

Studies on Transfection and Transformation of Protoplasts of *Bacillus larvae*, *Bacillus subtilis*, and *Bacillus popilliae*

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Protoplasts of *Bacillus larvae* NRRL B-3555 and *Bacillus subtilis* RM125 (restrictionless, modificationless mutant) were transfected with DNA from the *B. larvae* bacteriophage PBL1c in the presence of polyethylene glycol. *B. subtilis* 168 and *Bacillus popilliae* NRRL B-2309M protoplasts could not be transfected with PBL1c DNA. Protoplasts of *B. larvae* NRRL B-3555 were transformed with plasmids pC194 and pHV33 in the presence of polyethylene glycol. The frequency of transformation was much higher when the plasmids were isolated from *B. larvae* NRRL B-3555 transformants than when they were isolated from *B. subtilis* 168. These results indicate that the restriction-modification systems found in *B. larvae* NRRL B-3555 and *B. subtilis* 168 may be different. Conditions for protoplast formation and cell wall regeneration were developed for *B. popilliae* NRRL B-2309S. However, no transformation occurred with plasmids pC194 and pHV33 (isolated from *B. subtilis* 168).

Bacillus larvae is a pathogen that causes a widespread fatal disease of honeybees called American foulbrood. Only spores are able to transmit the disease (24). *B. larvae* sporulates efficiently in the hemolymph of honeybees but with difficulty in broth media (5). This characteristic is shared by *Bacillus popilliae*, a pathogen of Japanese beetles that is used for biological control of its host. Another unusual characteristic shared by these two species is that they are catalase negative, based on the commonly used loop test of placing a drop of H₂O₂ on a colony and observing for oxygen bubble formation (13). It is now known that both species contain catalase during sporulation but at extremely low levels (3, 6). Because of the unusual characteristics of sporulation in *B. larvae* and *B. popilliae*, only some of which were alluded to above, and because of the economic importance of these bacteria as insect pathogens, we have decided to study these organisms from both physiological and genetic perspectives.

Recent advances in protoplast transformation (2) have opened up the possibility of conducting gene transfer (and cloning) experiments in organisms that have no known "normal" methods of gene transfer. This study deals with use of the protoplast transformation procedure for transfection and plasmid transformation. As indicated here, we have been successful in demonstrating these processes in *B. larvae* but not as yet in *B. popilliae*.

MATERIALS AND METHODS

Microorganisms and bacteriophage used. The bacterial strains used in this study were *B. larvae* NRRL B-3555, *B. popilliae* NRRL B-2309M, *B. popilliae* NRRL B-2309S, *Bacillus subtilis* 168, *B. subtilis* RM125 (*arg15 leuA8* r_M m_M), *B. subtilis* BGSC IE17 (*trpC2*) (pC194), and *B. subtilis* 65(pHV33). *B. subtilis* RM125 and *B. subtilis* 65 were kindly provided by T. Uozumi (University of Tokyo) and Inge Mahler (Brandeis University), respectively. The bacteriophage used in this study was PBL1c, a clear-plaque mutant of PBL1, a phage originally isolated from *B. larvae* NRRL B-3553 (4, 10, 11).

Culture conditions. Unless otherwise specified, the bacteria were grown in MYPGP broth in a rotary shaker or on MYPGP agar (4) at 37°C (*B. larvae* and *B. subtilis*) or 30°C (*B. popilliae*). *B. subtilis* cultures were grown for plasmid isolation in L broth supplemented with 5 µg of chloramphenicol per ml. L broth contains per liter, tryptone (10 g), yeast extract (5 g), NaCl (5 g), and glucose (1 g).

Growth of PBL1c. Titters of 10¹⁰ PFU of PBL1c per ml were obtained by infecting *B. larvae* NRRL B-3555 growing in MYPGP broth (at a cell density of ca. 2 × 10⁸ cells per ml) with PBL1c at a multiplicity of infection of 10.0. Phage titers were determined by a standard soft-agar overlay technique (4).

Purification of PBL1c and isolation of PBL1c DNA. PBL1c from lysates of *B. larvae* NRRL B-3555 was purified on CsCl step gradients as previously described (4). PBL1c DNA was isolated by the procedure of Ito et al. (15).

Protoplast preparation. Cells to be used for protoplast preparation were grown in MYPGP broth with shaking to a cell density of ca. 5 × 10⁷ cells per ml (20 Klett units; no. 54 filter). Cell pellets resulting from the centrifugation of 25 ml of culture were suspended in 4 ml of SMM (+BSA)-MYPGP. This reagent consists of equal volumes of 2X SMM (+BSA) and 2X MYPGP broth. 2X SMM is 0.8 M sucrose, 0.04 M maleic acid, and 0.04 M MgCl₂ (adjusted to pH 6.5 with NaOH). 2X SMM (+BSA) is 2X SMM supplemented with 2% bovine serum albumin (2).

Protoplasts of *B. larvae* NRRL B-3555, *B. subtilis* 168, and *B. subtilis* RM125 were produced by treatment of cells with 20 mg of egg white lysozyme (Sigma Chemical Co.) per ml for 2 to 3 h at 37°C with gentle shaking. Protoplasts of *B. popilliae* NRRL B-2309S and NRRL B-2309M were prepared by treatment of cells with 1,430 U of mutanolysin (Sigma Chemical Co.) per ml for 2 to 3 h at 30°C. The protoplast suspensions were centrifuged at 1,900 × g for 10 min at 25°C. The protoplasts were washed in 10 ml of SMM-MYPGP. The pellets were resuspended in 1 ml of SMM-MYPGP. These protoplast suspensions were used for transfection, polyethylene glycol-mediated phage infection, and transformation.

Procedure for transfection of protoplasts. Transfection of protoplasts with PBL1c DNA was performed at room tem-

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perature by use of a modification of the plasmid transformation technique described by Chang and Cohen (2). Phage DNA (5 μ g in 0.1 ml of 20 mM Tris-hydrochloride-1 mM EDTA, pH 7.4) and 0.1 ml of 2X SMM were placed in a sterile 25-ml Corex centrifuge tube. Protoplast suspension (0.5 ml) was added, immediately thereafter 1.5 ml of 40% polyethylene glycol 1000 (dissolved in SMM) was added, and the suspension was mixed. After 2 min, 5 ml of SMM (+BSA)-MYPGP was added, and the suspension was centrifuged. The protoplast pellet was washed with 10.0 ml of SMM (+BSA)-MYPGP and resuspended in 1.0 ml of the same medium. The protoplast suspensions were incubated with gentle shaking at 37°C (*B. larvae* and *B. subtilis*) or 30°C (*B. popilliae*). Samples (0.1 ml) were withdrawn at specific times, and dilutions were made in water. Protoplast lysis occurs in water, and PBL1c is stable therein (4). PBL1c was assayed as described above.

Procedure for polyethylene glycol-mediated infection of protoplasts with PBL1c. PBL1c (10^3 PFU) was added to 1 ml of protoplast suspension just before polyethylene glycol was added. The subsequent dilution of protoplasts, washing, incubation, sample dilution, and phage assay were performed as described above for transfection.

Procedure for plasmid transformation of protoplasts. The transformation procedure was basically the same as the procedure used for transfection. Plasmid DNA (22 μ g) was added to the protoplast suspension. After transformation was completed in the presence of polyethylene glycol, the protoplasts were washed as described above and suspended in 1.0 ml of SMM (+BSA)-MYPGP. The protoplasts were incubated at 37°C with gentle shaking for 2.25 h to allow expression of the plasmid-borne antibiotic resistance genes. After the incubation period, the protoplast suspension was plated on regeneration medium (MYPGP agar plus 0.4 M sucrose for *B. larvae* and MYPGP agar plus 0.2 M sucrose for *B. popilliae*) supplemented with the appropriate antibiotic. The colonies that appeared represented presumptive transformants. Confirmation that transformation had occurred was obtained by isolation of plasmids from the cells.

Isolation and purification of plasmids. Plasmids pC194 and pHV33 were first isolated from *B. subtilis* BGSC IE17 (*trpC2*) (pC194) and *B. subtilis* 65(pHV33), respectively. Both strains are derivatives of *B. subtilis* 168. The cultures were grown in 1 liter of L broth supplemented with 5 μ g of chloramphenicol per ml. After centrifugation, the cell pellet was suspended in 100 ml of 20 mM Tris-hydrochloride (pH 7.4)-10 mM EDTA. Lysozyme and RNase were added to concentrations of 0.5 mg/ml and 20 μ g/ml, respectively, and the suspension was incubated at 37°C for 40 min. Next, 7.6 ml of Triton lysis buffer (1.32% Triton X-100, 0.16 M Tris-hydrochloride [pH 8.0], 0.2 M EDTA) was added, and the cell debris was removed by centrifugation at $27,100 \times g$ for 20 min. The supernatant was shaken with an equal volume of phenol (equilibrated with 20 mM Tris-hydrochloride [pH 7.4]-10 mM EDTA). DNA was precipitated from the aqueous phase by the addition of 3 volumes of 95% ethanol. The mixture was kept overnight at -20°C. The DNA pellet resulting from centrifugation at $33,800 \times g$ was dissolved in 4.15 ml of 20 mM Tris-hydrochloride (pH 7.4)-1 mM EDTA. CsCl (4.25 g) was dissolved in the DNA solution, and 0.165 ml of ethidium bromide (10 mg/ml in 20 mM Tris-hydrochloride [pH 7.4]) was added. The solution was placed in a Beckman 5-ml Quick-seal tube and centrifuged at 55,000 rpm for 7 h in a VTi65 rotor. The bottom plasmid band was removed, and ethidium bromide was extracted with five 1-ml portions of *n*-butanol (previously

equilibrated with a solution containing 40 g of CsCl in 40 ml of 20 mM Tris-hydrochloride [pH 7.4]-1 mM EDTA). The CsCl was removed from the DNA by sequential dialysis against three 1-liter portions of 20 mM Tris-hydrochloride (pH 7.4)-1 mM EDTA for 12 h each.

The method used for isolating plasmids from *B. larvae* was a scaled-up version of the procedure used by Williams et al. (26) for plasmid isolation from 1-ml cultures. Bacteria were grown in 1 liter of MYPGP broth supplemented with 5 μ g of chloramphenicol per ml. The culture was centrifuged, and the pellet was suspended in 100 ml of lysing buffer. After the addition of potassium acetate and centrifugation, the supernatant was extracted with an equal volume of phenol. DNA in the aqueous phase was precipitated by the addition of 3 volumes of 95% ethanol. The plasmids were further purified by cesium chloride-ethidium bromide ultracentrifugation as described above.

RESULTS

Transfection of protoplasts. DNA from PBL1c was used to transfect protoplasts of *B. larvae* NRRL B-3555, *B. popilliae* NRRL B-2309M, and *B. subtilis* 168. Intact PBL1c caused lysis of only *B. larvae*, but it is possible that the lack of productive infection of the other species was due to the lack of binding of the phage to the cells or to the lack of injection of DNA. It was considered possible that the protoplast transfection procedure would bypass these barriers. When

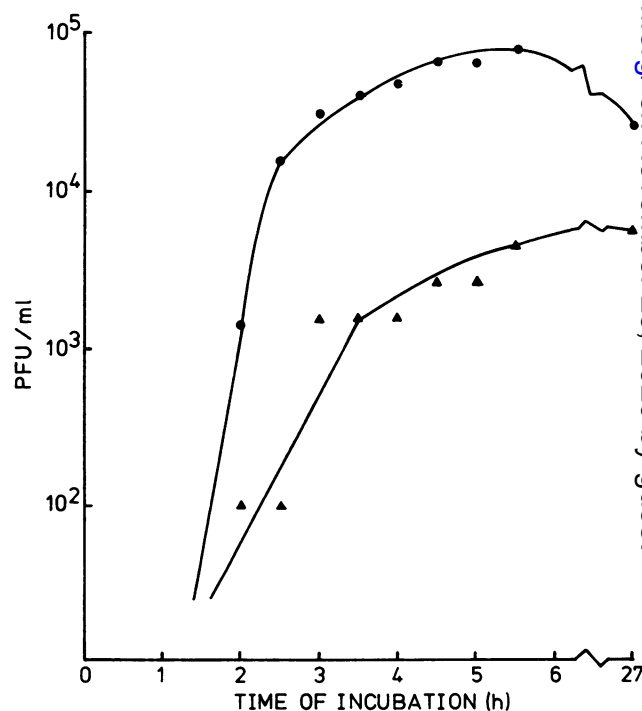


FIG. 1. Transfection of protoplasts of *B. larvae* NRRL B-3555 (●) and *B. subtilis* RM125 (▲) with PBL1c DNA. Cultures of both strains were treated with lysozyme for protoplast generation. PBL1c DNA (5 μ g) and polyethylene glycol were added. After being washed and suspended, the protoplasts were incubated at 37°C with gentle shaking, and samples were removed periodically for plaque assaying. The samples were diluted in water before being assayed, a procedure that causes lysis of protoplasts. Therefore, the titers represent total phage per milliliter (extracellular and intracellular). The indicator strain was *B. larvae* NRRL B-3555.

protoplasts of *B. larvae* were transfected with 5.0 μg of PBL1c DNA in the presence of polyethylene glycol, a maximum yield of 8.2×10^4 PFU/ml was observed (Fig. 1). No transfection of protoplasts of *B. subtilis* 168 or *B. popilliae* NRRL B-2309M occurred (data not shown).

B. subtilis 168 contains a restriction endonuclease, *Bsu*M (16), which may have prevented transfection. To test this hypothesis, we repeated the transfection experiment with *B. subtilis* RM125, a mutant lacking a restriction-modification system. Transfection of protoplasts with 5 μg of PBL1c DNA in the presence of polyethylene glycol resulted in a maximum yield of 5.8×10^5 PFU/ml (Fig. 1). *B. subtilis* RM125 cells were not infected by intact PBL1c.

Protoplasts of *B. popilliae* NRRL B-2309M were prepared by treatment of cells with mutanolysin, whereas lysozyme was used for *B. larvae* NRRL B-3555 and *B. subtilis* 168. It is possible that the mutanolysin treatment rendered the protoplasts unable to take up DNA. To test this hypothesis, we generated protoplasts of *B. larvae* by treating the cells with mutanolysin. Transfection of these protoplasts with 5 μg of PBL1c DNA yielded a maximum phage titer of 8.8×10^4 PFU/ml, a result comparable to that obtained with lysozyme-generated protoplasts. Therefore, the lack of transfection of *B. popilliae* protoplasts probably was not due to the mutanolysin treatment.

Infection of protoplasts with PBL1c. An experiment was performed to determine whether polyethylene glycol could cause intact phage particles to enter protoplasts and cause a productive infection. The experiment, involving protoplasts of *B. larvae* NRRL B-3555, *B. popilliae* NRRL B-2309M, and *B. subtilis* RM125, was essentially the same as the transfection experiments described above, except that intact PBL1c phage was used. Over a period of 7 h, there was no significant increase in the phage titer of the samples (data not shown).

Development of media suitable for detection of plasmid transformants of *B. larvae* and *B. popilliae*. The plasmids used were pC194, originally a *Staphylococcus aureus* plasmid (7), and pHV33, a chimeric plasmid constructed in vitro from pC194 and pBR322 (21). pC194 has a chloramphenicol resistance gene. pHV33 has genes coding for resistance to chloramphenicol, tetracycline, and ampicillin. The medium used to detect transformants must contain one or more antibiotics. In addition, it must prevent lysis of protoplasts and permit regeneration of cell walls.

To decide what concentration of antibiotics to use, we found it necessary to know the natural limit of tolerance to the antibiotics referred to above. *B. larvae* NRRL B-3555 on MYPGP agar was inhibited by 10 μg of chloramphenicol per ml, 5 μg of tetracycline per ml, or 100 μg of ampicillin per ml in the medium. The highest concentrations of the antibiotics that allowed growth were 5 μg of chloramphenicol per ml, 1 μg of tetracycline per ml, and 50 μg of ampicillin per ml. The concentrations of chloramphenicol, tetracycline, and ampicillin used to detect *B. larvae* transformants were 10, 5, and 100 $\mu\text{g}/\text{ml}$, respectively. The natural limit of tolerance of *B. popilliae* NRRL B-2309S was determined for chloramphenicol only. Growth on MYPGP agar was inhibited by 10 μg of chloramphenicol per ml. The highest concentration of chloramphenicol that allowed growth was 5 $\mu\text{g}/\text{ml}$. Therefore, the concentration of chloramphenicol used to detect *B. popilliae* transformants was 10 $\mu\text{g}/\text{ml}$.

Development of a medium that would allow cell wall regeneration was first attempted with *B. larvae* NRRL B-3555. Media used by other investigators to promote cell wall regeneration were tested first. Some modifications were

TABLE 1. Transformation of protoplasts of *B. larvae* NRRL B-3555 with plasmids isolated from *B. subtilis*

Plasmid	No. of transformants per ml ^a	% Transformation ^b
pC194	23	7.7×10^{-5}
pHV33	1	3.3×10^{-6}

^a The number of transformants per milliliter of transformation mixture was calculated from the number of colonies appearing after plating on MYPGP agar plus 0.4 M sucrose and 10 μg of chloramphenicol per ml.

^b The percentage of transformation was calculated as follows: (number of transformants per milliliter/total number of regenerated cells per milliliter) \times 100. The number of regenerated cells was determined by plating the nontransformed protoplasts on regeneration medium without added antibiotics. In this experiment, the number of regenerated cells per milliliter was 3.0×10^7 . The percentage of regeneration of protoplasts in this experiment was 6.5%.

made to make them nutritionally adequate for the growth of *B. larvae*. None of the following media permitted significant colony development of protoplasts of *B. larvae*: (i) the medium of Gray and Chang (14) for *B. subtilis*; (ii) the medium of Fodor and Alföldi (9) for *Bacillus megaterium*; (iii) the R2 medium of Okanishi et al. (20) for *Streptomyces griseus* and *Streptomyces venezuelae*; (iv) the medium of Stahl and Pattee (23) for *S. aureus*; and (v) the medium of Landman et al. (18) for *B. subtilis*, modified by the inclusion of 64.46 g of sucrose per liter instead of succinate.

Allcock et al. (1) reported that of several osmotic stabilizers tested in regeneration media for *Clostridium acetobutylicum* protoplasts, sucrose supported the highest frequency of regeneration. A concentration of 0.5 M was optimal. Therefore, we conducted a study on the effect of supplementation of MYPGP agar with different concentrations of sucrose on the growth of *B. larvae* NRRL B-3555 and then on cell wall regeneration plus growth. The highest sucrose concentration permitting good growth was 0.4 M. When protoplasts were plated on MYPGP agar plus 0.4 M sucrose, 5% regeneration occurred. This was determined by comparing the number of colonies after incubation with the number of protoplasts plated. The protoplast count was made with a Petroff-Hauser counting chamber. No colonies appeared when protoplasts were plated on MYPGP agar not supplemented with sucrose. A similar study was made with *B. popilliae* NRRL B-2309S. The highest sucrose concentration permitting good growth was 0.2 M. When protoplasts were plated on MYPGP agar plus 0.2 M sucrose, 6.4% regeneration occurred. Nonsupplemented MYPGP agar and MYPGP agar plus 0.4 M sucrose resulted in 0.8 and 1.3% regeneration, respectively.

Transformation of protoplasts with plasmid DNA. Table 1 shows the results of a transformation experiment in which *B. larvae* NRRL B-3555 protoplasts were transformed with pC194 and pHV33 (both isolated from *B. subtilis*). The DNA concentration in the transformation mixtures was 10 $\mu\text{g}/\text{ml}$. Transformation with both plasmids did occur, but the percentages of transformation were extremely low.

One possible explanation for the extremely low percentages of transformation is that *B. larvae* NRRL B-3555 may have a restriction-modification system that differs from that of *B. subtilis* 168 strains, the source of the plasmids. Therefore, we decided to isolate plasmids from the *B. larvae* transformants and to use them for transformation of *B. larvae*.

pHV33 and pC194 were isolated from the *B. larvae* transformants by the procedure described by Williams et al. (26) (see above). No difference was observed in the mobility of the plasmids isolated from *B. larvae* and *B. subtilis* upon

agarose gel electrophoresis (data not shown). No plasmids were detected in nontransformed *B. larvae* NRRL B-3555.

pHV33 and pC194 preparations obtained from *B. larvae* NRRL B-3555 transformants were used to transform *B. larvae* NRRL B-3555 protoplasts. The number of pC194 transformants per ml was 9.6×10^2 (a percent transformation of 3.2×10^{-3}). The number of pHV33 transformants per ml was 6.0×10^2 (a percent transformation of 2.0×10^{-3}). The percentages of transformation observed, although still low, were 42 and 600 times higher than when pC194 and pHV33 were isolated from *B. subtilis* (Table 1). These data strongly support the hypothesis that there are different restriction-modification systems in *B. subtilis* and *B. larvae*.

pHV33 and pC194 (both isolated from *B. subtilis*) did not transform *B. popilliae* NRRL B-2309S protoplasts. The DNA concentration in the transformation mixtures was 10 $\mu\text{g/ml}$. The medium used for the detection of transformants was MYPGP agar plus 0.2 M sucrose and 10 μg of chloramphenicol per ml.

Expression of pHV33 antibiotic resistance genes in *B. larvae*. As stated previously, pHV33 is a chimeric plasmid containing the chloramphenicol resistance gene of pC194, an *S. aureus* plasmid, and the ampicillin and tetracycline resistance genes of pBR322, an *Escherichia coli* plasmid. The pHV33 chloramphenicol resistance gene was expressed in *B. larvae*. In fact, transformants were selected as isolates resistant to 10 μg of chloramphenicol per ml. The transfer of transformant colonies to MYPGP agar containing either ampicillin (100 $\mu\text{g/ml}$) or tetracycline (5 $\mu\text{g/ml}$) resulted in no growth. These results indicate that the antibiotic resistance genes of the pBR322 portion of pHV33 are not expressed in *B. larvae*.

DISCUSSION

Before this investigation, no genetic exchange systems were known to exist in *B. larvae*. We have been successful in developing transfection and plasmid transformation systems in *B. larvae*. The transfection system also functioned in *B. subtilis*, and the plasmid transformation system has the potential of permitting genetic exchange among *B. larvae*, *B. subtilis*, and *E. coli*. Attempts to develop a genetic transfer system in *B. popilliae* have thus far been unsuccessful.

Intact phage PBL1c infected *B. larvae* NRRL B-3555 but not *B. subtilis* 168, *B. subtilis* RM125, or *B. popilliae* NRRL B-2309M. Part of the explanation for host specificity is that specific sites on the surfaces of bacteria must exist for phage binding to occur. In transfection, phage receptor sites do not need to be present. If the regulated expression of the phage genes can occur intracellularly, phage production should result. PBL1c DNA transfected protoplasts of *B. larvae* NRRL B-3555 but not protoplasts of *B. subtilis* 168 or *B. popilliae* NRRL B-2309M.

At least in the case of *B. subtilis* 168, the failure to achieve transfection was probably due to the presence in *B. subtilis* 168 of a restriction-modification system different from that in *B. larvae* NRRL B-3555. Evidence supporting this conclusion is that PBL1c DNA did transfect protoplasts of *B. subtilis* RM125, a restrictionless, modificationless mutant strain (25). The lack of transfection in *B. popilliae* NRRL B-2309M also could be due to different restriction-modification systems. Alternatively, it could be that in *B. popilliae* the regulated expression of PBL1c genes does not occur. A restrictionless mutant of *B. popilliae* would be helpful in testing these hypotheses.

Plasmids pC194 and pHV33 isolated from *B. subtilis* transformed *B. larvae* protoplasts, but the percentages of

transformation were extremely low. When plasmids isolated from the *B. larvae* transformants were used, the percentages of transformation were higher by factors of 42 for pC194 and 600 for pHV33. These results are consistent with the hypothesis that *B. larvae* and *B. subtilis* have different restriction-modification systems. Attempts to transform protoplasts of *B. popilliae* were not successful. This could have been due to *B. subtilis* and *B. popilliae* having different restriction-modification systems. Alternatively, *B. popilliae* may be incapable of replicating pC194 and pHV33.

pHV33 is a chimeric plasmid made by joining pC194 (an *S. aureus* plasmid) and pBR322 (an *E. coli* plasmid). Our results indicated that the tetracycline and ampicillin resistance genes on the pBR322 portion of pHV33 are not expressed in *B. larvae*. This situation is the same as that observed in *B. subtilis* (8, 17, 22). In fact, most *E. coli* genes are not expressed in *B. subtilis* (17), whereas most *B. subtilis* genes are expressed in *E. coli*. The blockage in the expression of *E. coli* genes in *B. subtilis* may be at the level of transcription in a few cases. However, most studies indicate that the blockage is at the level of translation (12, 19).

The transfection and plasmid transformation systems developed in this study provide the means by which genes can be cloned in *E. coli* or *B. subtilis* and hybrid molecules can be transferred into *B. larvae*. The possibility of constructing cosmids and phasmids as cloning vectors also exists.

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