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NOTES

Molecular Cloning with a pMEA300-Derived Shuttle Vector and Characterization of the *Amycolatopsis methanolica* Prephenate Dehydratase Gene

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An efficient restriction barrier for methylated DNA in the actinomycete *Amycolatopsis methanolica* could be avoided by using a nonmethylating *Escherichia coli* strain for DNA isolations. The *A. methanolica* prephenate dehydratase gene was cloned from a gene bank in a pMEA300-derived shuttle vector in *E. coli* and characterized.

Transformation of *Amycolatopsis methanolica* (6, 15) with various actinomycete vectors was unsuccessful (24). An indigenous, integrative, *A. methanolica* plasmid (pMEA300; 13.3 kb) (23) was subsequently selected for the construction of cloning vectors. *A. methanolica* WV1, devoid of both integrated and free pMEA300 sequences, was used to establish an efficient transformation protocol (24).

We are interested in the enzymology and regulation of aromatic amino acid biosynthesis in *A. methanolica* (1, 8, 10). Prephenate dehydratase (PDT) is a key regulatory enzyme in L-phenylalanine (L-Phe) biosynthesis in *A. methanolica* (6). The PDT protein has been purified to homogeneity and characterized as a homotetrameric enzyme with 34-kDa subunits (10).

Here we report the construction of a gene library of *A. methanolica* total DNA in *Escherichia coli* with a pMEA300-derived shuttle vector and the characterization of the *pdt* gene.

Growth, DNA manipulations, and transformation. *A. methanolica* and *E. coli* strains and plasmids used are shown in Table 1. The complex (23) and mineral (7) media and transformation protocols used have been described elsewhere (22, 24). DNA sequencing was done as previously described (23). Open reading frames were identified with a *Streptomyces* codon usage table (26).

Transformation with (non)methylated DNA. Attempts to transform strain WV1 with plasmids pWV102 and pWV110, isolated from strain HB101, were unsuccessful. The possible presence of a restriction barrier between *E. coli* and *A. methanolica*, as reported for several actinomycetes (2, 3, 5, 16, 17), was investigated. The *Streptomyces avermitilis* restriction barrier can be avoided by performing DNA isolations from nonmethylating strains, e.g., *E. coli* JM110 (*dam*, *dcm*) and *Streptomyces lividans* (16). Reisolation of pWV102 and pWV110 from strain JM110 indeed resulted in successful transformation of strain WV1 (approximately 10^5 pocks per μg of DNA). The effect of pMEA300 methylation on the transformation frequency was further studied by treating pMEA300 DNA with *Bam*HI (GGATC^{m}C) and *Sss*I (C^{m}G) methylases. The methylation efficiency was checked by the (in)ability of the appropriate restriction enzymes, *Bam*HI and *Sal*I, respectively, to digest the in vitro-methylated DNA. Treatment of pMEA300 with *Bam*HI methylase caused a 90% reduction in transformation frequency with strain WV1. The *Sss*I methylase recognizes many sites on pMEA300 with its high G+C content (68%; GenBank accession no. L36679), reducing the transformation frequency even more severely (by 99.8%).

Construction of a gene library in pWV138. Attempts to construct a gene bank of *A. methanolica* DNA in pWV137 failed; apparently, this vector could not stably maintain DNA fragments of *A. methanolica*. Cloning of DNA fragments may be greatly facilitated by the use of transcriptional terminators (4). Therefore, the tetracycline resistance marker flanked by omega (Ω) fragments from pHP45Ω-Tc (11) was cloned into the *Bam*HI site of pWV136, yielding pWV138 (Fig. 1). This plasmid contains a unique *Bam*HI site that can be used for cloning, resulting in insertional inactivation of the Tc^r gene in *E. coli*. The ligation mixture of pWV138 DNA digested with *Bam*HI and a partial *Sau*3A digest (>3 kb) of total DNA of *A. methanolica* was used to transform strain MC1061, yielding approximately 15,000 independent transformants in six different batches. The average insert frequency was 84%; plasmid DNA of 34 Tc^s colonies was analyzed by restriction analysis, showing an average insert size of 4 kb.

Cloning of the *A. methanolica pdt* gene. The oligonucleotide pdt3 (38 nucleotides, 256-fold degenerate) (TTCATGGC[CG]GC[CG]GG[CG]GACGA[AG]CT[CG]GT[CG]GC[CG]GC[GC]AGAC), based on the N-terminal amino acid sequence of the PDT protein of *A. methanolica* (10), was used as a probe to screen total DNA of *A. methanolica* and the pWV138 gene library for the *pdt* gene. pdt3 was labelled with the D.I.G. oligonucleotide tailing kit (Boehringer, Mannheim, Germany). Positive signals were observed with total DNA, digested with *Bam*HI (9.0 kb), *Bcl*I (5.5 kb), and *Pvu*II (3.4 kb), and in two

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TABLE 1. *A. methanolica* and *E. coli* strains and pMEA300-derived plasmids used

Strain or plasmid	Description	Reference
Strains		
A. methanolica	NCIB 11946 wild-type strain	6
WV1	pMEA300-free derivative strain of *A. methanolica*	24
WV2	Spectinomycin-resistant (150 μg/ml) derivative of strain WV1	24
GH71	PDT-negative mutant, derivative of WV2	9
E. coli		
MC1061	*hsdR mcrB araD139* Δ(*araABC-leu*) 7679 Δ*lacX74 galU galK rspL thi*	
DH5α	*supE44* δ*lacU169* (Ω80*lacZ*δM15) *hsdR17 recA1 gyrA96 thi-1 relA1*	
JM110	*dam dcm supE44 hsdR thi leu rpsL lacY galK galT ara tonA thr tsx* Δ(*lac-proAB*) F′(*traD36 proAB*$^+$ *lacZ* ΔM15)	
HB101	*supE44 hsdS20 recA 13 ara-14 proA2 lacY1 galK 2 rpsL20 xyl-5 mtl-1*	19
Plasmids		
pMEA300	Wild-type plasmid of *A. methanolica*	23
pWV102	pUC18 (18) containing *Bam*HI-digested pMEA300 in the *Bam*HI site	This work
pWV110	pUC18 containing *Bgl*II-digested pMEA300 in the *Bam*HI site	This work
pWV136	Integration- and conjugation-deficient derivative of pMEA300 with the pHSS6 ColE1 replicon	22
pWV137	pWV136 containing the *lacZ* region of pUC18 as a *Hae*II fragment in the *Eco*RI site	This work
pWV138	pWV136 containing the tetracycline resistance gene flanked by transcription terminators of pHP45Ω-Tc (11) as a *Hin*dIII fragment in the *Eco*RI site	This work
pNAT115	pWV138 containing a 15-kb chromosomal insert encoding the *pdt* gene	This work

batches of the gene library, digested with *Pvu*II (3.4 kb). The results indicated that an internal 3.4-kb chromosomal fragment had been cloned entirely. Batch 1 (approximately 2,500 plasmids) was transformed to strain DH5α. After replica plating of the transformants, plasmid DNA from pooled colonies was isolated, digested with *Pvu*II, and hybridized with pdt3. A positive signal was obtained in two subsets of batch 1. In three consecutive steps, a colony that contained a single plasmid (pNAT115, 27 kb [Fig. 1]) that hybridized strongly to pdt3 and contained a chromosomal DNA insert of 15 kb with a 3.5-kb *Pvu*II fragment was isolated. Unmethylated plasmid DNA isolated from strain JM110 (pNAT115) subsequently was used to transform *A. methanolica* GH71, a PDT-deficient mutant (10). Strain GH71 (pNAT115) grew on glucose mineral media without requiring L-Phe as supplement. The specific activities of PDT assayed (10) in crude extracts of *A. methanolica* wild type and GH71 (pNAT115) were 17 and 16 nmol/mg/min, respec-

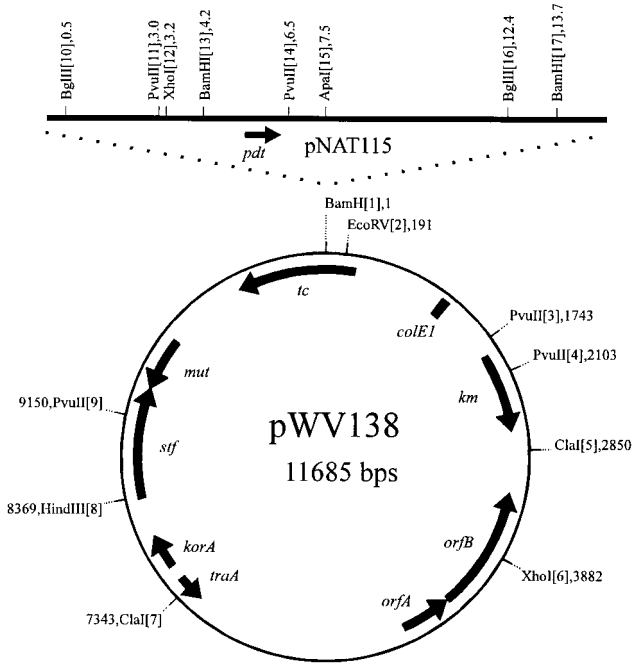

FIG. 1. Restriction maps of the plasmids pWV138 and pNAT115. Numbers between brackets indicate various restriction sites on the plasmids. Other numbers indicate distances from the unique *Bam*HI site on pWV138 and pNAT115. Arrows on pNAT115 and pWV138 indicate open reading frames encoding functions required for PDT activity (*pdt*), maintenance and autonomous replication (*korA*, *orfA*, *orfB*) (22), conjugational transfer (*traA*) (25), stimulation of transformation frequency (*stf*) (24), and high mutation frequency (*mut*) (21).

```
1   GTGTCACGGA TCGCATATTT CGGGCCGGTC GGGACGTTCA CCGAGCAGGC GGCGCGCACT
      fM  S  R  I  A  Y  F  G  P  V  G  T  F  T  E  Q  A  A  R  T
61  TTCATGGCGG CGGGGGACGA ACTCGTCGCC GCCGAGACGA TTCCGAAGGC GCTGGACGCC
      F  M  A  A  G  D  E  L  V  A  A  E  T  I  P  K  A  L  D  A
121 GTGCGGCGCG GCGAAGCGGA CGCGGCGTGC GTGCCGGTCG AAAACTCCGT CGAGGGCGCG
      V  R  R  G  E  A  D  A  A  C  V  P  V  E  N  S  V  E  G  A
181 GTTCCCGCGA CGCTGGACAG CCTCGCCGTC GGCGAGCCGT TGATCGGTGT TGCGGAAGCC
      V  P  A  T  L  D  S  L  A  V  G  E  P  L  I  G  V  A  E  A
241 TTGCTGCCAG TGCATTTCAG CGTTCTCACG CGGGACGACG TCGGCGAAAT CCGTACTGTG
      L  L  P  V  H  F  S  V  L  T  R  D  D  V  G  E  I  R  T  V
301 GCGAGCCATC CGCACGCGCT CGCGCAGGTC CGGAAGTGGC TGGAGGACAA CCTGCCCGGC
      A  S  H  P  H  A  L  A  Q  V  R  K  W  L  E  D  N  L  P  G
361 GCCCGCGTGG TGGCGGCCGG CTCGACCGCG GCGGCCGCGG TCGCCGTGCA GGCGGGGGAG
      A  R  V  V  A  A  G  S  T  A  A  A  A  V  A  V  Q  A  G  E
421 TTCGACGCCG CCGTGACCGC GCCGGTCGCC GTCGAGCACT ACCCGCTCAA GGTACTGGCG
      F  D  A  A  V  T  A  P  V  A  V  E  H  Y  P  L  K  V  L  A
481 ACGGAGGTCG CCGACGTCCG CGACGCCCGC ACCCGGTTCC TGCTGATGCG GCGGCCCCCG
      T  E  V  A  D  V  R  D  A  R  T  R  F  L  L  M  R  R  P  P
541 GTGGTGCTGC CGGAGCCGAC CGGCGCCGAC CGCACCTCGA TCGTCGCCGC GGCCGCCAAC
      V  V  L  P  E  P  T  G  A  D  R  T  S  I  V  A  A  A  A  N
601 CGCACCGGCA CGCTCGCCGA ACTGCTCACC GAACTCGCGA CCCGCGGCAT CAACCTGACC
      R  T  G  T  L  A  E  L  L  T  E  L  A  T  R  G  I  N  L  T
661 CGCCTGGACG CCCGCCCGCA CAAGCAGAAC TTCGGCGAGT ACCGGTTCTT CATCGACTTC
      R  L  D  A  R  P  H  K  Q  N  F  G  E  Y  R  F  F  I  D  F
721 GAGGGACACG TCGCCGAGCC GCGCATCGCC GACGCGCTCG CCGCGCTGCG CCGCCGTTGC
      E  G  H  V  A  E  P  R  I  A  D  A  L  A  A  L  R  R  R  C
781 CGGGATGTCC GGTTCCTCGG TTCGTTCGCC CGGGCCGACG GCGTGGCGGC GACCATCGAA
      R  D  V  R  F  L  G  S  F  A  R  A  D  G  V  A  A  T  I  E
841 CCGGCCGCCC GCAACGAAGA CTTCACCGAC GCCGCGGACT GGGTCGCCGC GGTGCAGAGA
      P  A  A  R  N  E  D  F  T  D  A  A  D  W  V  A  A  V  Q  R
901 GGGGAGCAGG CGTGA
      G  E  Q  A  -
```

FIG. 2. Nucleotide sequence of the *pdt* gene of *A. methanolica*. The deduced amino acid sequence (single-letter code) is shown below the DNA sequence. The nucleotide sequence data have been deposited in the GenBank database under the accession number L47666.

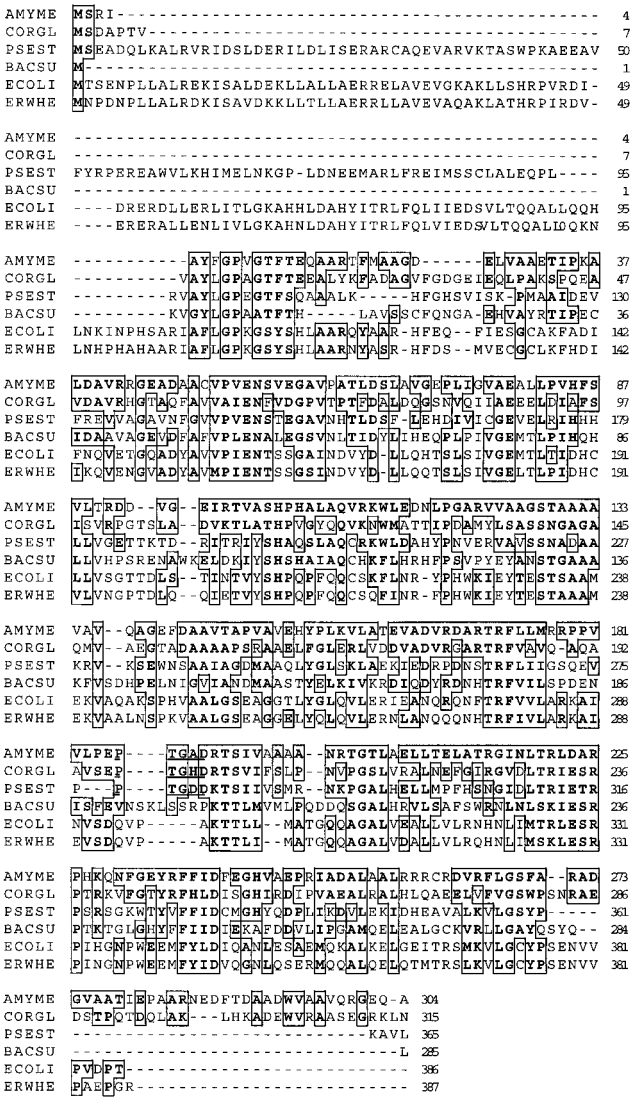

FIG. 3. Alignment of the amino acid sequences of the PDT proteins of *E. coli* (ECOLI) (14), *Erwinia herbicola* (ERWHE) (27), *C. glutamicum* (CORGL) (13), *Pseudomonas stutzeri* (PSEST) (12), *Bacillus subtilis* (BACSU) (20), and *A. methanolica* (AMYME) (this work). Alignments were constructed with the program CLUSTAL from the PC/GENE software package (IntelliGenetics, Mountain View, Calif.). Boxes indicate identical or similar amino acids according to the scheme RK, NQ, DE, PAGST, VILM, FYW, H, and C. Underlined amino acid residues indicate the putative amino acid box for L-Tyr stimulation of PDT enzymes, as observed in *A. methanolica*, *C. glutamicum*, and *P. stutzeri.*

tively. These results confirmed that the entire *A. methanolica pdt* gene had been cloned.

Nucleotide sequence of the *pdt* gene. An 0.9-kb DNA fragment was sequenced (GC content, 73%), revealing the presence of a single large open reading frame that would encode a protein of 304 amino acids (Fig. 2). Its deduced amino acid sequence was nearly identical (33 of 35 residues correct) with the previously determined N-terminal amino acid sequence of the *A. methanolica* PDT protein (10). Also, the calculated molecular mass of 32,288 Da is very close to the estimated molecular mass (34 kDa) of the purified PDT protein (10). The deduced amino acid sequence aligned well with known sequences of other PDT enzymes (Fig. 3) and showed highest similarity (61%) with PheA of *Corynebacterium glutamicum* (13).

PDT of *A. methanolica* is allosterically inhibited by L-Phe and activated by L-tyrosine (L-Tyr) (10). Interestingly, the short PTGXD amino acid sequence (starting at position 186, *A. methanolica* PDT protein) is only present in all PDT proteins that are activated by tyrosine (Fig. 3). Analysis of the amino acid sequences of deregulated mutant PDT proteins (10) will provide further information about factors determining L-Phe inhibition and L-Tyr stimulation.

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