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Published in:
Journal of Bacteriology

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1995

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Vrijbloed, J. W., Hylckama Vlieg, J. V., Put, N. M. J. V. D., Hessels, G. I., & Dijkhuizen, L. (1995). Molecular Cloning with a pMEA300-Derived Shuttle Vector and Characterization of the *Amycolatopsis methanolica* Prephenate Dehydratase Gene. *Journal of Bacteriology*, 177(22), 6666-6669.

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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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Received 26 June 1995/Accepted 10 September 1995

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^mC) and *Sss*I (C^mG) methylases. The methylation efficiency was checked by the (in)ability of the appropriate restriction enzymes, *Bam*HI and *Sall*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 pHP45 Ω -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 *pdt*3 (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. *pdt*3 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. methanolic* and *E. coli* strains and pMEA300-derived plasmids used

Strain or plasmid	Description	Reference
Strains		
<i>A. methanolic</i>	NCIB 11946 wild-type strain	6
WV1	pMEA300-free derivative strain of <i>A. methanolic</i>	24
WV2	Spectinomycin-resistant (150 µg/ml) derivative of strain WV1	24
GH71	PDT-negative mutant, derivative of WV2	9
<i>E. coli</i>		
MC1061	<i>hsdR mcrB araD139 Δ(araABC-leu) 7679 ΔlacX74 galU galK rspL thi</i>	
DH5α	<i>supE44 ΔlacU169 (Ω80lacZΔM15) hsdR17 recA1 gyrA96 thi-1 relA1</i>	
JM110	<i>dam dcm supE44 hsdR thi leu rpsL lacY galK galT ara tonA thr tsx Δ(lac-proAB) F'(traD36 proAB⁺ lacZ ΔM15)</i>	
HB101	<i>supE44 hsdS20 recA 13 ara-14 proA2 lacY1 galK 2 rpsL20 xyl-5 mtl-1</i>	19
Plasmids		
pMEA300	Wild-type plasmid of <i>A. methanolic</i>	23
pWV102	pUC18 (18) containing <i>Bam</i> HI-digested pMEA300 in the <i>Bam</i> HI site	This work
pWV110	pUC18 containing <i>Bgl</i> II-digested pMEA300 in the <i>Bam</i> HI site	This work
pWV136	Integration- and conjugation-deficient derivative of pMEA300 with the pHSS6 <i>ColE1</i> replicon	22
pWV137	pWV136 containing the <i>lacZ</i> region of pUC18 as a <i>Hae</i> II fragment in the <i>Eco</i> RI site	This work
pWV138	pWV136 containing the tetracycline resistance gene flanked by transcription terminators of pHP45Ω-Tc (11) as a <i>Hind</i> III fragment in the <i>Eco</i> RI site	This work
pNAT115	pWV138 containing a 15-kb chromosomal insert encoding the <i>pdt</i> 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. methanolic* 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. methanolic* wild type and GH71 (pNAT115) were 17 and 16 nmol/mg/min, respec-

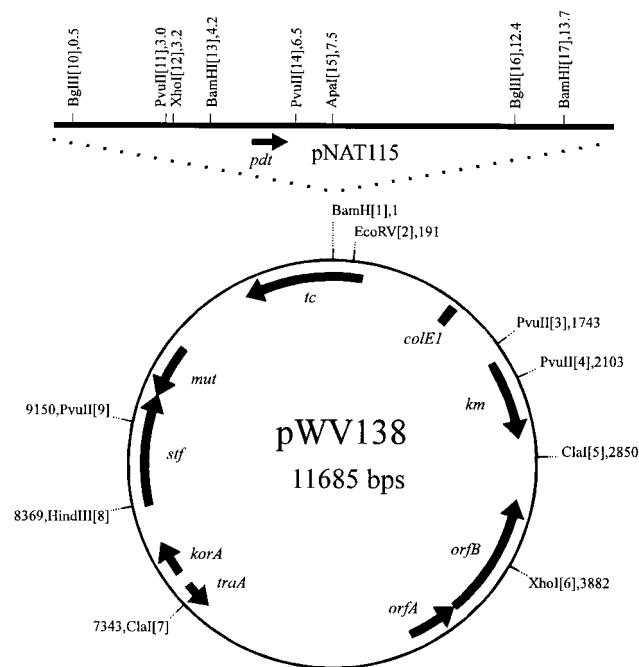


FIG. 1. Restriction maps of the plasmids pWV138 and pNAT115. Numbers between brackets indicate various restriction sites on the plasmids. Other numbers indicate distances from the unique *Bam*HI site on pWV138 and pNAT115. Arrows on pNAT115 and pWV138 indicate open reading frames encoding functions required 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).

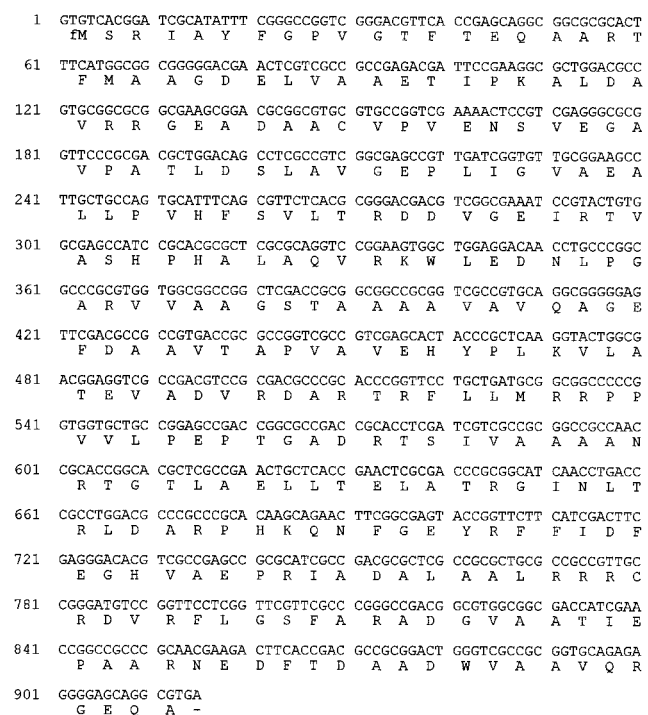


FIG. 2. Nucleotide sequence of the *pdt* gene of *A. methanolic*. The deduced amino acid sequence (single-letter code) is shown below the DNA sequence. The nucleotide sequence data have been deposited in the GenBank database under the accession number L47666.



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.

This study was supported by grant no. GBI81.1510 from the Netherlands Technology Foundation (STW), which is subsidized by the Netherlands Organization for the Advancement of Pure Research (NWO).

We are indebted to Peter Terpstra for assistance in the sequence analysis.

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