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 MgCl₂) 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 pPN101 (24) DNA isolated from *B. megaterium* was 3 × 10⁸ 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 Sau3A fragment containing the N-terminal end of the cryIIA gene from HD263 (8) into the BamHI site of the lac fusion vector pSK10ΔΔ6, replacing the first eight codons of lacZ with the first 45 codons of cryIIA. Plasmid pSK10ΔΔ6 (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 EcoRI fragment from pBC16 (3) containing the origin of replication and a tetracycline resistance (Tc') gene was introduced into the unique EcoRI site of the fusion plasmid such that the direction of transcription of the Tc' 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' gene from pBC16, but pEG409 also has the replication origin and ampicillin resistance (Ap') 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 either 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 Dam− Dcm− strains, or Dam− Dcm− *E. coli* strain (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::Tn9 allele present in GM2163 was transferred to the isogenic pEG409-containing strains EG1884 and EG1885 by transduction with P1vir (27), giving strains EG7311 (Dcm− Dam−) and EG7312 (Dcm− Dam−). The Dam− and Dcm− phenotypes of the strains were verified by *MboI* and *EcoRI* 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Construction, reference, or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM30</td>
<td>dcm&lt;sup&gt;+&lt;/sup&gt; dam&lt;sup&gt;+&lt;/sup&gt;</td>
<td>M. G. Marinus</td>
</tr>
<tr>
<td>GM31</td>
<td>dcm-6 dam&lt;sup&gt;+&lt;/sup&gt;</td>
<td>21</td>
</tr>
<tr>
<td>GM2163</td>
<td>dcm-6 dam&lt;sup&gt;-&lt;/sup&gt;:Tn9 hsdR2</td>
<td>22</td>
</tr>
<tr>
<td>RR1</td>
<td>dcm&lt;sup&gt;+&lt;/sup&gt; dam&lt;sup&gt;-&lt;/sup&gt; hsdS20</td>
<td>4</td>
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<tr>
<td>EG1418</td>
<td>dcm&lt;sup&gt;-&lt;/sup&gt; dam&lt;sup&gt;-&lt;/sup&gt; hsdS20(pEG409)</td>
<td>TRF of RR1</td>
</tr>
<tr>
<td>EG1883</td>
<td>dcm-6 dam&lt;sup&gt;-&lt;/sup&gt;:Tn9 hsdR2(pEG409)</td>
<td>TRF of GM2163</td>
</tr>
<tr>
<td>EG1884</td>
<td>dcm&lt;sup&gt;+&lt;/sup&gt; dam&lt;sup&gt;-&lt;/sup&gt;(pEG409)</td>
<td>TRF of GM30</td>
</tr>
<tr>
<td>EG1885</td>
<td>dcm-6 dam&lt;sup&gt;-&lt;/sup&gt;(pEG409)</td>
<td>TRF of GM31</td>
</tr>
<tr>
<td>EG7311</td>
<td>dcm&lt;sup&gt;-&lt;/sup&gt; dam&lt;sup&gt;-&lt;/sup&gt;:Tn9(pEG409)</td>
<td>TDX (GM2163 × EG1884)</td>
</tr>
<tr>
<td>EG7312</td>
<td>dcm-6 dam&lt;sup&gt;-&lt;/sup&gt;:Tn9(pEG409)</td>
<td>TDX (GM2163 × EG1885)</td>
</tr>
</tbody>
</table>

B. thuringiensis

HD73-26 | subsp. kurstaki | 9 |
HD1    | subsp. kurstaki | 11 |
HD2    | subsp. thuringiensis | 11 |
HD3    | subsp. finitimus | 11 |
HD4    | subsp. alesi    | 11 |
HD7    | subsp. dendrolimus | 11 |
HD201  | subsp. toumanoffi | 11 |
HD263  | subsp. kurstaki | 11 |
HD521  | subsp. indiana  | 11 |
HD536  | subsp. ostriinae | 11 |
HD567  | subsp. israelensis | 11 |
HD567-56 | subsp. israelensis | 12 |

* All of the E. coli strains, with the exception of RR1 and EG1418, are isogenic [F<sup>-</sup> thr<sup>-</sup> leu<sup>-</sup> lys<sup>-</sup> met<sup>-</sup> tyl<sup>-</sup> thi<sup>-</sup> lacY<sup>+</sup> tks<sup>-</sup> supE<sup>+</sup> (gln<sup>V</sup>44) galK<sup>2</sup> galK<sup>t22</sup> hisG<sup>4</sup> rpsL<sup>36</sup> wts<sup>-</sup> iti<sup>-</sup> (mcr<sup>C</sup> mer<sup>B</sup>)] except for the differences noted above and except for the fact that GM2163 and EG1883 are also Thr<sup>+</sup>. The hsdR2 allele confers an r<sub>Y</sub> m<sub>Y</sub> phenotype; the hsdS20 allele confers an r<sub>T</sub> m<sub>Y</sub> phenotype.

* TRF, Transformation; TDX, P1 transduction.

HD73-26 was high with the transformation efficiency when pEG409 was isolated from either a Dam<sup>+</sup> Dcm<sup>+</sup> or a Dam<sup>-</sup> Dcm<sup>-</sup> 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).

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 (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; however, 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. HD3 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<sup>-</sup> Dcm<sup>-</sup> 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 3. Electroporation of B. thuringiensis with plasmid DNA isolated from E. coli DNA methylation mutants

<table>
<thead>
<tr>
<th>DNA source</th>
<th>Relevant phenotype</th>
<th>No. of Tc&lt;sup&gt;c&lt;/sup&gt; transformants for the following recipient B. thuringiensis strain*:</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD73-26</td>
<td>HD263</td>
<td>HD1</td>
</tr>
<tr>
<td>EG1884</td>
<td>Dam&lt;sup&gt;-&lt;/sup&gt; Dcm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>EG1885</td>
<td>Dam&lt;sup&gt;-&lt;/sup&gt; Dcm&lt;sup&gt;-&lt;/sup&gt;</td>
<td>542</td>
</tr>
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<td>EG7311</td>
<td>Dam&lt;sup&gt;+&lt;/sup&gt; Dcm&lt;sup&gt;-&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>EG7312</td>
<td>Dam&lt;sup&gt;-&lt;/sup&gt; Dcm&lt;sup&gt;-&lt;/sup&gt;</td>
<td>431</td>
</tr>
</tbody>
</table>

* 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.

* Number of transformants for strains HD3, HD4, HD7, and HD567-56.

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

<table>
<thead>
<tr>
<th>Recipient strain(s)</th>
<th>DNA source*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD73-26</td>
<td>HD3</td>
</tr>
<tr>
<td>HD73 and HD9</td>
<td>+</td>
</tr>
<tr>
<td>HD73 and HD201</td>
<td>+</td>
</tr>
<tr>
<td>HD567</td>
<td>-</td>
</tr>
<tr>
<td>HD4, HD7, HD521, and HD536</td>
<td>-</td>
</tr>
</tbody>
</table>

* Symbols: +, transformation efficiency was greater than 10<sup>4</sup> Tc<sup>c</sup> transformants per μg of pEG409 DNA; -, transformation efficiency was less than 200 Tc<sup>c</sup> transformants per μg of plasmid DNA.
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 (HD73-26pEG204) 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<sup>−</sup> Dcm<sup>−</sup> *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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