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 pME300-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).

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 107 poxcs per μg of DNA).

The effect of pMEA300 methylation on the transformation frequency was further studied by treating pMEA300 DNA with BamHI (GGATCmC) and SssI (CmG) methylases. The methylation efficiency was checked by the (in)ability of the appropriate restriction enzymes, BamHI and Sall, respectively, to digest the in vitro-methylated DNA. Treatment of pMEA300 with BamHI methylase caused a 90% reduction in transformation frequency with strain WV1. The SssI 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 BamHI site of pWV136, yielding pWV138 (Fig. 1). This plasmid contains a unique BamHI site that can be used for cloning, resulting in insertional inactivation of the Tc gene in E. coli. The ligation mixture of pWV138 DNA digested with BamHI and a partial Sau3A 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 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) (TTCTATG[C][G]GC[G][C][G][C][G][C][C]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 BamHI (9.0 kb), BclI (5.5 kb), and PvuII (3.4 kb), and in two
batches of the gene library, digested with PvuII (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 PvuII, 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 PvuII fragment was isolated. Unmethylated plasmid DNA isolated from strain JM110 (pNAT115) subsequently was used to transform A. methanolica wild type and 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, respectively.

**FIG. 1.** Restriction maps of the plasmids pWV138 and pNAT15. Numbers between brackets indicate various restriction sites on the plasmids. Other numbers indicate distances from the unique BamHI site on pWV138 and pNAT15. Arrows on pNAT15 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 (orf) (24), and high mutation frequency (mut) (21).

**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.
enzymes, as observed in *pdt* A. *methanolica* 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 PTGD 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. GB811510 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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