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Species-Specific Type II Restriction-Modification System of *Xylella fastidiosa* Temecula1[▽]

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The transformation efficiency of *Xylella fastidiosa* can be increased by interfering with restriction by the strain-specific type II system encoded by the PD1607 and PD1608 genes. Here, we report results for two strategies: *in vitro* methylation using M.SssI and isolation of DNA from an *Escherichia coli* strain expressing the methylase PD1607.

Xylella fastidiosa is a fastidious, xylem-limited, Gram-negative bacterium and the causative agent of Pierce's disease (PD) of grapevines (1, 12). *X. fastidiosa* is transmitted from infected plants to susceptible plant species, like grapevines, by xylem-feeding insects. Once inside the grapevine xylem, *X. fastidiosa* impedes the flow of sap, thereby producing the characteristic symptoms of PD. Studies of the virulence and basic biology of *X. fastidiosa* have been facilitated by the availability of genetic and molecular tools (6, 8–10, 20, 22, 23). In each case, their successful use has been dependent on the ability to efficiently introduce DNA into *X. fastidiosa*.

In most bacteria, efficient acquisition of foreign DNA is limited by the presence of host-encoded restriction-modification (R-M) systems (14, 25). The restriction enzyme (REase) cleaves incoming DNA at specific sequences unless its recognition site has been modified by its cognate methyltransferase (MTase). R-M systems are divided into four categories (types I to IV) based on their mode of action and the distribution of the restriction, modification, and specificity functions within the enzyme subunits. Analysis of the *X. fastidiosa* Temecula1 genome has uncovered a number of potential R-M systems based on their overall sequence similarities to known systems (25, 28). According to the Restriction Enzyme Database (REBASE) (25), *X. fastidiosa* Temecula1 is predicted to encode four functional R-M systems, of the following types (with the following locus tags): type I (PD2070-PD2072 and PD2074-PD2076), type II (PD1607-PD1608), and type III (PD0833-PD0835). Restriction by these systems, particularly the type I systems, has been shown to have a major impact on the stable acquisition of foreign DNA by *X. fastidiosa* (9).

The focus of the manuscript is the type II R-M system encoded by the PD1607 and PD1608 genes (28). This system, which is present only in *X. fastidiosa* subsp. *fastidiosa*, does not impact the incidence of infection or the ability of *X. fastidiosa* to multiply within grapevines (17). The PD1607-PD1608 system is located within the prophage-like region Xpd8 (4) between the hypothetical protein PD1606 and $tRNA^{Asn}$ (Fig. 1). Analysis of their genetic organization suggests that the PD1608 and PD1607 genes are transcribed as a single polycistronic mRNA with the stop codon of the upstream REase overlapping the start codon of the downstream MTase. Based on naming conventions, the REase encoded by the PD1608 gene has been named XfaTORF1607P, and the MTase encoded by the PD1607 gene has been named M.XfaTORF1607P (REBASE) (25). For simplicity, we will refer to them as REase-PD1608 and MTase-PD1607, respectively. REase-PD1608 exhibits 58% amino acid identity to NspV from *Nostoc* sp. strain PCC7524 (27) and is predicted to recognize the sequence TTCGAA (REBASE) (25). The associated MTase-PD1607 exhibits 45% amino acid identity to M.NspV from *Nostoc* sp. strain PCC7524 (27). The similarity between the PD1607-PD1608 and NspV systems is further supported by the observation that *X. fastidiosa* Temecula1 genomic DNA is resistant to digestion with NspV (data not shown). The simplest interpretation for this result is that MTase-PD1607 methylates one of the bases within the sequence TTCGAA, thereby blocking cleavage by NspV.

To determine if *X. fastidiosa* transformation efficiency is lower for exogenous plasmids containing NspV sites, we conducted a series of experiments by using pBBR1MCS-5, a broad-host-range plasmid that contains a single NspV site and replicates in both *E. coli* and *X. fastidiosa* (15, 23). Plasmid DNA isolated from either *X. fastidiosa* or *E. coli* was introduced into *X. fastidiosa* electrocompetent cells as previously

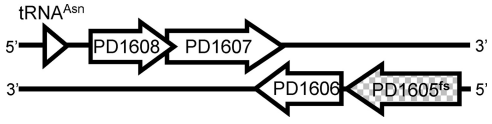

FIG. 1. Chromosomal region surrounding the *X. fastidiosa* type II R-M system. The orientations and locations of the putative open reading frames (ORFs; arrows) and $tRNA^{Asn}$ (triangle) are indicated. The PD1608 gene is predicted to encode a subtype P type II REase; the PD1607 gene is predicted to encode a subtype gamma type II MTase (REBASE) (25). The PD1608-PD1607 operon is located within prophage-like region Xpd8 (4) and adjacent to $tRNA^{Asn}$. The PD1605 ORF contains a frameshift mutation, which disrupts the putative integrase of Xpd8 (4). PD1606 extends toward and overlaps the 3′ end of PD1607 by 143 bp. The hypothetical protein encoded by the PD1606 gene shows some similarity in its N terminus (4e-10) to proteins belonging to the bacteriophage P4-like integrase subfamily (cd00801 sequence cluster) (19). However, PD1606 does not resemble PD1605 or other *X. fastidiosa* integrases.

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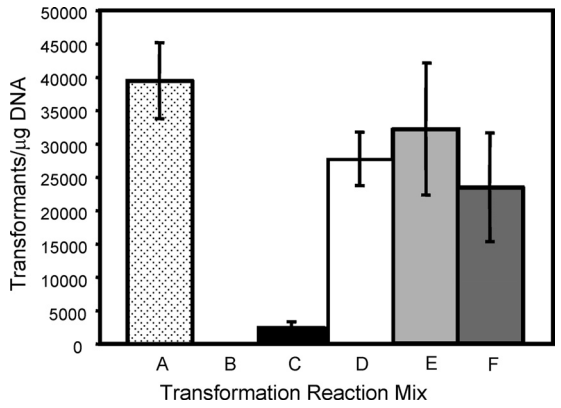

FIG. 2. Transformation efficiency of plasmids into *X. fastidiosa*. Efficiency of transformation was calculated as the number of transformants per microgram of DNA. The amount of DNA was quantified by measuring absorbance at 260 nm by using a NanoDrop spectrophotometer (Thermo Scientific). The means of the results for three independent experiments and their standard errors are indicated. (A) *X. fastidiosa*-isolated pBBR1MCS-5. (B) DH5α-isolated pBBR1MCS-5 alone. (C) DH5α-isolated pBBR1MCS-5 with TRI. (D) DH5α-isolated pAM232 with TRI. (E) DH5α-isolated pBBR1MCS-5 treated with M.SssI according to manufacturer's instructions before coelectroporation with TRI. (F) EAM1-isolated pBBR1MCS-5 with TRI.

described (20, 26), and the resulting transformants were selected on PD3 plates containing 5 μg/ml gentamicin. Transformation efficiencies were then calculated by dividing the total number of transformants by the amount of DNA (μg) added. When *X. fastidiosa*-isolated pBBR1MCS-5 was used, approximately 4.0×10^4 transformants/μg DNA were obtained (Fig. 2, column A). In contrast, electroporation using *E. coli* DH5α-isolated pBBR1MCS-5 resulted in only 1.7 transformants/μg DNA (Fig. 2, column B). As anticipated (9), coelectroporation of DH5α-isolated pBBR1MCS-5 with TypeOne restriction inhibitor (TRI) from Epicentre Biotechnologies enhanced transformation, resulting in 2.3×10^3 transformants/μg DNA (Fig. 2, column C) and indicating that type I restriction has a major impact on pBBR1MCS-5 transformation efficiency. Therefore, we included TRI in all subsequent transformation experiments using *E. coli*-isolated DNA. Lastly, to determine if an NspV site on a piece of DNA impacts its transformation efficiency, we disrupted the unique NspV site in pBBR1MCS-5, generating pAM232 (Table 1). As shown in Fig. 2 (column C versus D), removal of the NspV site resulted in a 12-fold increase in the transformation efficiency of pAM232 relative to that of pBBR1MCS-5. The simplest explanation for this result is that the NspV site on the incoming DNA is recognized and cleaved by the endogenous REase-PD1608.

Strategies for overcoming restriction by REase-PD1608. A common strategy for enhancing transformation involves prior *in vitro* methylation of foreign DNA by using methylases present in cell extracts (5, 7) or commercially available site-specific methylases (2, 16, 29). To determine if prior methylation would increase *X. fastidiosa* transformation efficiency, DH5α-isolated pBBR1MCS-5 was treated with M.SssI (New England Biolabs), which methylates DNA at the cytosine residue within its dinucleotide recognition sequence 5′-CG-3′ (24). To verify pBBR1MCS-5 methylation, treated and control plasmids were digested with two enzymes sensitive to M.SssI methylation (NspV and SalI) and one enzyme that is resistant (SphI). As expected, unmethylated pBBR1MCS-5 was linearized by all three enzymes, whereas CpG-methylated pBBR1MCS-5 was resistant to NspV and SalI treatments but not SphI (Fig. 3). When introduced into *X. fastidiosa*, CpG-methylated pBBR1MCS-5 exhibited approximately 14-fold higher transformation efficiency than did unmethylated pBBR1MCS-5 (Fig. 2, column C versus E), an efficiency similar to that observed for *X. fastidiosa*-isolated pBBR1MCS-5 (Fig. 2, column A). Thus, pretreatment of DNA containing an NspV site with M.SssI enhances its transformation efficiency, mostly likely by blocking cleavage by REase-PD1608.

TABLE 1. Bacteria, plasmids, and oligonucleotides used in this study

Strain, plasmid or oligonucleotide	Description	Reference or source
Strains		
Xylella fastidiosa Temecula1	*X. fastidiosa* subsp. *fastidiosa*; PD isolate ATCC 700964	8
Escherichia coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15*) recA1 endA1 hsdR17 thi-1 gyrA96 relA1*	Lab collection
DH5α λ*pir*	DH5α lysogenized with λ *pir* bacteriophage	Lab collection
EAM1	DH5α derivative; Sp^r St^r $attP_{HK022}$::($P_{LlacO-1}$-PD1607)	This study
Plasmids		
pAH69	Ap^r; CRIM helper plasmid; *oriR101* Int_{HK022}	11
pAH144	Sp^r St^r; R6K γ *ori* $attP_{HK022}$ plasmid dependent upon pir^+ in host	11
pAM217	Ap^r; PD1607 in pJET1.2/blunt	This study
pAM218	Ap^r; PD1607 from pAM217 in pZE12	This study
pAM224	Sp^r St^r; $P_{LlacO-1}$-PD1607 from pAM218 in pAH144	This study
pAM232	Gm^r; disrupted NspV site on pBBR1MCS-5 by filling-in with Klenow	This study
pBBR1MCS-5	Gm^r; pBBR1 replicon; broad-host-range cloning vector	15
pJET1.2/blunt	Ap^r; blunt PCR cloning vector	Fermentas
pZE12	Ap^r; ColE origin; $P_{LlacO-1}$ promoter	18
Oligonucleotides		
PD1607-Kpn	5′-GGTACCGTGAACGAAGCAAAGAAACG-3′ (KpnI site is underlined)	
PD1607-Xba	5′-TCTAGACTATGCGGCGCGGCGCTTGTGCGG-3′ (XbaI site is underlined)	

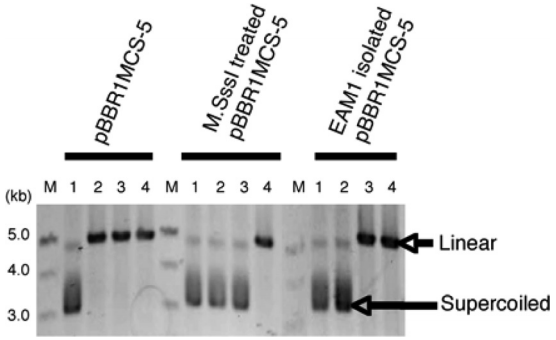

FIG. 3. Impact of *in vitro* and *in vivo* methylation on restriction by NspV. Plasmid DNA was isolated from either *E. coli* strain DH5α or EAM1 and, where indicated, treated with methylase M.SssI. The plasmid DNA (DH5α-isolated pBBR1MCS-5; DH5α-isolated pBBR1MCS-5 methylated using M.SssI; EAM1-isolated pBBR1MCS-5) was then subjected to restriction analysis. Lane 1, undigested plasmid DNA; lane 2, NspV digestion; lane 3, SalI digestion; lane 4, SphI digestion; lane M, Fermentas GeneRuler DNA ladder. Relevant molecular size markers are indicated.

Another strategy for preventing restriction involves *in vivo* methylation of DNA by passage through an *E. coli* strain expressing the appropriate MTase (3, 21). The vectors, intermediate plasmids, strains, and oligonucleotides that we used in this construction are listed in Table 1. To create this strain, the PD1607 gene was amplified by PCR using oligonucleotides PD1607-Kpn and PD1607-Xba and placed downstream of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible $P_{LlacO-1}$ promoter in pZE12 (18). The resulting fusion was integrated into the *attHK* site in DH5α by using the CRIM system (11), generating EAM1. Following transformation of EAM1 with pBBR1MCS-5, expression of the MTase-PD1607 was induced by growth in LB containing 1 mM IPTG and 10 μg/ml gentamicin. As shown in Fig. 3, EAM1-isolated pBBR1MCS-5 was resistant to cleavage by NspV but not by either SalI or SphI. Furthermore, its transformation efficiency was 10-fold higher than that of the DH5α-isolated plasmid (Fig. 2, column C versus F).

Concluding remarks. Comparative genomics has revealed that *X. fastidiosa* strains are unusually rich in R-M systems (14, 25, 28) and that some systems, like PD1607-PD1608, are found only in specific *X. fastidiosa* subspecies. Strain-specific R-M systems, which influence interstrain genetic exchange, are thought to contribute to the genetic isolation necessary for pathogens to successfully colonize a new host (13, 14). At a practical level, these systems present obstacles for genetic manipulations that require the transformation of *X. fastidiosa* with DNA from a foreign source. In this paper, we described two different methods for overcoming restriction by REase-PD1608 that involve prior methylation of the DNA. Similar strategies could be developed to overcome the restriction by species-specific REase in other *X. fastidiosa* subspecies.

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