Construction of pMEKm12, an expression vector for protein production in *Pseudomonas syringae*

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Abstract

Characterization of the biological roles of proteins is essential for functional genomics of pseudomonads. Heterologous proteins overproduced in *Escherichia coli* frequently fail to exhibit biological function. To circumvent this problem, vector pMEKm12 was constructed and used to overexpress proteins in *Pseudomonas*. The vector contains the pRO1600 replication origin, the maltose-binding protein (MBP) fusion system, and an inducible tac promoter. The pMEKm12 was successfully used to overexpress the syringomycin synthetase SyrB1 protein fused to MBP in *Pseudomonas syringae* pv. *syringae*. Furthermore, expression of the MBP-SyrB1 protein in the *syrB1* mutant BR132A1 resulted in the restoration of syringomycin production. This vector will facilitate confirmation of the biochemical roles of nonribosomal peptide synthetase genes in *Pseudomonas syringae*, and studies of gene function from a wide spectrum of pseudomonads. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: Replication origin; Overexpression vector; Protein purification; Phytotoxin

1. Introduction

With the development of *Pseudomonas* genomics, functional analysis of annotated and unknown proteins is becoming more critical to confirm and characterize their biological or biochemical roles. Traditionally, *Escherichia coli* is the most common host to produce heterologous proteins of prokaryotes. However, the overproduced proteins frequently do not function normally for a variety of reasons, which may include improper modification, abnormal folding, and proteolysis [1,2]. Recently, the *syr* and *syp* gene clusters were characterized that contain nonribosomal peptide synthetase genes required for biosynthesis of the syringomycin and syringopeptin phytotoxins in *Pseudomonas syringae* pv. *syringae* [3–5]. It is essential to purify the catalytic modules of these synthetases in order to determine their substrate specificity and biochemical functions in toxin biosynthesis. Furthermore, investigation of the DNA–protein interaction between synthetase genes and regulatory proteins, such as the SyrF and SalA proteins [6], is likely to require purified proteins overproduced in *P. syringae*, the native host organism.

Increasing interest in the genus *Pseudomonas* has led to the development of a few expression vectors. Plasmid pMBPMU, a chimeric vector of pRK415 [7] and pMAL-c2x (New England Biolabs, Schwalbach, Germany), was constructed to overproduce a translational fusion between the *corR* gene of *P. syringae* pv. *glycinea* and the *malE* gene of *E. coli* [1]. The *malE* gene encodes a maltose-binding protein (MBP) that has specific affinity to amylose resin [8]. The MBP affinity to amylose resin was used to purify the MBP-CorR fusion protein. Use of this vector is limited by the instability of pRK415 and its propensity to form deletions [7]. A second vector system, designed for overexpression of a 6×His-PfrA siderophore regulatory protein in *Pseudomonas aeruginosa*, is the pLAFR3-based pLAH series [9]. A Ni\(^{2+}\) affinity column was employed to...
purify the His-tagged protein in this system. However, the 6×His strategy is not recommended for overexpression of insoluble proteins, as exemplified by the CorR and CII proteins of *P. syringae* pv. *glycinea* [1]. Finally, the pYanni series of expression vectors for Gram-negative bacteria combines broad-host-range, inducible expression from the tac promoter and diverse antibiotic resistance determinants [10]. However, this expression vector system lacks a means for tagging the expressed target protein to facilitate purification.

A combination of the DNA replication origin of *Pseudomonas* plasmid pRO1600 and the MBP purification system of *E. coli* could facilitate the construction of a new expression vector that circumvents problems associated with the existing overexpression vectors for *Pseudomonas*. A 1.8-kb *Pst*I fragment of plasmid pRO1600 [11] conferred the ability of CoE1 replicons to replicate and be maintained in *Pseudomonas* [12–14]. Sequence analysis of the *Pst*I fragment revealed significant similarity to known replication origins [15]. The *E. coli* expression vector pMAL-c2x contains the *malE* gene coding for the MBP protein enabling a one-step column purification of fusion proteins. The *tac* (*trp-lac*) promoter [16] is functional in *P. syringae* pv. *glycinea* [1] and *Pseudomonas stutzeri* strain LO147 [10] and is highly induced by addition of isopropyl β-D-thiogalactoside (IPTG). In this study, we describe the construction of vector pMEKm12 used for improved overproduction of proteins in *Pseudomonas*. The vector contains the pRO1600 replication origin, the MBP fusion purification system, and kanamycin resistance selection. In addition, the biological function of SyrB1 overproduced in *P. syringae* using the vector system was evaluated for syringomycin production in vivo.

### Table 1

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH10B</td>
<td>F−, mcrA ΔlacZΔM15 Δmarg−hsdRMS−mcrB (deo R recA1 endA1 araD139 Δ[ara, leu])</td>
<td>[18]</td>
</tr>
<tr>
<td>B301D</td>
<td>Wild-type from pear</td>
<td></td>
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<tr>
<td><strong>Plasmid</strong></td>
<td></td>
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<tr>
<td>pMAL-c2x</td>
<td><em>E. coli</em> overexpression vector; ApR</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>pUCP26</td>
<td>Broad-host-range vector; TeR</td>
<td>[15]</td>
</tr>
<tr>
<td>pBSL15</td>
<td>Carrying a kanamycin resistance gene cassette; ApR, KmR</td>
<td>[19]</td>
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<tr>
<td>p601D-1-R</td>
<td>pRK415 carrying the 8-kb HindIII fragment of pYM101 with deletion of two EcoRV fragments upstream from syrB1; TeR</td>
<td>[6]</td>
</tr>
<tr>
<td>pME10</td>
<td>pMAL-c2x inserted with the 2.0-kb EcoRV-HindIII fragment at the MsiI restriction site; ApR</td>
<td>This study</td>
</tr>
<tr>
<td>pMEKm12</td>
<td>pME10 with the 1.2-kb napU gene inserted at the BamHI site outside of the MCS; KmR, ApR</td>
<td>This study</td>
</tr>
<tr>
<td>pSL61</td>
<td>pMEKm12 carrying the syrB1 gene inserted at the MCS; KmR, ApR</td>
<td>This study</td>
</tr>
<tr>
<td>pSL62</td>
<td>pMEKm12 carrying the syrB12 operon inserted at the MCS; KmR, ApR</td>
<td>This study</td>
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into a noncoding region of expression vector pMAL-c2x (New England Biolabs, Beverly, MA, USA) (Fig. 1). A 2.0-kb EcoRV-HindIII fragment containing the ori_{1600} was isolated from pUCP26 (GenBank accession No. U07168) and was polished by T4 DNA polymerase. The blunt-end fragment was then inserted into pMAL-c2x, previously linearized with MscI, resulting in the construct pME10. Plasmid pME10 was electroporated into *P. syringae* pv. *syringae* strain B301D to evaluate replication and maintenance.

The overexpression vector pMEKm12 was constructed by insertion of the *nptII* gene from pBSL15 [19] into plasmid pME10 (Fig. 1). A 1.2-kb EcoRI fragment containing the *nptII* gene (GenBank accession No. M17626) was isolated from pBSL15 and polished with T4 DNA polymerase. Restriction mapping demonstrated that a BamHI site located between the *ori_{1600}* replication origin and the *rop* gene in pME10 was generated when the EcoRV-HindIII fragment containing the *ori_{1600}* was cloned into pMAL-c2x. The blunt-end EcoRI fragment that confers kanamycin resistance from pBSL15 was cloned into the second BamHI site of the pME10 previously polished by T4 DNA polymerase to generate pMEKm12. Maintenance and replication of pMEKm12 in bacterial cells (*E. coli* or *P. syringae*) carrying the plasmid were confirmed by culturing on LB or NBY medium supplemented with kanamycin and by restriction mapping of the plasmid isolated from the cells.

### 2.4. Construction of pSL61 and pSL62 for overproduction of MBP-SyrB1

To test the utility of pMEKm12 in *P. syringae*, the *syrB1* synthetase gene was translationally fused to *malE* to generate plasmid pSL61. To amplify the *syrB1* gene and to ascertain an in-frame fusion to the 3′ end of *malE*, specific primers B1F (5′-GCG AAC ACT GAC GAA, forward) and B1R (5′-TGA ACG AAG CTT GCT GTT CAG CAG TTA TGA, reverse) were synthesized. The primers B1F and B1R contain EcoRI and HindIII restriction endonuclease sites, respectively, for direct cloning. Plasmid pSL61 was generated by insertion of the PCR product (1.7 kb) into the multiple cloning site of pMEKm12 in order to express an MBP-SyrB1 fusion protein. Sequencing was employed to confirm the in-frame insertion of *syrB1* at the 3′ end of *malE*.

Plasmid pSL62 was constructed by insertion of the complete *syrB* operon in pMEKm12 in order to determine the biological activity of the expressed MBP-SyrB1 fusion protein. A 2.8-kb fragment carrying the complete *syrB* operon containing the *syrB1* and *syrB2* genes was amplified using forward primer B1F and reverse primer B2R (5′-TCA GTA AGC TTG CGC GAG GCA GTA TTT GTG TAG), and then cloned into pMEKm12 to generate plasmid pSL62.

### 2.5. Protein expression and purification

Proteins were expressed from cultures of *E. coli* with addition of IPTG to a final concentration of 0.3 mM and purified with maltose affinity chromatography as recommended by the manufacturer (New England Biolabs, Beverly, MA, USA) except that TBG medium was used. Fusion proteins were induced and purified from cultures of *P. syringae* with the same methods as used for *E. coli* except that *P. syringae* cells were induced with 5 mM IPTG at 25°C for 6 h. Protein yields were measured using the Bradford assay [20].

### 2.6. Analysis of *P. syringae* pv. *syringae* strains for phytoxin production

To determine the biological activity of MBP-SyrB1 fusion protein overproduced in *P. syringae*, construct pSL62 was electroporated into *P. syringae* pv. *syringae* strain BR132A1. Production of syringomycin was evaluated using the indicator organism *Geotrichum candidum* as described previously [5]. *P. syringae* pv. *syringae* strains B301D, BR132A1, and BR132A1 carrying pMEKm12 were used as positive and negative controls, respectively, for syringomycin production.

### 3. Results and discussion

#### 3.1. Construction of overexpression vector pMEKm12

Vector pMEKm12 was constructed by insertion of the replication origin *ori_{1600}* and the kanamycin resistance gene (*nptII*) into the noncoding region of *E. coli* expression vector pMAL-c2x (Fig. 1). Restriction mapping of pMEKm12 revealed that the expression vector was stably replicated and maintained in both *E. coli* strain DH10B and *P. syringae* pv. *syringae* strain BR132A1. The *ori_{1600}* replication origin conferred the ability of ColEI replicons to replicate in *Pseudomonas* and was widely used to construct broad-host-range vectors, such as pUCP18 [13], pPZ vector series [14], and pUCP26 [15]. However, all the plasmids listed above are of limited use as expression vectors in *Pseudomonas* because expression proteins of interest could not be easily purified. In addition, since many pseudomonads frequently possess natural resistance to β-lactamase [21], the presence of the *nptII* gene in the pMEKm12 vector improved screening of transformants in *P. syringae*.

Vector pMEKm12 possesses all the components of pMAL-c2x, which allow efficient cloning, convenient purification and controlled expression of target proteins. The most striking feature of pMEKm12 is the translation of a fusion protein between MBP and the protein of interest. The MBP fusion proteins can be isolated readily with one-step amylose affinity chromatography. The MBP does not
contain cysteine residues that could interfere with disulfide bond formation within the target peptide [8]. Another important feature of pMEKm12 vector is that the multiple cloning sites downstream from malE contain the sequence coding for the recognition site of the specific protease factor Xa [22]. This recognition site allows the MBP to be cleaved from the protein of interest after purification. Finally, the presence of lacI in the vector enables expression to be controlled by induction with IPTG.

The tac promoter and the MBP fusion strategy were shown previously to function in P. syringae by Peñaloza-Vázquez et al. [1]. In that study, the E. coli expression vector pMAL-c2x and the broad-host-range vector pRK415 [7] were digested with BamHI and ligated to generate a 17.2-kb chimeric plasmid called pMBPMU. The pMBPMU construct was mobilized into P. syringae pv. glycinea by a triparental mating procedure. Peñaloza-Vázquez et al. [1] successfully purified the MBP-CorR fusion protein from P. syringae pv. glycinea. However, if DNA fragments larger than approximately 5 kb are cloned into pRK415, over 50% of the P. syringae exconjugants suffered deletions in the inserted DNA [7]. In contrast, the pMEKm12 at 9.9 kb in size is significantly smaller than pMBPMU (17.1 kb). As a result, pMEKm12 is easily transformed by heat shock or electroporation into competent cells. More importantly, no deletions were observed in the study when the pMEKm12 was used to overexpress proteins in either E. coli or P. syringae.

3.2. Expression of translational fusions harbored in pMEKm12

No effect due to insertion of the replication origin ori1600 and the nptII gene on expression of the malE gene in pMEKm12 was observed in E. coli DH10B by analysis of protein expression. The results from both total protein and purified protein analyses indicated that the expected 50-kDa MBP-LacZα fusion protein was overproduced from both the pMEKm12 and pMAL-c2x vectors and that there was no significant difference of the protein yields overproduced from the two vectors in E. coli (Fig. 2).

Fig. 1. Construction of overexpression vector pMEKm12 used in P. syringae. A 2.0-kb EcoRV-HindIII fragment containing ori1600 was isolated from pUCP26 and then polished by T4 DNA polymerase. This blunt-end fragment was inserted into pMAL-c2x previously linearized with MscI, to result in plasmid pME10. A 1.2-kb EcoRI fragment containing the nptII gene was isolated from pBSL15 and polished with T4 DNA polymerase. The blunt-end EcoRI fragment that confers kanamycin resistance was cloned into the extra BamHI site of the pME10, which was previously polished by T4 DNA polymerase, to generate pMEKm12.

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lacZ DH10B. Thus, expression of the fusion protein was approximately 20% compared to that in strain BR132A1, the yield of the MBP-LacZ fusion protein purified with affinity chromatography averaged 22 mg l\(^{-1}\) using pMEKm12 in E. coli. When overproduced from pMEKm12 in P. syringae pv. syringae strain BR132A1, the yield of the MBP-LacZα protein was approximately 20% compared to that in E. coli DH10B. Thus, expression of the malE gene fused to lacZα was at a lower level in P. syringae as compared to E. coli. Nevertheless, the protein yield (approximately 5 mg l\(^{-1}\) MBP-LacZα in strain BR132A1) is adequate for most functional studies [4,23].

Analysis of overproduced MBP-SyrB1 fusion protein revealed the utility of pMEKm12 in both E. coli and P. syringae pv. syringae strain BR132A1. Total protein analysis by SDS-PAGE demonstrated overproduction of a 110-kDa MBP-SyrB1 fusion protein in both E. coli and P. syringae, which corresponds to the predicted size of a fusion protein between MBP (42.7 kDa) and SyrB1 (68 kDa) (Fig. 2). Furthermore, the same size protein was recovered by amylose affinity chromatography from total proteins of the induced cells. These results indicate the pMEKm12 is functional in both E. coli and P. syringae.

Translational fusions of the target protein to MBP significantly improved solubility. As shown in Fig. 2, a 110-kDa MBP-SyrB1 fusion protein was separated and purified from supernatant fractions of cell lysates of E. coli and P. syringae after induction by IPTG at 0.3 and 5 mM, respectively. However, when a SyrB1 protein fused to the glutathione S-transferase was overproduced in E. coli, most of the product was insoluble after induction with a high concentration of IPTG (> 0.1 mM) [4]. Similarly, the solubility of transcriptional factors NifA of Klebsiella pneumoniae [23] and VirG of Agrobacterium tumefaciens [24] was increased when fused to MBP. In contrast, when the soluble MBP-CorR fusion was cleaved with factor Xa, the freed CorR regulatory protein was completely insoluble [1].

Overproduction and purification of proteins from native organisms are especially important for identification of the substrate specificity of nonribosomal peptide synthetases. Each nonribosomal peptide synthetase consists of one or more catalytic modules [25]. Individual modules activate their cognate amino acids in a two-step reaction using a pair of closely coupled domains. An adenylation domain selects the cognate amino acid as substrate and forms the corresponding aminoacyl adenylate by hydrolysis of ATP. The aminoacyl moiety is then covalently tethered to the sulfhydryl group of a phosphopantetheinyl (Ppant) prosthetic group on an adjacent thiolation domain. More importantly, the thiolation domains are post-translationally modified by Ppant transferases [25]. As a result, purified catalyzation modules of synthetases overproduced in E. coli showed lower activity in ATP-PP\(_i\) exchange reactions [26], and this was attributed to lower efficiency of the post-translational modification of the purified protein in E. coli as compared to the native organism. The vector pMEKm12 will be useful in identifying the biochemical functions of the peptide synthetase modules dedicated to syringomycin and syringopeptin biosynthesis.

3.3. Biological activity of MBP-SyrB1 fusion protein

Plasmid pSL62 carrying the syrB operon under the control of the tac promoter effectively complemented the syrB1-recA mutant (BR132A1, J.H. Zhang and D.C. Gross, unpublished) in trans to restore syringomycin production (Fig. 3). The syrB1 gene was predicted to code for a nonribosomal peptide synthetase [3] that activates the last amino acid (L-Thr) in syringomycin biosynthesis [4]. The syrB2 gene, which is required for syringomycin production, is located downstream from syrB1 in the syrB operon [3,4]. Consequently, expression of the complete syrB operon is required for complementation of a polar mutation of the syrB1 gene. Mutation of the recA gene in the syrB1 mutant resulted in cells defective in DNA recombination to facilitate stable maintenance of plasmid pSL62 carrying genomic DNA in trans. Interestingly, syringomycin biosynthesis was restored in strain BR132A1 containing plasmid pSL62 as observed by the production of an 8-mm zone inhibitory to growth of G. candidum. This was comparable to the zone of inhibition produced by wild-type strain B301D. As expected, BR132A1 and BR132A1 (pMEKm12) did not produce syringomycin. These results suggest that the overproduced MBP-SyrB1 fusion protein is biologically comparable to the native SyrB1 protein.

The ability of the MBP-SyrB1 fusion protein to restore syringomycin production in strain BR132A1 indicated...
that the MBP portion of the fusion protein did not interfere with SyrB1 activity in vivo. Similarly, overproduced MBP-CorR and MBP-CII, respectively, complemented in vivo a corR (regulatory gene for coronatine production) mutant and a cff (coronafacate ligase gene) mutant of P. syringae pv. glycinea to restore coronatine production [1]. Likewise, MBP fusions to NilA and VirG, which are transcriptional activators in K. pneumoniae and A. tumefaciens, respectively, exhibited their biological functions [23,24]. These results indicate that MBP is unlikely to interfere with functional folding of the proteins targeted for over-expression.

In conclusion, a new expression system for producing native proteins in pseudomonads was developed that will be useful for functional characterization of biosynthesis and regulatory genes located in the syr and syp gene clusters of P. syringae pv. syringae. Compared to expression vectors reported previously, the pMEKm12 vector system has significantly improved features including easy manipulation and stable maintenance of this plasmid in Pseudomonas and inducible production and one-step purification of target proteins. Currently, this vector is being used to overproduce SyrF and SalA proteins for characterization of the regulatory functions in syringomycin biosynthesis, and to overexpress catalytic modules of peptide synthetases required for biosyntheses of syringomycin and syringopeptin for characterization of substrate specificity.

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