Expression of *Pseudomonas* phosphotriesterase activity in the fall armyworm confers resistance to insecticides[1]

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Summary. The gene encoding for the phosphotriesterase *(opd)* from *Pseudomonas diminuta* has been subcloned into a baculovirus expression system. Functional enzyme is produced when the recombinant baculovirus is used to infect either cultured *Spodoptera frugiperda* sf9 cells or the larval stage of the fall armyworm. The LD_{50} for paraoxon toxicity was found to increase 280-fold in the larvae after infection with the recombinant baculovirus and expression of the functional phosphotriesterase.
Key words. Phosphotriesterase; fall armyworm; pesticide resistance; paraoxon.

The resistance of bacteria to antibiotics is due in many cases to the existence of a gene which encodes for an enzyme that catalyzes the chemical modification of the particular antibiotic to a nontoxic molecule[2]. The hydrolytic action of the various β-lactamases with the penicillin-type antibiotics is a classic and well-characterized example. A *Pseudomonas diminuta* gene (*opd*, organophosphorus degrading) encoding an enzyme that catalyzes the rapid hydrolysis of many of the phosphotriesters currently used as commercial insecticides[3-5] has been successfully cloned[6,7]. These insecticides are highly toxic due to their ability to rapidly inactivate the enzyme acetylcholinesterase from a variety of sources[8]. The problem to be addressed in this report is whether the expression of this gene in insects could confer reduced toxicity to these phosphotriester-type insecticides due to the chemical detoxification of the pesticide within the insect itself. The baculovirus expression system developed by Summers et al.[9-11] appeared to be a well-suited model for this hypothesis since the cloned *opd* gene could be inserted into the baculovirus and functionally expressed either in cultured *Spodoptera frugiperda* sf9 cells or, more appropriately, into the larval caterpillar stage of the fall armyworm.

Materials and methods

All kinetic measurements were performed at 25 °C using a Gilford 260 spectrophotometer. Enzymatic activity was measured by monitoring the absorbance of p-nitrophenol at 400 nm generated upon the hydrolysis of diethyl-p-nitrophenylphosphate (paraoxon) at pH 9.0 in 150 mM 2-[cyclohexylamino]-ethanesulfonic acid (CHES). A unit of activity is defined as the amount of enzyme needed to hydrolyze 1 μmole of paraoxon per min.

A recombinant baculovirus containing the *opd* gene was constructed as in Harper et al.[12] using standard recombinant DNA techniques as described by Summers and Smith[9,10]. The construction of this baculovirus utilized the insertion of the *opd* gene into the baculovirus transfer vector pVL941 to give the recombinant transfer vector pLH1 170*opd*. pVL941 is a derivative of pAC311 in which the ATG start has been changed to an ATT triplet by site directed mutagenesis[11]. The translation of a foreign gene is thus expressed from the polyhedron protein promoter and initiated at the first codon in the open reading frame of that gene. Recombinant virus containing the *opd* gene was obtained by the co-transfection of *Spodoptera frugiperda* sf9 cells with pLH1 170*opd* and wild-type *Autographa californica* nuclear polyhedris virus (AcNPV) viral DNA followed by the isolation of those cells which were able to hydrolyze paraoxon to diethylphosphate and p-nitrophenol. Among the viral lines capable of expressing the phosphotriesterase, isolate A5B-*opd* was shown to produce the greatest amount of phosphotriesterase activity[12].

The fall armyworms used in this study were cultured from eggs provided by Dr James Fuxa of Louisiana University. These eggs were from a strain originally isolated in Hammond, Louisiana. The larvae of the fall armyworms were raised in 1-oz plastic creamer cups with cardboard lids, and fed a commercially prepared diet (Bio-Serv, Inc. Frenchtown, NJ). The temperature was maintained at 27 °C with a 16-h photoperiod. After pupation, the insects were transferred to oviposition cages made from 3-gallon ice cream cylinders covered with cheese cloth. Upon emergence, the moths fed on a solution of beer and honey (12 oz beer, 30 ml honey, and 10 g ascorbic acid diluted to 1l). Eggs were harvested daily from the cheese cloth and placed in a sealed container with food until hatching when they were separated into plastic creamer cups.

In order to assess whether the expression of a functional phosphotriesterase by a baculovirus within living insects would protect against the lethal effects of an insecticide, the recombinant baculovirus was injected directly into the larval stage of the fall armyworm after stupification of the insect in carbon dioxide and disinfection by dipping in 2% bleach followed by dipping in 70% ethanol and finally submerged for 5–10 min in 0.9% NaCl. The caterpillars were injected late in the third instar by taking

up 5 µl of either 2×10^8/ml A5B-*opd* or 2×10^8/ml AcNPV virus into a fine capillary needle and injecting s.c. into the hemolymph through one of the prolegs.

The expression of phosphotriesterase activity was assessed by the sacrifice of ten caterpillars each day for several consecutive days by forcing the insects individually through the small orifice of a disposable 10-ml syringe. The crushed caterpillars were centrifuged and the supernatant fluid was decanted and recentrifuged to further separate the body fluids from the fat and body parts that remained after the first centrifugation. After preparing the body fluids, enzymatic activity was determined on an aliquot of the solution without further dilution and the values for the ten caterpillars were averaged.

The larvae were exposed to different concentrations of paraoxon by placing 1-µl droplets of paraoxon/acetone solutions on the top of the last five segments of the insect. Above 0.5 mg, paraoxon was placed directly on the back of the caterpillar. Mortality was defined as a moribund state 24 h after exposure when the caterpillar could no longer roll onto its legs when placed on its back. A total population of 222 A5B-*opd* infected caterpillars were used to construct the mortality curve of infected insects, while 331 caterpillars were used to determine LD_{50} of the control group. This control group included 90 caterpillars that had been infected with the wild-type AcNPV baculovirus. All larvae were tested in the fifth instar of development, four days after infection. The median lethal dose was calculated using the computational method of Finney[13] on the corrected mortality as determined by Abbott's formula.

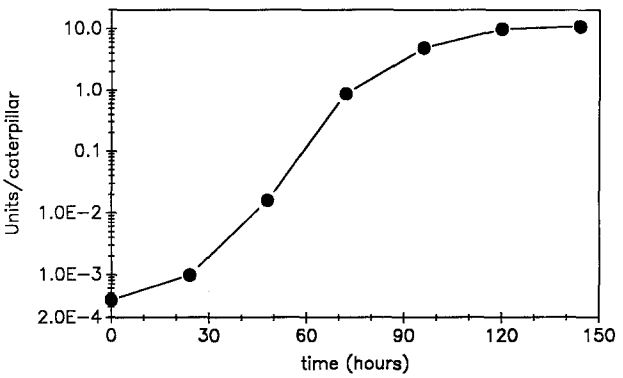

Figure 1. The time course for phosphotriesterase production was followed for up to six days after injection of A5B-*opd*. Each value represents the average of ten caterpillars prepared as described in the text.

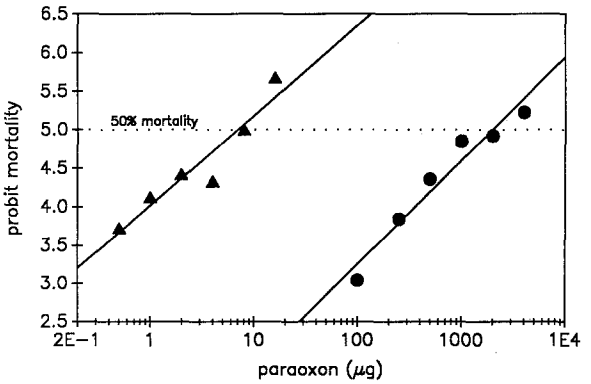

Figure 2. Mortality curve for infected and uninfected caterpillars is shown, where the triangles represent caterpillars that were never exposed to A5B-*opd* and the circles represent caterpillars which had been injected with A5B-*opd* four days prior to application of the paraoxon.

Results and discussion

The production of active phosphotriesterase in A5B-*opd* infected sf9 cells increased logarithmically after a short initial lag with maximum enzymatic activity of more than 10 units/ml per 10^6 cells being achieved 5–6 days after infection when the cells begin to lyse. This protein production mimics the expression of polyhedrin protein in the AcNPV wild-type baculovirus system[9], and represents an overproduction of the phosphotriesterase 40 times greater than the native expression system in *Pseudomonas diminuta*. While this is a substantial increase over the native system, the amount of phosphotriesterase produced (about 7 µg/ml per 10^6 cells) is on the low end of the 1–600 µg/l per 10^6 cells range reported for the baculovirus expression systems[11].

Shown in figure 1 is the time course for the appearance of phosphotriesterase activity in the caterpillars following injection. After a relatively short lag phase, enzymatic activity could be detected in the caterpillar when paraoxon was utilized as a substrate. For the first four days after injection the phosphotriesterase activity increased about one order of magnitude every 24 h until it reached a maximum of approximately 11 units of paraoxon hydrolyzing activity per caterpillar (based upon an average of 250 µl fluids recovered from each caterpillar). No phosphotriesterase activity ($< 5 \times 10^{-5}$ units/caterpillar) could be detected in uninfected caterpillars or caterpillars infected with the wild-type baculovirus. Therefore, the phosphotriesterase activity expressed within the A5B-*opd* infected caterpillars is due solely to the recombinant baculovirus containing the *opd* gene.

The effect of the insecticide paraoxon was determined on both the infected and uninfected caterpillars. Shown in figure 2 is a plot of probit mortality[14] versus the amount of paraoxon that could be applied directly to the caterpillars. The LD_{50} for the A5B-*opd* infected caterpillars was calculated to be 1800 ± 800 µg while the control group had an LD_{50} of 6.5 ± 3 µg.

This graph clearly demonstrates that those caterpillars containing a functional phosphotriesterase are resistant to all but the highest concentration of paraoxon that could be applied. The slopes of the two mortality curves, being roughly the same, demonstrate the death of caterpillars in the A5B-*opd* infected group occurs through a similar mode of action as for the control group[15]. The phosphotriesterase in the caterpillars infected with the recombinant virus is therefore hydrolyzing the paraoxon

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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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-