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Research Note

Use of Translational Fusions to the Maltose-Binding Protein to Produce and Purify Proteins in *Pseudomonas syringae* and Assess Their Activity in Vivo

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A simple approach is described for the production and purification of proteins in *Pseudomonas syringae*. The strategy involves the use of the *tac* promoter, the maltose-binding protein, and the broad-host-range vector, pRK415. This approach was used to partially purify two proteins involved in coronatine biosynthesis from *P. syringae*. The activity of the fusions was demonstrated in vivo in complementation experiments using the appropriate mutants.

Additional keywords: affinity chromatography, *malE*, phytotoxin.

The production of heterologous proteins in *Escherichia coli* using translational fusions has become increasingly popular as new methods have been developed which render these systems more efficient and practical. The carrier moiety selected for linkage to the protein of interest can confer a range of useful properties to the recombinant fusion, such as a simple, one-step affinity purification of the translational fusion. Several commonly used fusion proteins include β-galactosidase (Das 1990), glutathione-*S*-transferase (Smith and Johnson 1988), and the maltose-binding protein (MBP) (Guan et al. 1988). Unfortunately, the overexpression of foreign genes in *E. coli* may result in insoluble, unstable, or nonfunctional protein products for a variety of reasons, including improper folding, proteolysis, or the absence of associated prosthetic groups, respectively (Das 1990; Marston 1986; Rusnak et al. 1991). Frequently these obstacles necessitate purification of the protein from the native organism using conventional methodology.

A facile method for the controlled production and purification of proteins in the native organism would circumvent many of the problems mentioned above. In this study, we explored the potential use of the hybrid *tac* (*trp-lac*) promoter and the MBP for the production and purification of fusion proteins in *Pseudomonas syringae*. The *tac* promoter was previously shown to function in both *P. aeruginosa* and *P. putida* (Arai et al. 1991; Bagdasarian et al. 1983; de Lorenzo et al. 1993); however, we are not aware of reports documenting the use of *tac* in *P. syringae*. Furthermore, we are unaware of any studies where *tac*-MBP fusions have been used to purify proteins from an organism other than *E. coli*. In the present study, we show that such fusions can be purified directly from *P. syringae*. We also demonstrate that MBP fusions can be assessed for function in vivo prior to purification.

The protein fusion vector used in this study was pMAL-c2 (Table 1), which contains the *tac* promoter positioned to transcribe the MBP-encoding gene, *malE*. Protein fusions to MBP are easily purified by affinity chromatography on amylose resin and are subsequently eluted with buffers containing maltose. pMAL-c2 contains the *lacZα* gene, *tac* promoter, and factor X_a cleavage site which facilitate blue/white selection of recombinants, inducible expression of cloned sequences, and separation of fused proteins, respectively.

To examine the potential production of translational fusions to MBP in *P. syringae*, we first constructed a chimeric plasmid consisting of pMAL-c2, which contains a ColEI origin, and pRK415, a broad-host range vector derived from the Inc-P1 plasmid, RK2. This chimeric plasmid was constructed by digesting both pMAL-c2 and pRK415 with *Bam*HI, ligating the linearized products, and then screening transformants of *E. coli* DH5α for resistance to ampicillin and tetracycline at 100 and 25 μg/ml, respectively. pMBPMU, the chimeric plasmid obtained from this experiment, was then used to overproduce MBP in DH5α. Cells were grown at 37°C in Terrific Broth (TB) (Sambrook et al. 1989) to 0.4 to 0.5 OD_{600}, induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and incubated an additional 3 h. Aliquots of cells (1 ml) were removed before and after induction, pelleted by centrifugation, resuspended in lysis buffer (Sambrook et al. 1989), and incu-

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bated on ice for 30 min. The cell suspension was then sonicated as described previously (Riggs 1994) and centrifuged at 14,000 × *g* for 20 min at 4°C. The pellet was discarded, and the supernatant (which contained the soluble fraction of the crude extract) was analyzed by SDS-PAGE on a 10% polyacrylamide gel (Sambrook et al. 1989). DH5α cells containing pMBPMU produced the expected 42.7-kDa MBP when induced with IPTG (Fig. 1, lane 2).

pMBPMU was then mobilized into *P. syringae* pv. *glycinea* PG4180.N9 using a triparental mating procedure (Bender and Cooksey 1987), and transconjugants were selected on mannitol-glutamate (MG) medium (Keane et al. 1970) supplemented with tetracycline (25 μg/ml). The presence of pMBPMU in PG4180.N9 was confirmed by agarose gel electrophoresis of plasmid preparations (Kado and Liu 1981). To induce the *malE* gene, PG4180.N9 containing pMBPMU was incubated at 28°C in TB to 0.4 to 0.5 OD_{600}, induced with 5.0 mM IPTG, and incubated an additional 6 h. Aliquots of cells (1 ml) were removed before and after the induction period and total cellular proteins were analyzed as described above. For purification of MBP from PG4180.N9(pMBPMU), a 100-ml culture of IPTG-induced cells was pelleted, suspended in 8 ml of lysis buffer, and incubated on ice for 30 min. The soluble fraction of the crude cellular extract was recovered as described above and applied to the amylose resin as described previously (Riggs 1994).

The level of MBP produced in PG4180.N9(pMBPMU) (Fig. 1, lane 4) was lower than the amount produced in DH5α(pMBPMU) (Fig. 1, lane 2). However, MBP could be purified from PG4180.N9(pMBPMU) (Fig. 1, lane 5) by affinity chromatography on amylose resin. Possible explanations for the differential expression of MBP in the two strains include: (i) differential expression from the *tac* promoter; (ii) variation in the stability of MBP; or (iii) variation in the copy number of pMBPMU.

The results described above indicated that the *tac* promoter and the *lac* repressor (encoded by *lacI*^{q}) functioned in PG4180.N9 and could be used or the controllable production of fusion proteins in *Pseudomonas*. We investigated the potential of this approach by utilizing pMAL-c2 to overproduce CorR, a response regulator previously shown to be involved in the thermoregulated production of the phytotoxin coronatine in *P. syringae* pv. *glycinea* PG4180 (Ullrich et al. 1995). *corR* was cloned into the *Eco*RI site of pMAL-c2 as a 0.63-kb PCR product. Plasmid pMUP20 was used as template and the following oligonucleotides were used as primers in the PCR reaction: forward primer 5′-GAC**GAATTC**ATGCCGAGCTCTTCGATCTTG (the *Eco*RI recognition site is shown in bold; the following base pairs are nucleotides 813 to 833 in Ullrich et al. 1995) and reverse primer 5′-ATT**GAATTC**ATGAGTCACAGGACCTCC (the *Eco*RI site is shown in bold; the base pairs which follow are the complement of nucleotides 1430 to 1447 in Ullrich et al. 1995). Oligonucleotide primers were synthesized by the Oklahoma State University Recombinant DNA/Protein Resource Facility. Following amplification of the 0.63-kb PCR product, ligation into pMAL-c2, and transformation in *E. coli* DH5α, plasmid pAP06 was recovered (Fig. 2). DH5α cells containing pAP06 were induced as described for DH5α(pMBPMU), and total cellular proteins were analyzed by SDS-PAGE. DH5α(pAP06) produced a 64-kDa protein when induced with 0.3 mM IPTG; this band represents the predicted size of a fusion protein consisting of MBP (42.7 kDa) and CorR (21.5 kDa) (Fig. 3, lane 2).

To produce the MBP-CorR fusion protein in *P. syringae*, a chimeric plasmid consisting of pAP06 and pRK415 was constructed. Briefly, pAP06 was linearized with *Msc*I and then ligated to pRK415 which was previously digested with *Hin*dIII and *Eco*RI and end-filled with the Klenow fragment of DNA polymerase I (Fig. 2). The 17.7-kb chimeric plasmid which resulted was designated pAP06.415 (Fig. 2); this was electroporated into PG4180.N9 (Sambrook et al. 1989), and transformants were selected on KB medium containing ampicillin and tetracycline at 100 and 25 μg/ml, respectively. The presence of pAP06.415 in putative transformants of PG4180.N9 was confirmed by agarose gel electrophoresis. When PG4180.N9(pAP06.415) was induced with 5.0 mM IPTG as described above, a 64-kDa protein was observed corresponding to the predicted size of the MPB-CorR fusion (Fig. 3, lane 4). Furthermore, this band was partially purified by affinity chromatography on an amylose column (Fig. 2, lane 5) using conditions specified by the manufacturer (New England Biolabs, Beverly, MA).

To assess the function of MBP-CorR, we constructed a *corR* mutant. The gentamicin-resistance gene (Gm^r) from pMGm (Murillo et al. 1994) was cloned into the *Bam*HI site of *corR*. Insertion of the Gm^r cassette at this position disrupts

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Escherichia coli		
DH5α		Miller 1972
Pseudomonas syringae pv. *glycinea*		
PG4180.N9	COR^+; Km^r	Ullrich et al. 1994
PG4180.P2	COR^-; *corR*::Gm^r	This study
Plasmids		
pMAL-c2	Ap^r; ColEI origin, *tac* promoter, encodes *lacI*^{q} *malE lacZα*; contains factor X_a cleavage site	New England Biolabs
pRK415	Tc^r; IncP; RK2-derived vector	Keen et al. 1988
pMBPMU	Ap^r Tc^r; chimeric plasmid constructed from pMAL-c2 and pRK415; 17.1 kb	This study
pMUP20	Tc^r; contains *corR* as a 2.0-kb *Pst*I fragment in pRK415	Ullrich et al. 1995
pAP06	Ap^r; contains *corR* on a 0.63-kb *Eco*RI fragment; derived from pMUP20 by PCR cloning in pMAL-c2	This study
pAP06.415	Ap^r Tc^r; contains *corR*; chimeric plasmid constructed from pAP06 and pRK415; 17.7 kb	This study
pAPP20	Ap^r; contains *corR* and the N-terminal half of *corS* on a 2.0-kb *Pst*I-*Eco*RI fragment in pT7-7	This study
pAPP20.1	Ap^r; Gm^r; derived from pAPP20; contains *corR*::Gm^r	This study
pVM1	Ap^r; contains *cfl* as a 1.3-kb *Bam*HI/*Hin*dIII fragment in pMAL-c2	C. Bender
pVR1	Ap^r Tc^r; contains *cfl*; chimeric plasmid constructed from pVM1 and pRK415	This study

the helix-turn-helix motif at the C-terminus of the protein. This motif is absolutely required for transcriptional activation of CorR-regulated genes (A. Peñaloza-Vázquez and C. Bender, unpublished data). pAPP20.1, the clone containing the mutant allele (*corR*::Gm^r), was electroporated into PG4180.N9; this was followed by screening for Ap^s Gm^r *Pseudomonas* derivatives. Recombination of the Gm^r cassette into *corR* was verified by plasmid isolation and Southern blot analysis. PG4180.P2, the *corR* mutant resulting from this experiment, was analyzed for COR production as described previously (Palmer and Bender 1993) and shown to be completely defective in biosynthesis of COR, CFA, and all other coronafacoyl compounds.

pAP06.415 was transformed into PG4180.P2 to determine whether the MBP-CorR fusion could complement this mutant. PG4180.P2(pAP06.415) cells were grown at 18°C in Hoitink-Sinden medium optimized for COR production (HSC; Palmer and Bender 1993) and induced with 5 mM IPTG 48 h after inoculation. Supernatants were analyzed for COR production by HPLC 7 days after inoculation (Palmer and Bender 1993). PG4180.P2 cells containing pAP06.415 produced 31 mg COR/g protein, a level comparable to that produced by wild-type PG4180 cells (Ullrich et al. 1995).

We also evaluated the use of pMAL-c2/pRK415 chimeric plasmids for production of coronafacate ligase (Cfl) in *P. syringae*. Cfl, an enzyme required for coronatine biosynthesis, is thought to catalyze the ligation of coronafacic and coronamic acid, the two intermediates in the coronatine pathway (Liyanage et al. 1995). Cfl was previously cloned as a 1.3-kb PCR fragment in pMAL-c2 to yield the construct pVM1 (C. Bender and P. Reynolds, unpublished). Induction of DH5α(pVM1) with IPTG was previously shown to result in overproduction of a 95-kDa protein (C. Bender and P. Reynolds, unpublished), which corresponds to the predicted size of a protein fusion between MBP (42.7 kDa) and Cfl (53 kDa).

A chimeric plasmid consisting of pVM1 and pRK415 was constructed to facilitate production of a MBP-Cfl protein fusion in *P. syringae*. pVM1 was linearized with *Msc*I, and

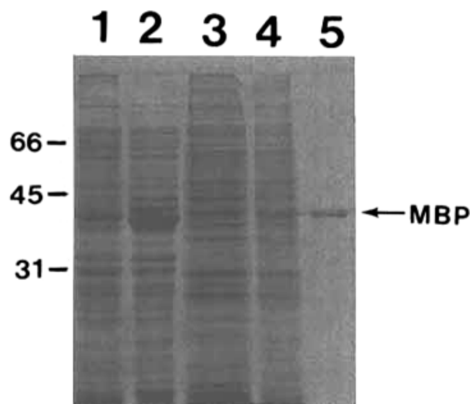

Fig. 1. SDS-PAGE analysis of *Escherichia coli* and *Pseudomonas syringae* pv. *glycinea* PG4180.N9 producing the maltose-binding protein (MBP). Lanes 1 to 4 contain total cellular proteins from the following strains: 1, DH5α(pMBPMU), uninduced; 2, DH5α(pMBPMU), induced with IPTG; 3, PG4180.N9(pMBPMU), uninduced; 4, PG4180.N9 (pMBPMU), induced with IPTG. Lane 5 contains MBP which was isolated from induced cells of PG4180.N9(pMBPMU) and purified by affinity chromatography on an amylose column. The migration of molecular weight markers is shown on the left.

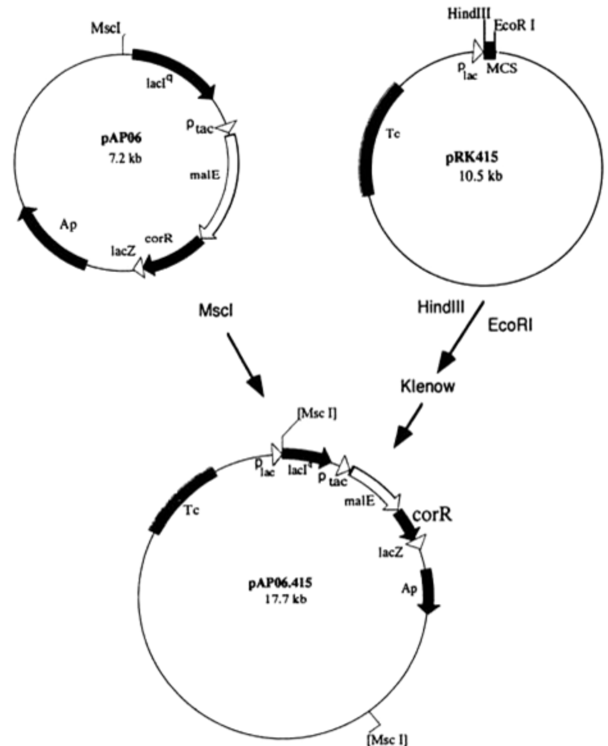

Fig. 2. Construction of pAP06.415, a fusion plasmid used for controllable expression of MBP-CorR in *Pseudomonas syringae*. Details regarding the construction of pAP06 are given in the text. To construct pAP06.415, pAP06 was linearized with *Msc*I and then ligated to pRK415 which was previously digested with *Hin*dIII and *Eco*RI and endfilled with the Klenow fragment.

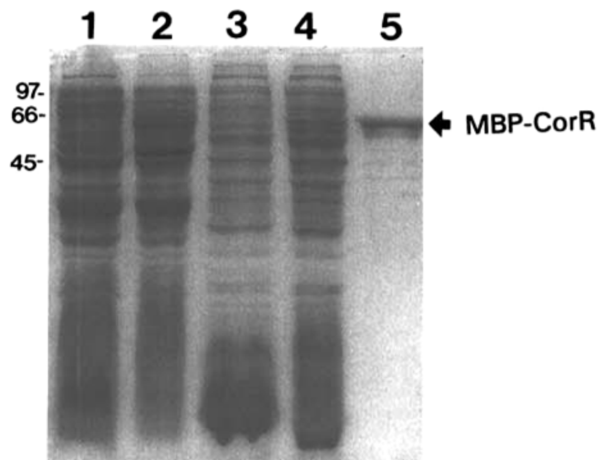

Fig. 3. SDS-PAGE analysis of *Escherichia coli* and *Pseudomonas syringae* pv. *glycinea* PG4180.N9 producing a translational fusion of the maltose-binding protein (MBP) and the response regulator, CorR. Lanes 1 to 4 contain total cellular proteins from the following strains: 1, DH5α(pAP06), uninduced; 2, DH5α(pAP06), induced with isopropyl-β-D-thiogalactopyranoside; 3, PG4180.N9(pAP06.415), uninduced; 4, PG4180.N9(pAP06.415), induced with IPTG. Lane 5 contains the MBP-CorR fusion which was isolated from induced cells of PG4180.N9(pAP06.415) and purified by affinity chromatography on an amylose column. The migration of molecular weight markers is shown on the left.

pRK415 was digested with *Eco*RI and end-filled with the Klenow fragment. The two linearized plasmids were ligated and transformed into *E. coli* DH5α. *E. coli* DH5α cells containing the resulting construct, designated pVR1, produced a 95-kDa fusion protein when induced with IPTG (Fig. 4, lane 2).

pVR1 was then mobilized into PG4180.N9 by triparental mating, and transconjugants were verified by agarose gel electrophoresis of plasmid preparations (Kado and Liu 1981). When PG4180.N9(pVR1) was induced with 5.0 mM IPTG as described above, a 95-kDa fusion protein was apparent (Fig. 4, lane 4). The fusion protein, which constituted approximately 7% of the total soluble protein, was purified on an amylose column (Fig. 4, lane 5), and then cleaved with factor X_a protease as specified by the manufacturer. After cleavage with factor X_a, affinity chromatography on amylose resin was repeated; the eluate contained a 53-kDa band which is the predicted size of Cfl (Fig. 4, lane 6).

The chimeric pMAL-c2/pRK415 plasmids constructed in the present study were used to successfully express and purify proteins from *P. syringae* pv. *glycinea*. When our objective was to overproduce a MBP fusion protein in *P. syringae*, we found it quite helpful to initially generate the MBP fusion in pMAL-c2 and check for production in *E. coli* before constructing the pMAL-c2/pRK415 chimera. A single *Msc*I site in pMAL-c2 facilitated the ligation of this plasmid to linearized, end-filled pRK415.

One advantage of producing fusion proteins in the native host is the ability to ascertain the functionality of the engineered protein. For example, we showed the functionality of MBP-CorR by using pAP06.415 to complement PG4180.P2, a *corR* mutant. Similarly, the MBP-Cfl fusion contained in pVR1 was used to complement *cfl* mutants for COR production (V. Rangaswamy and C. Bender, unpublished data). Thus,

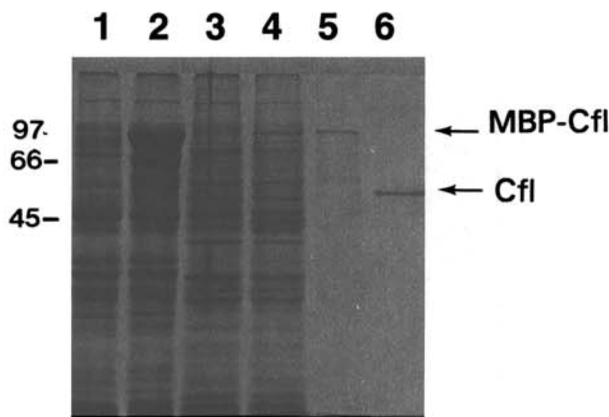

Fig. 4. SDS-PAGE analysis of *Escherichia coli* and *Pseudomonas syringae* pv. *glycinea* PG4180.N9 producing a translational fusion of the maltose binding protein (MBP) and coronafacate ligase (Cfl). Lanes 1 to 4 contain total cellular proteins from the following strains: 1, DH5α(pVR1), uninduced; 2, DH5α(pVR1), induced with isopropyl-β-D-thiogalactopyranoside; 3, PG4180.N9(pVR1), uninduced; 4, PG4180.N9 (pVR1), induced with IPTG. Lane 5 contains the MBP-Cfl fusion which was isolated from induced cells of PG4180.N9(pVR1) and purified by affinity chromatography on an amylose column. Lane 6 contains a 53-kDa protein which is the predicted size of Cfl. This protein was isolated by digesting the MBP-Cfl fusion product with factor X_a and purifying the reaction products on an amylose column. The 53-kDa protein was recovered from the column eluate. The migration of molecular weight markers is shown on the left.

in both examples, the 42.7-kDa MBP did not interfere with CorR or Cfl activity in vivo. Consequently, it was possible to assess the function of these proteins using a genetic approach in vivo before undertaking the more difficult in vitro experiments with the purified proteins. Furthermore, the production of proteins in their native host helps circumvent some of the problems which arise when expression is undertaken in a foreign background. For example, environmental factors which affect protein function and yield in the natural host background can be easily studied. This was particularly evident in the studies with MBP-CorR where the fusion protein constituted 25% of the total soluble protein when purified from cells grown at 18°C; however, when cells were grown at 28°C, MBP-CorR was only 11% of the total soluble protein recovered. The former value of 25% is comparable to the yields of 20 to 40% which were reported for MBP fusions in *E. coli* (Julien and Calendar 1995; Rhyum et al. 1994). The difference in recovery of MBP-CorR at 18 and 28°C may reflect its function as a response regulator which modulates coronatine biosynthesis in a temperature-dependent manner (Ullrich et al. 1995).

It is interesting to note that translational fusions to MBP can actually increase the solubility of foreign proteins. This has been noted for MBP fusions to VirG and NifA, which are transcriptional activators in *Agrobacterium tumefaciens* and *Klebsiella pneumoniae*, respectively (Han and Winans 1994; Lee et al. 1993). For example, when the soluble MBP-CorR fusion was cleaved with factor X_a, CorR was totally insoluble (data not shown), a result also obtained for the MBP-NifA fusion (Lee et al. 1993). Solubility was previously shown to be a problem for the overexpression of *cfl* in *E. coli*; for example, the cloning and overexpression of *cfl* in pGEX-2T (Pharmacia LKB, Piscataway, NJ) and pET-22b(+) (Novagen, Madison, WI) resulted in the production of insoluble fusion proteins (Liyanage et al. 1995; H. Liyanage and C. Bender, unpublished). Fortunately, this problem was circumvented when Cfl was overproduced as an MBP fusion.

In summary, we have described a simple approach which can be used to overproduce and purify proteins from the native host bacterium. The strategy has broad applicability and can be expanded by incorporating other controllable promoters and fusion proteins; furthermore, cloning vectors can be chosen to optimize replication in the native bacterium. An improvement over the present approach would be the construction of a shuttle vector containing the *lacI*^{q} P_{tac} *malE lacZ*α portion of pMAL-c2 in a small, broad-host-range plasmid. It is important to mention that the *tac* promoter and MBP have been used to overproduce and purify proteins from *Xanthomonas campestris* pv. *vesicatoria* (A. Peñaloza-Vázquez and C. Bender, unpublished), and the approach is likely to work in other strains as well. In summary, the real merit of this approach is twofold: (i) the ability to assess function of an engineered protein in vivo; and (ii) the ease of protein purification using an affinity tag. Thus, this approach can substantially enhance the recovery of a functional protein and is cost effective when compared with conventional methods for protein purification.

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LITERATURE CITED

Arai, H., Igarashi, Y., and Kodama, T. 1991. Construction of novel expression vectors effective in *Pseudomonas* cells. Agric. Biol. Chem. 55:2431-2432.

Bagdasarian, M. M., Amann, E., Lurz, R., Rückert, B., and Bagdasarian, M. 1983. Activity of the hybrid *trp-lac* (*tac*) promoter of *Escherichia coli* in *Pseudomonas putida*. Construction of broad-host-range, controlled-expression vectors. Gene 26:273-282.

Bender, C. L., and Cooksey, D. A. 1987. Molecular cloning of copper resistance genes from *Pseudomonas syringae* pv. tomato. J. Bacteriol. 169:470-474.

Das, A. 1990. Overproduction of proteins in *Escherichia coli*: Vectors, hosts, and strategies. Methods Enzymol. 182:93-112.

de Lorenzo, V., Eltis, L., Kessler, B., and Timmis, K. N. 1993. Analysis of *Pseudomonas* gene products using *lacI*/P*trp-lac* plasmids and transposons that confer conditional phenotypes. Gene 123:17-24.

Guan, C.-d., Li, P., Riggs, P. D., and Inouye, H. 1988. Vectors that facilitate the expression and purification of foreign peptides in *Escherichia coli* by fusion to maltose-binding protein. Gene 67: 21-30.

Han, D. C., and Winans, S. C. 1994. A mutation in the receiver domain of the *Agrobacterium tumefaciens* transcriptional regulator VirG increases its affinity for operator DNA. Mol. Microbiol. 12:23-30.

Julien, B., and Calendar, R. 1995. Purification and characterization of the bacteriophage P4 δ protein. J. Bacteriol. 177:3743-3751.

Kado, C. I., and Liu, S. T. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365-1373.

Keane, P. J., Kerr, A., and New, P. B. 1970. Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. Aust. J. Biol. Sci. 23:585-595.

Keen, N. T., Tamaki, S., Kobayashi, D., and Trollinger, D. 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene 70:191-197.

Lee, H.-S., Berger, D. K., and Kustu, S. 1993. Activity of purified NIFA, a transcriptional activator of nitrogen fixation genes. Proc. Natl. Acad. Sci. USA 90:2266-2270.

Liyanage, H., Penfold, C., Turner, J., and Bender, C. L. 1995. Sequence, expression, and transcriptional analysis of the coronafacate ligase-encoding gene required for coronatine biosynthesis by *Pseudomonas syringae*. Gene 153:17-23.

Marston, F. A. O. 1986. The purification of eukaryotic polypeptides synthesized in *Escherichia coli*. Biochem. J. 240:1-12.

Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Murillo, J., Shen, H., Gerold, D., Sharma, A. K., Cooksey, D. A., and Keen, N. T. 1994. Characterization of pPT23B, the plasmid involved in syringolide production by *Pseudomonas syringae* pv. *tomato*. Plasmid 31:275-287.

Palmer, D. A., and Bender, C. L. 1993. Effects of environmental and nutritional factors on production of the polyketide phytotoxin coronatine by *Pseudomonas syringae* pv. glycinea. Appl. Environ. Microbiol. 59:1619-1626.

Rhyum, S. B., Jin, B. R., Park, H. R., and Hong, H. Y. 1994. High level expression of hepatitis B virus preS1 peptide in *Escherichia coli*. J. Biotechnol. 36:221-230.

Riggs, P. 1994. Expression and purification of maltose-binding protein fusions. Pages 16.6.1-16.6.13 in: Current Protocols in Molecular Biology. F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Siedman, J. A. Smith, and K. Struhl, eds. Wiley, New York.

Rusnak, F., Sakaitani, M., Drueckhammer, D., Reichert, J., and Walsh, C. T. 1991. Biosynthesis of the *Escherichia coli* siderophore enterobactin: Sequence of the *entF* gene, expression and purification of EntF, and analysis of covalent phosphopantetheine. Biochemistry 30:2916-2927.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Smith, D. B., and Johnson, K. S. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione-*S*-transferase. Gene 67:31-40.

Ullrich, M., Guenzi, A. C., Mitchell, R. E., and Bender, C. L. 1994. Cloning and expression of genes required for coronamic acid (2-ethyl-1-aminocyclopropane 1-carboxylic acid), an intermediate in the biosynthesis of the phytotoxin coronatine. Appl. Environ. Microbiol. 60:2890-2897.

Ullrich, M., Peñaloza-Vázquez, A., Bailey, A. M., and Bender, C. L. 1995. A modified two-component regulatory system is involved in temperature-dependent biosynthesis of the *Pseudomonas syringae* phytotoxin coronatine. J. Bacteriol. 177:6160-6169.