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Feeding Behavior of *Aedes aegypti* Larvae and Toxicity of Dispersed and of Naturally Encapsulated *Bacillus thuringiensis* var. *israelensis*

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Two modes of feeding behavior, filtering and scavenging, of *Aedes aegypti* larvae were investigated. Ingestion rates of *Bacillus thuringiensis* var. *israelensis* spores and filtering rates of spore suspensions were determined. A rise in filtering rate and a reduction of ingestion rate were observed as the spore concentration was increased. Higher scavenging was found when the carcass density was lower or when another food source was abundant. Consumption of *B. thuringiensis* var. *israelensis*-killed larvae caused death of the scavengers. When the toxicity of the scavengers' carcasses was quantified and compared with that of *B. thuringiensis* var. *israelensis* powder, the latter seemed to be more effective than an equivalent number of spores ingested with a carcass, whether intact or homogenized. Great variability in toxicity of single carcasses was observed and can be explained by a similar variation in the number of spores per carcass at the end of a recycle.

KEY WORDS: *Bacillus thuringiensis* var. *israelensis; Aedes aegypti* larvae; δ-endotoxin; biological control; toxicity quantitation; filter feeding; scavenging behavior.

INTRODUCTION

Dipteran larvae ingest small particles from their aquatic environment by filter feeding (Clements, 1963). We recently described an additional mode of feeding acquired by *Aedes aegypti* larvae, in which they were shown to gnaw and ingest carcasses of their own and of related species (Zaritsky and Khawaled, 1986). This scavenging behavior, which can support development to adults without an additional food source, is fatal if the carcasses are of larvae killed by *Bacillus thuringiensis* var. *israelensis* (Zaritsky and Khawaled, 1986; Zaritsky et al., 1986). The toxicity of these carcasses to scavenging larvae is a result of recycling of *B. thuringiensis* var. *israelensis* spores in the dead larvae (Aly et al., 1985; Ohana, 1985).

Here, we further describe these two modes of feeding behavior. The influence of various factors such as concentration of *B. thuringiensis* var. *israelensis* spores, of the carcasses, and of another particulate food source on feeding behavior was investigated. In addition, multiplication of *B. thuringiensis* var. *israelensis* in larval carcasses and toxicity of these carcasses were quantified.

MATERIALS AND METHODS

Rearing mosquito larvae. Dry strips of paper bearing eggs of *A. aegypti* (provided by Dr. J. Margalit) were submerged for 1 day in 1 liter of sterile tap water supplemented with 1 g of Pharmamedia (Traders). Third instar larvae used in this study were selected after 3–4 days of incubation at 30°C. All further incubations were performed in an open-air water bath at 27° ± 2°C.

Bioassays. Two procedures were employed, one (a) for carcasses of *B. thuringiensis* var. *israelensis*-infected larvae and the other (b) for *B. thuringiensis* var. *israelensis* powder, as follows:

(a) Third instar larvae were introduced into a suspension of *B. thuringiensis* var. *israelensis* powder (R-153-78, Roger Bellon Laboratories, 4 μg/ml). The carcasses of larvae which died during 30 min were introduced (after 30 hr of incubation at 30°C), each to a single larva (primary scavenger), either first or third instar, in 10 ml of sterile

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tap water. The carcasses of the primary scavengers were incubated for 5 days and then introduced singly, intact or homogenized, to varying numbers of third instar larvae (secondary scavengers) in 10 ml of sterile tap water. Mortality of the secondary scavengers was determined 24 hr later. In control experiments, either uninfected larvae (decapitated or homogenized) or *B. thuringiensis* var. *israelensis* powder (70,000 spores/ml) were introduced.

(b) Third instar larvae, treated as in (a) with *B. thuringiensis* var. *israelensis* powder, were washed with sterile tap water at intervals during 20 min and each transferred to a beaker containing 10 ml of sterile tap water. The percentage mortality after 12 hr of incubation and the time between transferring the larvae and their death were monitored.

Filter feeding behavior. Thirty third instar larvae in 300 ml of sterile tap water were treated with various concentrations of *B. thuringiensis* var. *israelensis* powder suspension (1–5 × 10^3 spores/μl). At intervals, they were washed with sterile tap water, homogenized in 2 ml of 1% Tween 80, sonicated for 4 min at 4°C at maximum energy (MSE sonifier), and heat shocked for 10 min at 70°C. The number of *B. thuringiensis* var. *israelensis* colonies formed after 24 hr of incubation at 37°C was determined by plating the sonicated homogenate on LB plates (Miller, 1972) at the appropriate dilutions. The colonies were identified by their morphology as well as by their toxicity to *A. aegypti* larvae (Ohana et al., 1985). *Initial ingestion rates* were calculated using the number of colonies formed per larva for each concentration as a function of time. *Filtering rates* were determined using the ingestion rates and spore concentrations, assuming a 100% efficiency of filtration.

Scavenging Behavior

(*a*) *As a function of Pharmamedia concentration.* One 30-hr-old carcass of a *B. thuringiensis* var. *israelensis*-killed third instar larva was introduced to a third instar larva in a 10-ml sterile tap water suspension of various Pharmamedia concentrations. The time between introduction and scavenging and the percentage of scavengers were monitored.

(*b*) *As a function of carcass density.* Varying number of 24-hr-old carcasses of *B. thuringiensis* var. *israelensis*-killed third instar larvae were introduced to a single third instar larva in 10 ml of sterile tap water. The time between introduction and scavenging was determined.

RESULTS

Filter feeding. The initial rate of ingestion of *B. thuringiensis* var. *israelensis* spores by *A. aegypti* larvae increased with spore

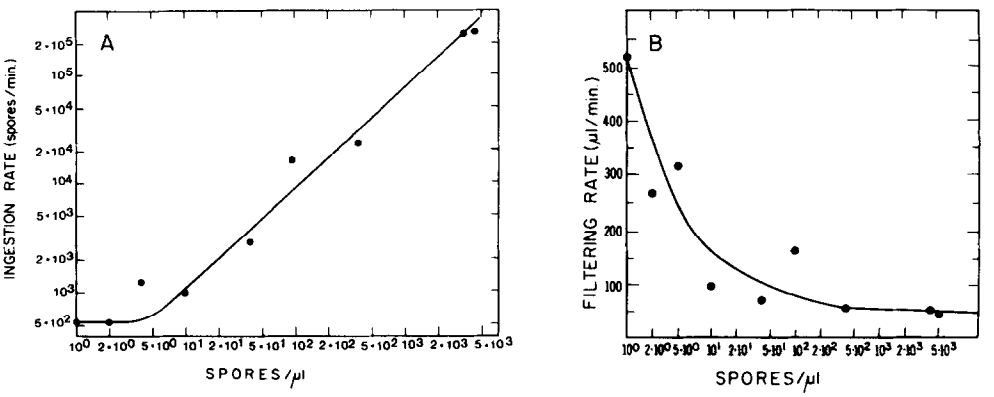

FIG. 1. Rates of spore ingestion (A) and of filtering (B) as functions of spore concentration. Third instar *Aedes aegypti* larvae were used at about 4 hr after molting.

concentration (Fig. 1A), while total filtering rate was decreased from 500 μl/min at 1 spore/μl to a minimum of 50 μl/min at above 200 spores/μl (Fig. 1B). For example, at 250 *B. thuringiensis* var. *israelensis* spores/μl, the number of spores ingested by an early third instar larva increased linearly during the first 7 min and reached 10^5 (Fig. 2), indicating a filtration rate of about 60 μl/min. Larvae started to die at about 15 min. Late third instar larvae filtered about sixfold slower (Fig. 2), and consequently died later (Fig. 3). The number of *B. thuringiensis* var. *israelensis* spores per larva started to decline (Fig. 2) before larval death. The same rate of decline was obtained whether or not the larvae were washed off the spore suspension at 15 min and transferred to sterile water, suggesting that they stopped ingestion before then. Decrease in the death time associated with increase of percentage mortality were observed as the number of ingested spores increased (Fig. 3). The data can be translated to the lethal dose (LD) as follows: 6000 spores ingested within 3 min (Fig. 2) were sufficient to induce 100% mortality within 1.5 hr (Fig. 3).

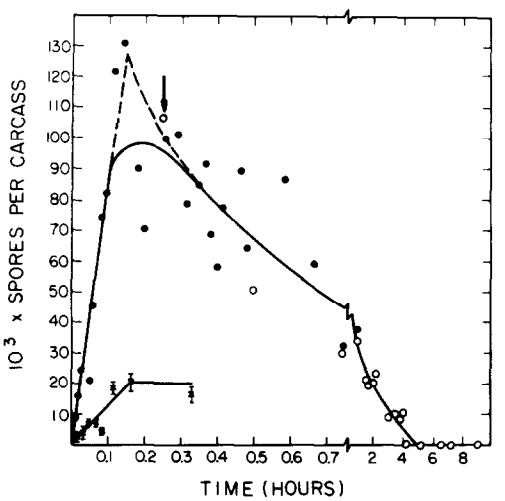

FIG. 2. The fate of *Bacillus thuringiensis* var. *israelensis* spores in larvae treated with *B. thuringiensis* var. *israelensis* powder in the first (○, ●, two similar experiments) or third (x) quarter of third instar. Spore number in single larvae or carcasses. Arrow, beginning of larval death.

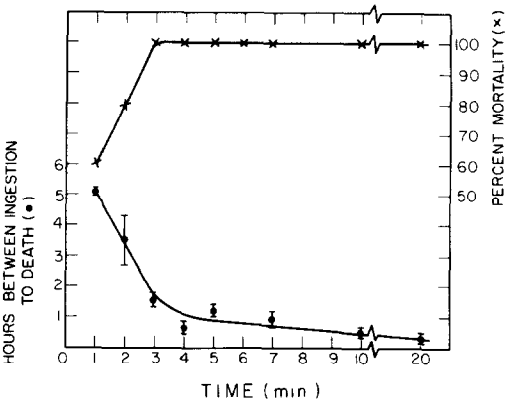

FIG. 3. Influence of period of *Bacillus thuringiensis* var. *israelensis* treatment on larval death. One-hundred third instar larvae (third quarter) were treated in 1 liter of sterile tap water with *B. thuringiensis* var. *israelensis* powder as in Fig. 1. At the indicated times, five larvae were rinsed and incubated. Percentage mortality (x) was determined 12 hr afterward. Death time (●) is the average time of death during the incubation period. Bars represent standard errors.

Scavenging behavior. The time it took a third instar scavenger to effectively approach and scavenge a third instar carcass was determined previously to be 7 min when the carcass' age was 6 hr (Zaritsky et al., 1986). This time increased with the concentration of Pharmamedia (Fig. 4), but decreased with increased carcass concentration (1 min at more than 6 in 10 ml) (Fig. 5).

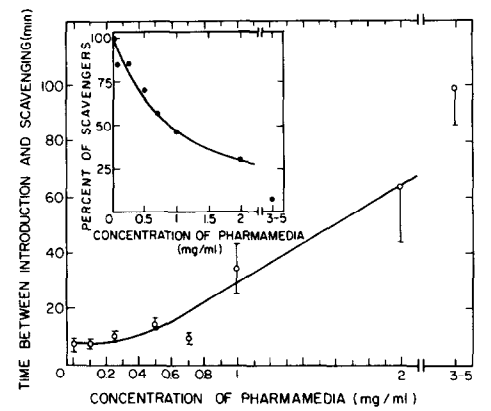

FIG. 4. Scavenging behavior as a function of Pharmamedia concentration (13–15 larvae for each concentration). ○, Average time between introduction and scavenging among the first six scavengers. Inset (●), percentage of scavengers during the first 2 hr. Bars represent standard errors.

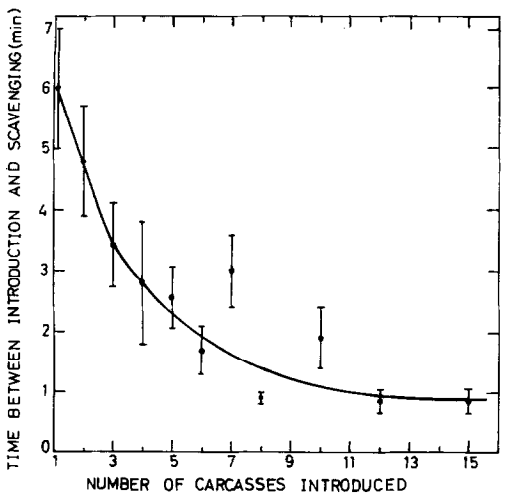

FIG. 5. Scavenging behavior as a function of carcass density. Each point is an average of five repetitions. Bars represent standard errors.

In addition, the percentage of scavengers was reduced upon raising the particulate food concentration (Fig. 4, inset).

Toxicity of carcasses. The toxicity to secondary scavengers of an intact or homogenized carcass of primary scavenger larva is expressed as the number of scavengers that died as a function of their density (Fig. 6). A homogenized carcass of first and of third instar larva caused death of 80–100% of scavengers at concentrations of 15 and 40 secondary scavengers/10 ml, respectively. The killing potential of intact carcasses was lower. It was never reached with an intact carcass of a first instar larva and was reduced fourfold with a carcass of a third instar larva (12 compared to 45). A high degree of variability was observed in toxicity of these carcasses. The possibility that this great heterogeneity in carcass toxicity is due to differences in the scavenging activity of the larvae was ruled out because a similar variability of toxicity was induced by *homogenized* carcasses, which were introduced in the same manner (Fig. 6). The simplest explanation for the observed heterogeneity is a similar variability in the number of *B. thuringiensis* var. *israelensis* spores per carcass. Three levels of spore yields were indeed observed in carcasses of third instar larvae as follows: $3.3 \pm 1.5 \times 10^4$, $3.0 \pm 0.87 \times 10^5$, and $1.75 \pm 0.63 \times 10^6$ spores per carcass (Fig. 7). These levels were reached 2 to 3 days after larval death and remained constant for at least 1 week. Introduction of uninfected larvae (decapitated or homogenized) did not induce any mortality.

A suspension of 700,000 spores/10 ml was sufficient to kill 70 of 100 larvae introduced (Fig. 8). A carcass of third instar *B.*

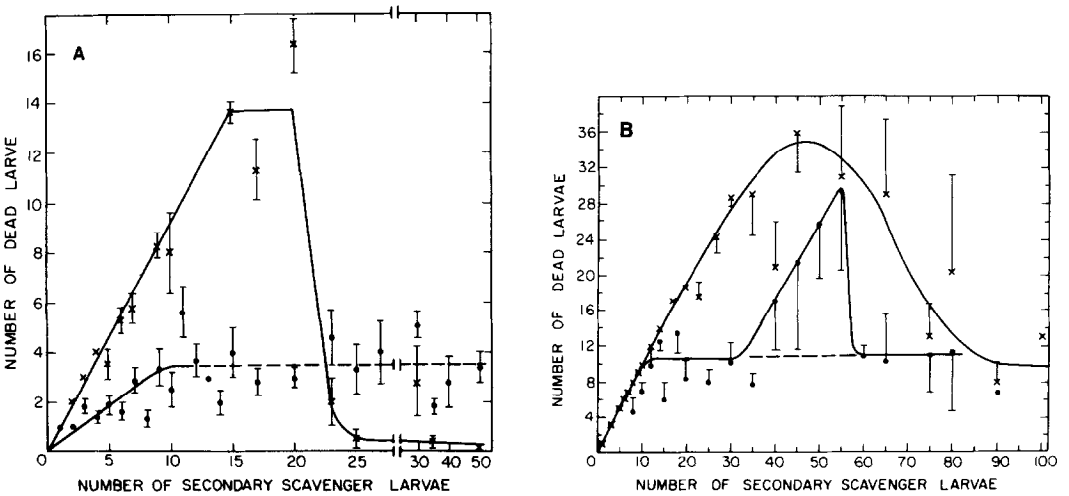

FIG. 6. Biological quantitation of the toxin produced in intact (●) and homogenized (x) carcasses of *Bacillus thuringiensis* var. *israelensis*-killed first instar (A) and third instar (B) *Aedes aegypti* larvae, introduced singly to the indicated numbers of secondary scavengers (third instar). Each point is an average of three experiments, each executed in five repetitions. Bars represent standard errors.

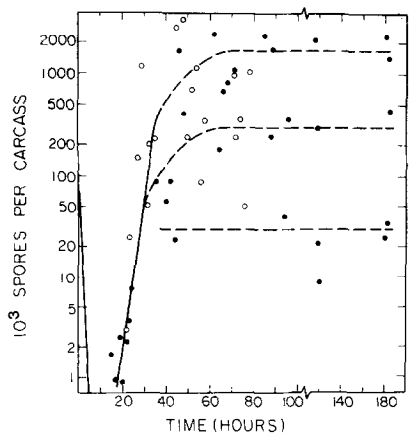

FIG. 7. The fate of *Bacillus thuringiensis* var. *israelensis* spores in larvae treated with *B. thuringiensis* var. *israelensis* powder in the first quarter of third instar. Data of the two experiments of Fig. 2 (○, ●, respectively).

thuringiensis var. *israelensis*-killed larva, including on the average a similar number of spores, resulted in 10 dead larvae out of 100 (Fig. 6B). In the same series of experiments, an average of 1.4 ± 0.7 larvae per 100 died after 24 hr of incubation in sterile water.

DISCUSSION

In our filtering studies, *B. thuringiensis* var. *israelensis* spores were used as tracers for water flow through the brushes of *A. aegypti* larvae (Surtees, 1959). Assuming that each spore collected by the brushes is consequently ingested (without any rejection), we conclude that filtering rate of early third instar larvae depends on spore concentration. An increased spore concentration led to a decrease in the filtering rate (Fig. 1B), but to a lesser degree than expected if ingestion rate is not altered. Hence, an increase in the ingestion rate is obtained (Fig. 1A). Extensive data regarding filter feeding of mosquito larvae (Gophen and Gophen, 1986), blackfly larvae (Hart and Steven, 1986), *Lampetra planeri* larvae (Malmquist and Bronmark, 1981), copepods (Frost, 1972; Lam and Frost, 1976), rotifers (Starkweather et al., 1979), and *Daphnia* (McMahon and Rigler, 1963) demonstrate that upon increasing the food concentration, these organisms increase their filtering rate or maintain it at a constant level until a critical food concentration is exceeded, above which filtering rate is reduced. The decrease in the filtering rate of *A. aegypti* larvae may therefore be one of the poisoning effects of the *B. thuringiensis* var. *israelensis* δ-endotoxin. The results of Fig. 2 support this conclusion since the filtering activity ceased 5–10 min following the introduction of the larvae into a highly concentrated spore suspension. Both early and late third instar larvae stopped ingestion at about the same time irrespective of the number of spores ingested (Fig. 2). This implies a fast response of filtering activity to a low yet toxic dose of δ-endotoxin.

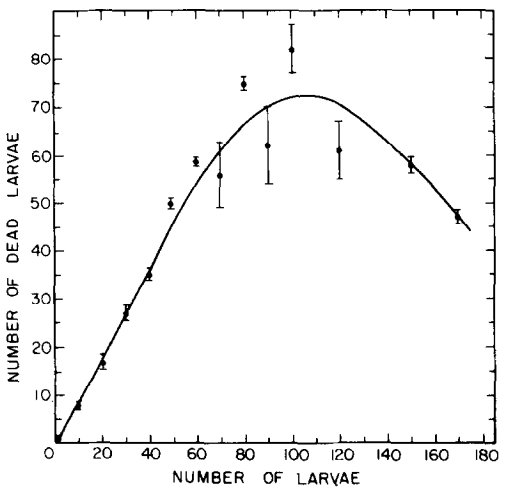

FIG. 8. Quantitation of the larvicidal activity of *Bacillus thuringiensis* var. *israelensis* powder, at 700,000 spores per 10 ml. Bars represent standard errors in three separate experiments.

A rapid and sensitive method for determination of toxicity of any *B. thuringiensis* var. *israelensis* powder thus arises: brief treatments of late third instar *A. aegypti* larvae, for which a concentration response curve (such as Fig. 1A) is yet to be described, and determination of death time and of percentage mortality (Fig. 3) 2–5 hr later, would allow computation of the powder's lethal dose.

The decrease in the number of spores per larva/carcass (Fig. 2) substantiates previ-

ous observations (Aly, 1985; Ohana, 1985; Barak et al., 1987). However, we are not convinced that the number of spores starts to decline before larval death as is shown by the dashed line in Fig. 2. If it is real, it may result of germination or of digestion. In order to discriminate between these possibilities, the total number of *B. thuringiensis* var. *israelensis* colony forming units should be determined by using a drug-resistant mutant of *B. thuringiensis* var. *israelensis* (Barak et al., 1987) to allow selection against other microorganisms.

Carcasses of *B. thuringiensis* var. *israelensis*-killed larvae have been shown to support growth and multiplication of this bacterium (Aly et al., 1985; Ohana, 1985; Barak et al., 1987), hence to become larvicidal to scavenging larvae (Zaritsky and Khawaled, 1986). This larvicidal activity was however less effective than that of lyophilized powder with an equivalent number of *B. thuringiensis* var. *israelensis* spores (Figs. 6B and 8). The lower activity may be due to incomplete homogenization of the carcasses so that doses higher than the lethal one are quickly ingested by a small number of larvae which will consequently die, and a concentration lower than the lethal concentration is left in suspension. Two lines of evidence support this interpretation (Fig. 6):

(a) Toxicity of carcasses of third instar larvae was only threefold higher than that of carcasses of first instar larvae despite the fact that spore numbers in them were sevenfold higher [A carcass of first instar larva contains $0.12 \pm 0.04 \times 10^6$ spores (Zaritsky et al., 1986)];

(b) Toxicity of intact carcasses was fourfold lower than of homogenized carcasses of the same origin.

A lower larvicidal activity of a carcass can also be explained by an efficient regeneration of damaged epithelial midgut cells at low toxin dosage in the presence of an excess amount of nutritional food source, as was observed in Lepidopteran larvae (Chiang et al., 1986). The carcass itself has been shown to serve as a nutritional food source for mosquito larvae (Zaritsky and Khawaled, 1986) and may thus allow some of the infected larvae to survive and thereby increase the lethal dose (Figs. 6B and 8). This hypothesis predicts that the lethal dose is higher in the presence of another food source, as has been reported before (Ramoska and Pacey, 1979; Ignoffo et al., 1981; Aly, 1983).

The scavenging activity of mosquito larvae allows "encapsulation" of *B. thuringiensis* var. *israelensis* spores inside carcasses of *B. thuringiensis* var. *israelensis*-killed larvae. However, a high efficacy of these "naturally encapsulated" *B. thuringiensis* var. *israelensis* spores depends on their completion of a full growth cycle inside the carcass *before* it is ingested by scavenging larvae or by other water-dwelling organisms.

The heterogeneity in carcass toxicity (Fig. 6) did not result from differences in scavenging behavior because homogenizing the carcasses did not reduce the variability. It stems apparently from the three sporulation levels observed inside the decaying carcasses of third instar *B. thuringiensis* var. *israelensis*-killed larvae (Fig. 7). Existence of three sporulation levels in *B. thuringiensis* var. *israelensis*-killed larvae can only be detected when a *single* carcass is homogenized at a time. Mean spore content per carcass among all 35 carcasses tested in this study (Fig. 7) is 8.8×10^5, similar to those obtained for carcasses of *A. aegypti* (1.45×10^5 and 0.7×10^5; Aly et al., 1985 and Barak et al., 1987, respectively) and of *Aedes albimanus* larvae (3.3×10^5) (Aly et al., 1985) when several carcasses were homogenized together.

Different levels of sporulation yield may arise when different quantities of *B. thuringiensis* var. *israelensis* spores are ingested if multiplication in the carcass is restricted due to a competition with other microorganisms, for example. This possibility can be tested by rearing the larvae in asep-

tic conditions or by using drug-resistant mutants of *B. thuringiensis* var. *israelensis* in the presence of the drug(s) to select against other infecting microorganisms (Barak et al., 1987).

Another factor that may contribute to this heterogeneity in toxicity of single carcasses is a variation in the scavenging activity of the secondary scavengers which may feed on carcasses that resulted during the bioassay. If these are scavenged at 10 hr or later following their death, they can cause death of the secondary scavengers (Zaritsky et al., 1986). To eliminate this presumed factor, the bioassay period must not exceed 17–18 hr, as was already recommended (Zaritsky and Khawaled, 1986).

Our observations regarding scavenging behavior of mosquito larvae imply that their biocontrol is not limited to small unicellular organisms due to an exclusive filter feeding. The genes encoding the *B. thuringiensis* var. *israelensis* cyrstal proteins could therefore be engineered into larger "delivery organisms" for an efficient expression. The likely organisms to be exploited should have readily manipulated genetical systems. Such candidates should also be checked for competitive consumption ability by the target organisms.

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