease in absorbance at 400 nm, with a corresponding increase in absorbance at 595 nm. Δ 400 values measured after 2, 4 and 6 h incubation were 0.61, 0.58 and 0.40, while ΔA_{595} values were 0.20, 0.34 and 0.56. These results show that Cr^{6+} was reduced and Cr^{3+} was formed. The Cr^{6+} reducing enzyme was constitutive. Fraction IV of the uninduced parent (3W) and the adapted strain (3M) could reduce Cr^{6+} (table 2). The computed t-values calculated by the difference method show that the observed differences (table 2) were highly significant ($p < 0.001$). The reducing activities of the chromate uninduced strains were less than those of the induced strains. A 3-fold increase in specific activity of the reducing enzyme was observed in the chromate-induced 3M when compared to the uninduced 3W.

1 To whom reprint requests should be addressed.
2 Romenokova, V. I., and Koren'kov, V. N., Mikrobiologiya *46* (1977) 414.
3 Luli, G. W., Talnagi, J. W., Strohl, W. R., and Pfister, R. M., Microbiology *46* (1983) 846.
4 Kvasnikov, E. I., Stepanyuk, V. V., Klyushnikova, T. M., Serpokrylov, N. S., Simonova, G. A., Kasatkina, T. P., and Pachenko, L. P., Mikrobiologiya *54* (1985) 83.
5 Gvozdyak, P. I., Mogilevich, N. F., Ryl'Skii, A. F., and Grishchenko, N. I., Mikrobiologiya *55* (1987) 962.
6 Aislabie, J., and Loutit, M. W., Mar. envir. Res. *20* (1986) 221.
7 Gruber, J. E., and Jennette, K. W., Biochem. biophys. Res. Commun. *82* (1978) 700.
8 Horitsu, H., Futo, S., Ozawa, K., and Kawai, K., Agric. biol. Chem. *47* (1983) 2907.
9 Horitsu, H., Futo, S., Miyasawa, Y., Ogai, S., and Kawai, K., Agric. biol. Chem. *51* (1987) 2417.
10 Bopp, L. H., and Ehrlich, H. L., Archs Microbiol. *150* (1988) 426.
11 Vogel, A. I., A Text Book of Quantitative Inorganic Analysis Including Elementary Instrumental Analysis, 3rd ed. Longmans & Co 1961.
12 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. biol. Chem. *193* (1951) 265.
13 Levanon, H., and Charbinaky, S., J. chem. Phys. *53* (1970) 3056.
14 Murray, R. G. E., in: Bacteria: Structure, vol. 1, p. 51. Eds Gunsalus and Stanier. Academic Press, New York 1960.

0014-4754/90/070731-03$1.50 + 0.20/0