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Efficient Transformation of *Bacillus thuringiensis* Requires Nonmethylated Plasmid DNA

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The transformation efficiency of *Bacillus thuringiensis* depends upon the source of plasmid DNA. DNA isolated from *B. thuringiensis*, *Bacillus megaterium*, or a Dam$^-$ Dcm$^-$ *Escherichia coli* strain efficiently transformed several *B. thuringiensis* strains. *B. thuringiensis* strains were grouped according to which *B. thuringiensis* backgrounds were suitable sources of DNA for transformation of other *B. thuringiensis* strains, suggesting that *B. thuringiensis* strains differ in DNA modification and restriction. Efficient transformation allowed the demonstration of developmental regulation of cloned crystal protein genes in *B. thuringiensis*.

Bacillus thuringiensis is a gram-positive bacterium that produces insecticidal crystal proteins during sporulation. Crystal protein genes have been cloned and characterized in *Escherichia coli*, *Bacillus subtilis*, and *Bacillus megaterium* (for recent reviews, see references 14 and 17) because of the lack of an efficient transformation protocol for *B. thuringiensis*. Transformation procedures for *B. thuringiensis* protoplasts (1, 7) and vegetative cells (13) are inefficient, tedious, and time-consuming. Electroporation is an efficient means of introducing plasmid DNA into a number of different bacteria (26), and recently, *B. thuringiensis* transformation by electroporation has been described (2, 5, 16, 20, 25). Here, we report that DNA modifications are important for efficient transformation of *B. thuringiensis*.

The electroporation procedure used for this work is an adaptation of the protocol for *Streptococcus faecalis* (11a). Stationary *B. thuringiensis* cultures grown overnight with shaking at 30°C in BHIG (brain heart infusion plus 0.5% [wt/vol] glycerol) were diluted 1:20 into BHIG and incubated for 1 h at 30°C with shaking. The cells were washed once in EB (0.625 M sucrose–1 mM $MgCl_2$) and suspended in 1/2 volume of EB. A 0.8-ml volume of cells was mixed with less than 10 μl of DNA in a 0.4-cm cuvette, and the mixture was chilled on ice for 5 min. A 5-Ω resistor was set in series between the cuvette and a Bio-Rad Gene-Pulser. A single discharge (2,500 V, 25 μF) was used for electroporation. The cells were incubated on ice for 5 min, diluted into 1.6 ml of BHIG, and incubated with shaking at 30°C for 1 h. The transformation efficiency of the acrystalliferous *B. thuringiensis* subsp. *kurstaki* HD73-26 with pNN101 (24) DNA isolated from *B. megaterium* was 3×10^6 transformants per μg of plasmid DNA.

Bacterial strains used in this work are listed in Table 1. The plasmid used in this work was constructed by inserting the 1.3-kb *Sau*3A fragment containing the N-terminal end of the *cryIIA* gene from HD263 (8) into the *Bam*HI site of the *lac* fusion vector pSK10Δ6, replacing the first eight codons of *lacZ* with the first 45 codons of *cryIIA*. Plasmid pSK10Δ6 (14a) is a 6.2-kb derivative of pMC1403 (6) in which *lacA* and the 3′ end of *lacY* have been deleted. The 3.1-kb *Eco*RI fragment from pBC16 (3) containing the origin of replication and a tetracycline resistance (Tc^r) gene was introduced into the unique *Eco*RI site of the fusion plasmid such that the direction of transcription of the Tc^r gene was opposite to that of the *cryIIA-lacZ* fusion. The resulting plasmid was designated pEG409.

The transformation efficiency of strain HD73-26 was dependent upon the source of plasmid DNA. The source was more important for the large plasmid pEG409 (10.5 kb) than for the smaller pBC16 (4.6 kb). These two plasmids are similar in that both plasmids have the replication origin and Tc^r gene from pBC16, but pEG409 also has the replication origin and ampicillin resistance (Ap^r) gene from pBR322, allowing replication in *E. coli*. The transformation efficiencies of HD73-26 with pBC16 isolated from *B. subtilis* BD170 (10), *B. megaterium* VT1660 (30), or *B. thuringiensis* subsp. *kurstaki* HD73-26 and with pEG409 isolated from *B. megaterium* or *B. thuringiensis* subsp. *kurstaki* were similar (Table 2). However, when pEG409 was isolated from *B. subtilis* and used to transform *B. thuringiensis* subsp. *kurstaki* HD73-26, the transformation efficiency decreased significantly (Table 2). These results might be expected if modification of plasmid DNA occurs in *B. subtilis* but not in *B. thuringiensis* or *B. megaterium* and if *B. thuringiensis* restricts modified DNA. The larger pEG409 might have more sites subject to modification than pBC16 and therefore more sites susceptible to restriction by *B. thuringiensis*.

The transformation efficiency of strain HD73-26 with pEG409 isolated from a Dam$^-$ (DNA adenine methylation negative) Dcm$^-$ (DNA cytosine methylation negative) *E. coli* strain was 500- to 1,000-fold higher than that with pEG409 isolated from EG1418 or EG1884 and was similar to the level observed for plasmid DNA isolated from either *B. thuringiensis* subsp. *kurstaki* or *B. megaterium* (Table 2). We constructed an isogenic set of pEG409-containing strains that differed at only the *dam* and *dcm* loci to determine whether both modifications interfered with efficient transformation. The *dam-13*::Tn*9* allele present in GM2163 was transferred to the isogenic pEG409-containing strains EG1884 and EG1885 by transduction with P1*vir* (27), giving strains EG7311 (Dcm$^+$ Dam$^-$) and EG7312 (Dcm$^-$ Dam$^-$). The Dam$^-$ and Dcm$^-$ phenotypes of the strains were verified by *Mbo*I and *Eco*RII digestion of pEG409 isolated from each strain. All of these strains are Hsd$^+$ and therefore methylate DNA by the *E. coli* K-12 modification system.

When pEG409 was isolated from either a Dam$^+$ Dcm$^-$ or a Dam$^-$ Dcm$^-$ *E. coli* strain, the transformation efficiency of

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TABLE 1. Bacterial strains used in this study

Strain	Description[a]	Construction, reference, or source[b]
E. coli		
GM30	*dcm*$^+$ *dam*$^+$	M. G. Marinus
GM31	*dcm-6 dam*$^+$	21
GM2163	*dcm-6 dam-13*::Tn*9 hsdR2*	22
RR1	*dcm*$^+$ *dam*$^+$ *hsdS20*	4
EG1418	*dcm*$^+$ *dam*$^+$ *hsdS20*(pEG409)	TRF of RR1
EG1883	*dcm-6 dam-13*::Tn*9 hsdR2*(pEG409)	TRF of GM2163
EG1884	*dcm*$^+$ *dam*$^+$(pEG409)	TRF of GM30
EG1885	*dcm-6 dam*$^+$(pEG409)	TRF of GM31
EG7311	*dcm*$^+$ *dam-13*::Tn*9*(pEG409)	TDX (GM2163 × EG1884)
EG7312	*dcm-6 dam-13*::Tn*9*(pEG409)	TDX (GM2163 × EG1885)
B. thuringiensis		
HD73-26	subsp. *kurstaki*	9
HD1	subsp. *kurstaki*	11
HD2	subsp. *thuringiensis*	11
HD3	subsp. *finitimus*	11
HD4	subsp. *alesti*	11
HD7	subsp. *dendrolimus*	11
HD201	subsp. *toumanoffi*	11
HD263	subsp. *kurstaki*	11
HD521	subsp. *indiana*	11
HD536	subsp. *ostriniae*	11
HD567	subsp. *israelensis*	11
HD567-56	subsp. *israelensis*	12

[a] All of the *E. coli* strains, with the exception of RR1 and EG1418, are isogenic [F$^-$ *thr-1 ara-14 leuB6 tonA31 lacY1 tsx-78 supE44* (*glnV44*) *galK2 galT22 hisG4 rpsL136 xyl-5 mtl-1 thi-1* (*mcrA*$^+$ *mcrB*)] except for the differences noted above and except for the fact that GM2163 and EG1883 are also Thr$^+$. The *hsdR2* allele confers an r_K^- m_K^+ phenotype; the *hsdS20* allele confers an r_B^- m_B^- phenotype.

[b] TRF, Transformation; TDX, P1 transduction.

HD73-26 was high compared with the transformation efficiency when pEG409 was isolated from either a Dam$^+$ Dcm$^+$ or a Dam$^-$ Dcm$^+$ strain (Table 3). Therefore, methylation by the *dcm* system, but not by the *dam* system, results in a decrease in the transformation efficiency of HD73-26. However, for two other *B. thuringiensis* subsp. *kurstaki* strains (HD1 and HD263) and for *B. thuringiensis* subsp. *thuringiensis* HD2, the methylation of plasmid DNA by either the *dam* or the *dcm* system in *E. coli* resulted in a decrease in transformation efficiency compared with that observed with plasmid DNA isolated from EG7312 (Table 3). The only other strain that has been reported to restrict DNA modified by either the Dam or the Dcm methylase is *Streptomyces avermitilis* (19). Finally, the transformation frequencies for four other strains (HD3, HD4, HD7, and HD567-56) were low regardless of the source of plasmid DNA (Table 3).

TABLE 2. Transformation of *B. thuringiensis subsp. kurstaki* HD73-26 with DNA from various sources

Transforming DNA source		Transformation efficiency[a] (Tc^r colonies/μg of DNA)
Organism	Plasmid	
E. coli		
EG1418 (r_B^- m_B^- Dam$^+$ Dcm$^+$)	pEG409	5.0×10^2
EG1884 (r_K^+ m_K^+ Dam$^+$ Dcm$^+$)	pEG409	2.2×10^2
EG7312 (r_K^+ m_K^+ Dam$^-$ Dcm$^-$)	pEG409	2.5×10^5
B. subtilis	pEG409	6.4×10^2
	pBC16	2.8×10^5
B. megaterium	pEG409	3.8×10^5
	pBC16	7.5×10^5
B. thuringiensis	pEG409	2.4×10^5
	pBC16	6.3×10^5

[a] *B. thuringiensis* subsp. *kurstaki* HD73-26 was transformed with 0.1 μg of plasmid DNA isolated from the indicated background. Transformants were selected on Luria-Bertani (LB) plates containing 10 μg of tetracycline per ml.

TABLE 3. Electroporation of *B. thuringiensis* with plasmid DNA isolated from *E. coli* DNA methylation mutants

DNA source	Relevant phenotype	No. of Tc^r transformants for the following recipient *B. thuringiensis* strain[a]:				
		HD73-26	HD263	HD1	HD2	HD3,4,7, 567-56[b]
EG1884	Dam$^+$ Dcm$^+$	0	0	0	0	0
EG1885	Dam$^+$ Dcm$^-$	542	0	0	0	0
EG7311	Dam$^-$ Dcm$^+$	0	0	0	0	0
EG7312	Dam$^-$ Dcm$^-$	431	188	127	30	0

[a] Each transformation included 0.06 μg of pEG409 DNA isolated from the indicated donor strain. The outgrowth of the transformation was in a total volume of 2.4 ml. A 0.1-ml volume of the outgrowth was plated on LB plates containing 10 μg of tetracycline per ml.

[b] Number of transformants for strains HD3, HD4, HD7, and HD567-56.

The transformation efficiencies of several strains that had very low transformation efficiencies with pEG409 isolated from EG7312 were improved with plasmid DNA isolated from HD73-26, HD3, or HD567 (Tables 3 and 4). Four different recipient classes of strains were identified: HD3 and HD9 accepted plasmid DNA isolated from HD73-26, HD3, or HD567; HD73 and HD201 accepted plasmid DNA isolated from HD73-26 and HD3 but not from HD567; HD567 accepted only plasmid DNA isolated from the HD567 background; and HD4, HD7, HD521, and HD536 did not accept plasmid DNA from any donor background tested. Plasmid DNA isolated from HD73-26 transformed the same strains as plasmid DNA isolated from HD3. However, HD3 and HD73-26 behaved differently as recipients. HD73 and HD201 were in the same recipient class with respect to the *B. thuringiensis* donor backgrounds tested; however, HD73 can be easily transformed with plasmid DNA isolated from a Dam$^-$ Dcm$^-$ *E. coli* strain, while HD201 cannot (data not shown). These data suggest that there are different modification and restriction systems among *B. thuringiensis* strains and that strains that modify DNA similarly do not necessarily have the same restriction systems.

TABLE 4. Transformation of *B. thuringiensis* strains with pEG409 isolated from different *B. thuringiensis* backgrounds

Recipient strain(s)	DNA source[a]		
	HD73-26	HD3	HD567
HD3 and HD9	+	+	+
HD73 and HD201	+	+	−
HD567	−	−	+
HD4, HD7, HD521, and HD536	−	−	−

[a] Symbols: +, transformation efficiency was greater than 10^4 Tc^r transformants per μg of pEG409 DNA; −, transformation efficiency was less than 200 Tc^r transformants per μg of plasmid DNA.

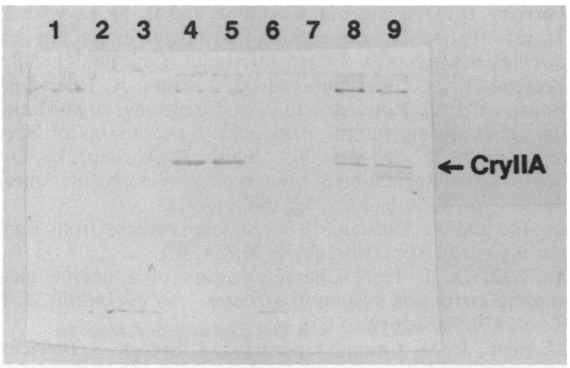

FIG. 1. Developmental expression of a cloned *cryIIA* gene in *B. thuringiensis*. Strains were grown in NSM plus 10 μg of tetracycline per ml to promote sporulation and assayed by the Western immunoassay. HD73-26 is a cured crystal-negative *B. thuringiensis* strain. Plasmid pEG204 carries the *cryIIA* gene from HD263; plasmid pEG213 is a similarly constructed plasmid that carries the *cryIIIA* gene from EG2158. Lane 1, Sporulated HD73-26(pEG213); lanes 2 through 5, mid-log-phase, late-log-phase, mid-stationary-phase, and sporulated samples of HD73-26(pEG204); lanes 6 through 9, mid-log-phase, late-log-phase, mid-stationary-phase, and sporulated samples of HD263.

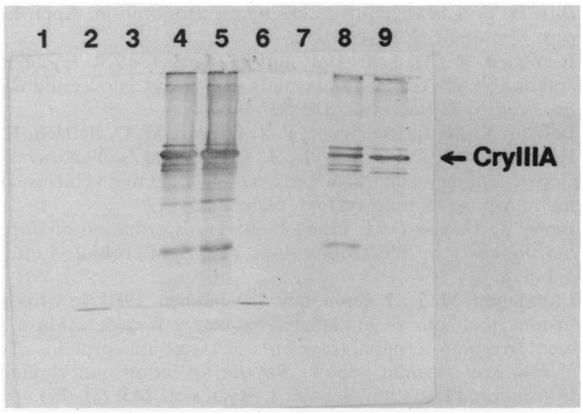

FIG. 2. Developmental expression of a cloned *cryIIIA* gene in *B. thuringiensis*. Strains were grown in NSM plus 10 μg of tetracycline per ml to promote sporulation and assayed by the Western immunoassay. HD73-26 is a cured crystal-negative *B. thuringiensis* strain. Plasmid pEG213 carries the *cryIIIA* gene from EG2158; pEG204 is a similarly constructed plasmid that carries the *cryIIA* gene from HD263. Lane 1, Sporulated HD73-26(pEG204); lanes 2 through 5, mid-log-phase, late-log-phase, mid-stationary-phase, and sporulated samples of HD73-26(pEG213); lanes 6 through 9, mid-log-phase, late-log-phase, mid-stationary-phase, and sporulated samples of EG2158.

The ability of *B. thuringiensis* to be readily transformed makes possible studies of crystal gene expression directly in *B. thuringiensis*. To study expression by using cloned crystal protein genes, it is necessary to determine whether these genes are developmentally regulated in *B. thuringiensis*, as are the genes when present on native plasmids. To determine this, we introduced pEG204 (8), which contained the *cryIIA* gene from HD263 (coding for a lepidopteran- and dipteran-active protein), and pEG213 (9), which contained the *cryIIIA* gene from EG2158 (coding for a coleopteran-active protein), into the acrystalliferous HD73-26 by electroporation.

The time course of expression of the cloned *B. thuringiensis cryIIA* gene present in strain EG7326 [HD73-26(pEG204)] was compared with that of the *cryIIA* gene present on a native plasmid in *B. thuringiensis* subsp. *kurstaki* HD263 (Fig. 1). The strains were grown in NSM (18) (plus 10 μg of tetracycline per ml for recombinant strains). Samples were prepared as previously described (23). Samples containing equal cell masses based on optical density at 600 nm were subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE) (15), blotted onto nitrocellulose (28), and probed with polyclonal antibody raised in mice (29) against SDS-PAGE-purified CryIIA protein. CryIIA antigen could be detected only in stationary-phase cells of both EG7326 and HD263 (Fig. 1). The antibody was specific for CryIIA and did not react with CryIIIA or other proteins in late-stationary-phase cells of strain EG7327 [HD73-26(pEG213)] (Fig. 1, lane 1). A similar analysis comparing EG7327 (containing the cloned *cryIIIA* gene) and EG2158 (containing *cryIIIA* on a native plasmid) demonstrated that the developmental expression of *cryIIIA* in EG7327 is similar to that of *cryIIIA* in EG2158 (Fig. 2). The additional bands seen in Fig. 2 are breakdown products of CryIIIA and are not seen in samples from log phase or in the strain which does not contain the *cryIIIA* gene (Fig. 2, lane 1). Thus, these cloned crystal protein genes are developmentally regulated in *B. thuringiensis*, as are the same genes when they are present on native plasmids in the strains from which they were originally isolated.

The isolation of transforming DNA from *B. megaterium* or a Dam^- Dcm^- *E. coli* strain resulted in a significant improvement of transformation of *B. thuringiensis* and should be applicable to protoplast transformation and all electroporation procedures. The demonstration that modification of plasmid DNA is important for transformation of *B. thuringiensis* provides a basis for strategies to improve the transformation of *B. thuringiensis* strains that are difficult to transform with DNA isolated from the hosts described above. We have demonstrated that the limitation for transformation of many *B. thuringiensis* strains is the source of plasmid DNA and not the electroporation procedure. It should be possible to extend the range of *B. thuringiensis* strains readily transformable via electroporation by isolating plasmid DNA from intermediate *B. thuringiensis* strains that are easy to transform or perhaps from *E. coli* strains that are defective in both DNA methylation and host restriction systems. Alternatively, *B. thuringiensis* mutants that are defective in the restriction of incoming DNA but which are still able to modify plasmid DNA may prove to be useful intermediates for the transformation of other, poorly transformable *B. thuringiensis* strains. These improvements in transformation of *B. thuringiensis* allow the introduction of cloned crystal protein genes back into *B. thuringiensis*. We have demonstrated that cloned genes are developmentally expressed, as are the genes when present on native plasmids. This finding opens the door for examining the regulation of crystal protein gene expression in *B. thuringiensis*.

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