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NOTE

Cloning of Steroid Δ^1-dehydrogenase Gene of *Arthrobacter simplex* IAM 1660

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To clone the gene coding for steroid Δ^1-dehydrogenase of *Arthrobacter simplex*, its genomic library was constructed with a λgt11 expression vector and immunoscreened with antiserum against the enzyme. One positive clone was found to carry a 1.6-kb *Eco*R I restriction endonuclease fragment of *A. simplex* DNA. The restriction map of the 1.6-kb *Eco*R I fragment was determined after cloning of the DNA into pBS vector.

Steroid Δ^1-dehydrogenase which introduces the C_1-C_2 double bond into steroid ring A mediates one of the critical steps for the microbiological steroid ring degradation. The enzyme has attracted considerable interest in the pharmaceutical industry because the 1-dehydro-derivatives of cortisone and cortisol (i.e. prednisone and prednisolone) showed increased anti-allergic and anti-rheumatic activities with significantly reduced side effects (11).

Δ^1-Dehydrogenase activities have been observed in many bacterial genera, including *Pseudomonas, Nocardia, Rhodococcus, Mycobacterium* and *Arthrobacter* (5, 6, 10, 13), but purification of the enzyme from Nocardia was only recent accomplishment (5). The gene encoding 3-oxosteroid Δ^1-dehydrogenase of *Pseudomonas testosteroni* has been cloned, sequenced and expressed in *Escherichia coli* (8). We recently purified and characterized 98-kDa steroid Δ^1-dehydrogenase from *Arthrobacter simplex* (1, 2, 3) which has been widely used in the steroid drug industry to produce Δ^1-dehydrogenated corticosteroids by introducing the C_1-C_2 double bond into the steroids. In this study we report cloning and preliminary characterization of a gene coding for the steroid Δ^1-dehydrogenase from *A. simplex*.

A. simplex IAM 1660 was grown at 30°C in a medium containing 0.1% NH_4NO_3, 0.025% $MgSO_4 \cdot 7H_2O$, 0.025% K_2HPO_4, 0.5% yeast extracts (pH 7.3) with vigorous agitation. *E. coli* Y1090 (*ΔlacU169 proA*$^+$ *Δlon araD139 strA supF* [*trpC22::*Tn10] [pMC9]) was used as a host for the bacteriophage λgt11 vector (4, 12), while *E. coli* DH5α (*supE44 ΔlacU169*[φ80d*lacZ*ΔM15] *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used for plasmid constructions. *E. coli* strains were grown at 37°C in Luria medium (9) under aerobic conditions. *E. coli* cells harboring plasmid pBS (Stratagene Co.) were cultured in Luria medium supplemented with ampicillin (50 μg/ml). Total genomic DNA of *A. simplex* was extracted according to the method described by Marmur (7) with some modifications. Other manipulations of DNA were carried out as described by Sambrook *et al.* (9).

For cloning of the steroid Δ^1-dehydrogenase gene at the unique *Eco*RI restriction site of λgt11, *A. simplex* chromosomal DNA was digested with *Eco*RI. The *A. simplex* chromosomal DNA, however, was not restricted by *Eco*RI possibly due to modification of DNA bases in a way different from that of *E. coli*. The chromosomal DNA was partially digested with another restriction enzyme, *Sma* I and fractionated by agarose gel electrophoresis. DNA fragments in the size from 2- to 7-kb were excised out and purified by Gene Clean Kit (Bio101 Inc.). These fragments were ligated with a 12-bp synthetic *Eco*RI linker. Then the DNA fragments treated with *Eco*RI again were ligated with λgt11 *Eco*RI arms and packaged into infective phage particles using Package extract (Promega). The resulting recombinant phage stocks were transfected into *E. coli* Y1090 and amplified before use.

The constructed genomic library was immunoscreened by using a polyclonal antibody against steroid Δ^1-dehydrogenase purified from *A. simplex* (4, 12). Amplified phage stocks were transfected into *E. coli* Y1090 in LB top agarose containing 10 mM $MgSO_4$. Nitrocellulose filters which had been saturated with 10 mM IPTG were overlayed and incubated on each plate at 37°C for another 3 to 5 h. The filter removed from the plate was treated with rabbit anti-steroid Δ^1-dehydrogenase an-

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tibody and then mouse anti-rabbit IgG-alkaline phosphatase conjugate (Sigma) as a secondary antibody (Fig. 1). As shown in Fig. 2, one positive clone was found and confirmed after tertiary iummunoscreenings. This positive clone contains insert DNA of about 1.6-kb in the λgt11 vector (Fig. 3).

For restriction analysis of the insert DNA, the λ DNA of the immunopositive clone was purified and completely digested with *Eco*RI. After the seperation of the DNA fragments the insert fragments were purified by Gene Clean Kit. The insert fragments were ligated into the *Eco*RI-cut pBS vector with T4 DNA ligase. The ligated plasmid DNA was used to transform $CaCl_2$-treated competent *E. coli* DH5α cells (9). The transformants were selected on LB plates which contained 50 μg/ml ampicillin and 40 μg/ml X-gal. The recombinant plasmid carrying the insert fragments was selected and designated as pBS21. The plasmid pBS21 was digested with several restriction enzymes and analyzed by agarose gel electrophoresis. Each of *Pst* I and *Kpn* I restriction sites was found on the insert DNA, but *Eco*RI, *Bam*HI, *Hin*dIII, *Pvu* I, *Stu* I, *Xba* I and *Xho* I did not cleave the insert DNA (Fig. 4).

Isolation of chromosomal DNA
↓
Restriction endonuclease (*Sma*I) digestion
↓
Size fractionation of DNA
↓
Ligation of *Eco*RI linker
↓
*Eco*RI digestion
↓
Ligation between λgt11 arms and insert DNA
↓
Packaging into phage paticle with package extract
↓
Immunoscreening of λgt11 library with antibody

- *Blotting onto nitrocellulose paper
- *Primary antibody (Antiserum raised against Δ^1-dehydrogenase in rabbit) reaction
- *Secondary antibody (Mouse antirabbit IgG-alkaline phosphatase conjugate) reaction
- *Visualization after alkaline phospatase reaction with nitroblue tetrazolium (NBT) and bromochloroindolyl phosphate (BCIP)

Fig. 1. Cloning scheme of steroid Δ^1-dehydrogenase gene of *A. simplex*.

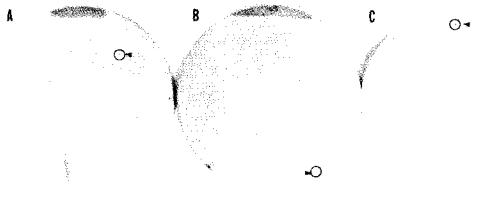

Fig. 2. Isolation of the genomic clones for Δ^1-dehydrogenase from *A. simplex* genomic library in λgt11 by immunoscreening.
A, primary immunoscreening; B, secondary immunoscreening of the clones picked from the primary screening; C, tertiary immunoscreening of the clones picked from the secondary screening. Arrows indicate the immunopositive plaques.

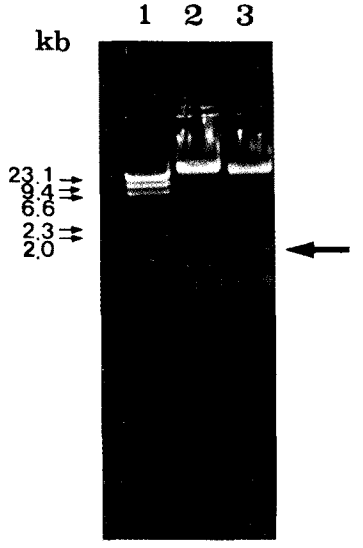

Fig. 3. Agarose gel (1%) electrophoresis of the recombinant λgt11 DNA digested with *Eco*RI.
Lane 1, λ *Hin*dIII size marker; lane 2, immunopositive recombinant λgt11 DNA without restriction; lane 3, immunopositive recombinant λgt11 DNA digested with *Eco*RI.

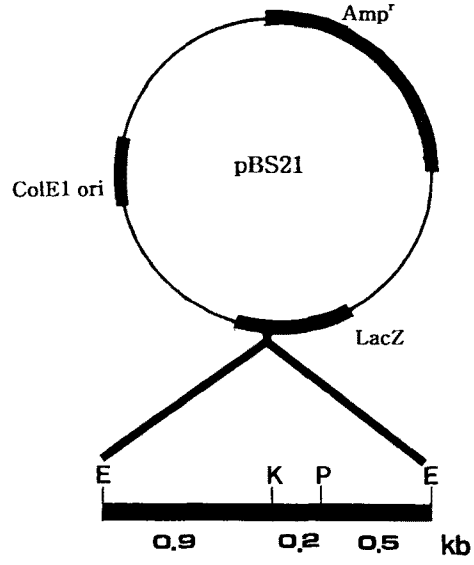

Fig. 4. Restriction map of pBS21.
E, P, and K represent *Eco*RI, *Pst*I, and *Kpn*I, respectively.

The molecular weight of Δ^1-dehydrogenase of *A. simplex* is about 98 kDa (3) and the estimated size of the coding region would be about 2.7 kb. Thus, the 1.6 kb insert DNA fragment could not contain the complete Δ^1-dehydrogenase gene of *A. simplex*. The 1.6-kb DNA, however, should comprise a gene segment encoding part of steroid Δ^1-dehydrogenase responsible for the antigenic determinant(s) which is reactive against the antibdy used in this work. The *A. simplex* chromosomal DNA flanking the 1.6-kb DNA needs to be isolated to get the whole operon coding for steroid Δ^1-dehydrogenase of *A. simplex*, which will be performed with the 1.6-kb DNA.

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