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Cloning, Sequencing, and Expression of the *Pseudomonas testosteroni* Gene Encoding 3-Oxosteroid Δ^1-Dehydrogenase

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Pseudomonas testosteroni **ATCC 17410 is able to grow on testosterone. This strain was mutagenized by Tn*5*, and 41 mutants defective in the utilization of testosterone were isolated. One of them, called mutant 06, expressed 3-oxosteroid Δ^1- and 3-oxosteroid Δ^4-5 α-dehydrogenases only at low levels. The DNA region around the Tn*5* insertion in mutant 06 was cloned into pUC19, and the 1-kbp *Eco*RI-*Bam*HI segment neighbor to the Tn*5* insertion was used to probe DNA from the wild-type strain. The probe hybridized to a 7.8-kbp *Sal*I fragment. Plasmid pTES5, which is a pUC19 derivative containing this 7.8-kbp *Sal*I fragment, was isolated after the screening by the 1-kbp *Eco*RI-*Bam*HI probe. This plasmid expressed Δ^1-dehydrogenase in *Escherichia coli* cells. The 2.2-kbp *Kpn*I-*Kpn*I segment of pTES5 was subcloned into pUC18, and pTEK21 was constructed. In *E. coli* containing the *lacI*^{q} plasmid pRG1 and pTEK21, the expression of Δ^1-dehydrogenase was induced by isopropyl-β-D-thiogalactopyranoside (IPTG). The induced level was about 40 times higher than the induced level in *P. testosteroni*. Δ^1-Dehydrogenase synthesized in *E. coli* was localized in the inner membrane fraction. The minicell experiments showed that a 59-kDa polypeptide was synthesized from pTEK21, and this polypeptide was located in the inner membrane fraction. The complete nucleotide sequence of the 2.2-kbp *Kpn*I-*Kpn*I segment of pTEK21 was determined. An open reading frame which encodes a 62.4-kDa polypeptide and which is preceded by a Shine-Dalgarno-like sequence was identified. The first 44 amino acids of the putative product exhibited significant sequence similarity to the N-terminal sequences of lipoamide dehydrogenases.**

Steroids are growth substrates for a wide variety of microorganisms (28). In the steroid catabolic pathways of these organisms, the degradation of the compounds is usually initiated by the introduction of unsaturated bonds into ring A. In *Pseudomonas testosteroni*, which can grow on testosterone (25), Δ^1-dehydrogenase (EC 1.3.99.4) and two Δ^4-dehydrogenases (Δ^4-5α-dehydrogenase and Δ^4-5β-dehydrogenase) which introduce double bonds into ring A at positions C1-C2 and C4-C5, respectively (10, 26), are induced by steroids. Unlike Δ^4-5β-dehydrogenase, which is a soluble protein, the Δ^1- and Δ^4-5α-enzymes are membrane bound (26). Δ^1-Dehydrogenases from other steroid-degrading bacteria are also membrane bound (33, 38, 47). The analysis of Δ^1-dehydrogenase in *P. testosteroni* has suggested that the major fraction of this enzyme is firmly attached to the inner membrane (44). Partial purification of Δ^1- and Δ^4-5α-dehydrogenases has revealed that these two activities are determined by different proteins (26). By analogy with isofunctional steroid dehydrogenase of *Bacillus sphaericus* (35), the reaction catalyzed by Δ^1-dehydrogenase of *P. testosteroni* is thought to occur by the direct and irreversible *trans*-diaxial removal of the 1α,2β hydrogens.

Very little genetic information is available on the steroid catabolic pathway in *P. testosteroni* (8, 25). The catabolic enzymes including Δ^1-dehydrogenase are expressed at 30°C but not at 37°C (45). Recently, the structural gene for Δ^5-3-ketoisomerase, has been cloned and sequenced (6, 7, 22). This paper describes the isolation and characterization of the structural gene for Δ^1-dehydrogenase of *P. testosteroni*.

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MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. Bacterial cells were grown in L broth (36), YE broth, or on Mueller-Hinton agar plates (Institut Pasteur Production, Paris, France). The composition of YE broth is similar to HC broth (31), but Casamino Acids were replaced by yeast extract at the same concentration. Minimal medium described by Davis (11), from which citrate was omitted, was used for minimal plates. Agar (1.5%, wt/vol) and an appropriate carbon source at a final concentration of 5 mM (except testosterone, which was 2.5 mM) were added. Amino acids were also added to the medium as required (18). For enzyme assays, cells of *P. testosteroni* were grown in YE broth containing testosterone at a final concentration of 100 μg per ml, while for biotransformation tests, YE broth containing 500 μg of a steroid per ml was used. All bacterial cultures were incubated at 30°C. Antibiotics were used when necessary at the following concentrations (in micrograms per milliliter): ampicillin, 25; kanamycin, 25 for *E. coli* and 500 for *P. testosteroni*.

Preparation of cell extracts and enzyme assays. Overnight cultures of *P. testosteroni* grown in YE broth were diluted 10-fold into fresh medium containing testosterone as an inducer and incubated with vigorous shaking until late-exponential growth phase. Recombinant cells of *E. coli* were grown in L broth supplemented with antibiotics. Overnight cultures were diluted approximately 15 times into fresh medium and cultivated for 2 h. If not stated, isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM was added to the cultures, which were further cultivated to late-exponential growth phase. For induction kinetic studies, 10-ml samples were harvested every hour from the cultures. Cells were washed twice in 50 mM

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Remarks and origin or reference
Strains		
E. coli		
DH5α	*supE44 endA1 hsdR17* (r_K^- m_K^+) *thi-1 recA1* Δ(*argF-lac zya*)U169 φ80 *lacZ* ΔM15	Recipient strain for cloning with pUC vectors (from GIBCO-BRL)
JM103	*supE thi* Δ(*lac-proAB*) F′(*traD36 proAB*$^+$ *lacI*^{q} *lacZ* ΔM15)	36
JM105	*supE endA sbcB15 hsdR4 rpsL thi* Δ(*lac-proAB*)	49
S17-1	*pro res mod*$^+$ Sm^r Tp^r, contains RP4-2 (Tc::Mu) (Km::Tn*7*) integrated in the chromosome	Mobilizing strain for pSUP2021 transfer (39)
TH912	F$^-$ *minA minB rpsL*	Minicell-producing strain (16)
P. testosteroni ATCC 17140	*tes*$^+$	N. J. Palleroni
Plasmids		
pRG1	*lacI*^{q}	pACYC177 derivative obtained from J. Clark (13)
pSUP2021	Ap^r Cm^r Km^r(Tn*5*) Tc^r	Suicide vector for Tn*5* mutagenesis (39)
pUC18, pUC19	Ap^r	Multipurpose cloning vectors (49)

HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonate, Na^+)] at pH 8.2, resuspended in 1 ml of the same buffer, and disrupted by sonication in a Soniprep 150 disintegrator (MSE Scientific Instruments, Crawley, England) for 2 min at 0°C. Protein concentrations in the crude extracts were determined by BCA protein assay reagent (Pierce) calibrated by serum albumin. The assay for Δ^1-dehydrogenase was performed by using Wurster's blue as an electron acceptor. This method was adapted from the assay methods of two other dehydrogenases of *P. testosteroni* (14, 34). In a 1-ml mixture consisting of 0.75 ml of 1.4 mM KCN in 50 mM HEPES (pH 8.2), 0.25 ml of Wurster's blue solution in distilled water (0.01% [wt/vol]), and lysate, 4-androstene-3,17-dione dissolved in 10 μl of dioxane (used for *P. testosteroni* extracts) or in 10 μl of methanol (used for extracts from recombinant strains) was added to a final concentration of 25 μM to initiate the reaction. The rate of the Wurster's blue reduction was measured at 600 nm, and this rate was subtracted by the rate observed with the reaction mixture containing no steroid. The assay was performed at 25°C. Reaction rates were calculated from speeds of initial decrease in A_{600}. One unit of activity was defined as the amount of enzyme producing a decrease of 0.001 in A_{600} per min. The assay based on the reduction of the Wurster's dye was also used to measure the activity of Δ^4-5α-steroid dehydrogenase (EC 1.3.99.5) using Δ^1-5α-androstene-3,17-dione as a substrate.

Assays for 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50), 3(17)β-hydroxysteroid dehydrogenase (EC 1.1.1.51), and 3-oxosteroid Δ^5-Δ^4 isomerase (EC 5.3.3.1) using androsterone, 1,4-androstadiene-17β-ol-3-one, and 5-androstene-3,17-dione as substrates, respectively, have previously been described (19, 30, 40).

Assays for the conversion of steroids. Bacterial conversion of steroids to their Δ^1-derivatives was demonstrated by thin-layer chromatography. Cultures (5 ml) were incubated overnight with substrates, and 500-μl samples were mixed to 100 μl of ethyl acetate. The resultant emulsion was separated into two phases by centrifugation, and 5 μl of the organic phase were spotted onto silica gel plates (type 60 F254; Merck Inc.). The solvent system used for the separation of metabolites was dichloromethane-dioxanne (95:5 [vol/vol]). After development, the plates were sprayed with a solution of 15% (vol/vol) sulfuric acid dissolved in ethanol and heated at 100°C for 20 min. Steroid compounds which appeared under UV light (365 nm) as spots of characteristic colors were identified by comparison with reference chemicals.

Genetic techniques. Matings were performed directly on Mueller-Hinton agar plates by spotting 20 μl each of donor and recipient strains. After incubation overnight at 30°C, bacterial spots were scrapped off and resuspended in saline solution, and appropriate dilutions were plated on selective media. Plasmid pSUP2021 (39) was used for Tn*5* insertion mutagenesis of *P. testosteroni*. This pBR325 derivative containing a *mob* site and Tn*5* was transferred from *E. coli* S17-1 to *P. testosteroni*, and Km^r derivatives of *P. testosteroni* were selected on minimal plates containing *p*-hydroxybenzoate and kanamycin (500 μg/ml).

DNA manipulations. Rapid preparation of plasmid DNA was performed as described by Birnboim (3). In subcloning experiments, DNA fragments were isolated from agarose gels by the Gene Clean kit (Bio 101 Inc., La Jolla, Calif.). Size selection of DNA restriction fragments from total genomic digests was carried out by centrifuging digested DNA at 100,000 × *g* for 16 h on a sucrose gradient (5 to 40% [wt/vol]). Fractions containing desired sizes were identified by agarose gel electrophoresis. Other DNA manipulations were carried out according to standard protocols (36).

DNA sequencing. The 2.2-kbp *Kpn*I insert of pTEK21 was subcloned into M13mp18 and M13mp19 vectors (49). Overlapping DNA fragments were generated by (i) subcloning of appropriate restriction fragments and (ii) unidirectional nested deletions using exonuclease III and S1 nuclease according to the manufacturer's recommendations (kit from Pharmacia, Inc.). The DNA sequences of these fragments were determined by the dideoxy-chain termination method of Sanger et al. (37) using Sequenase version 2 (U.S. Biochemical Corp., Cleveland, Ohio). The samples were separated by electrophoresis in 0.4-mm, 7% polyacrylamide gels and exposed to Fuji films. DNA sequences were edited by the DNASIS program from Pharmacia.

Preparation of minicells and analysis of plasmid-encoded proteins. The preparation of minicells and their incubation with [^{35}S]methionine were as described by Harayama et al.

(15) except that the minicell-producing strain was cultured overnight at 37°C in L broth. [^{35}S]methionine incorporations were carried out at 30°C for 30 min. Electrophoresis of minicell proteins was performed in denaturing 10% acrylamide-bisacrylamide (29:1) gels according to the method of Laemmli (23). After staining with Coomassie blue R, gels were dried and exposed to Fuji films.

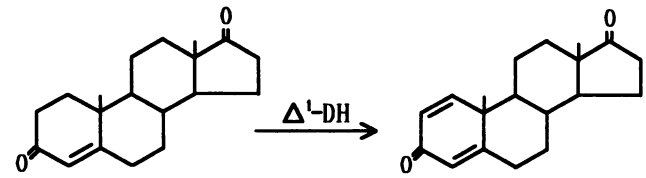
Isolation of subcellular fractions. The cellular distribution of Δ^1-dehydrogenase in *E. coli* JM105 cells containing recombinant plasmids was determined by measuring the Δ^1-dehydrogenase activity in different subcellular fractions. Spheroplasts were prepared from cells grown to mid-exponential growth phase in 200 ml of L broth by lysozyme and mild osmotic shock (46). Proteins released into osmotic shock fluids were considered to be periplasmic proteins. Spheroplasts were then disrupted by sonication for 2 min at 0°C. The cell debris were removed by low-speed centrifugation at 0°C (8 min, 3,000 × *g*). Membranes were sedimented by high-speed centrifugation (2 h, 100,000 × *g*) and subsequently separated by sucrose gradient centrifugation as previously described (41). Proteins in the 100,000 × *g* supernatant were defined as soluble proteins. Each of these fractions was then assayed for the Δ^1-dehydrogenase activity. The activity of NADH dehydrogenase, an enzyme bound to the inner membrane, was also determined in order to score cross-contamination of the inner membrane into other fractions (51). To prepare inside-out vesicles of the inner membrane, the cells were disrupted with a French press (American Instrument Company, Silver Spring, Md.) with a pressure ratio of 500 and fractionated by the procedures described above. The sensitivity of Δ^1-dehydrogenase to trypsin was determined by measuring residual activities of Δ^1-dehydrogenase after incubation of the vesicles (250 μg of proteins per ml) with trypsin (50 μg per ml) in 50 mM HEPES buffer (pH 8.2) at a room temperature.

Chemicals. Most of steroids used in this work were kindly provided by Roussel Uclaf Inc. (Romainville, France); other steroids were purchased from Sigma Chemical Co. Molecular biology products and enzymes, if not specified, were from GIBCO-BRL (Bethesda Research Laboratories), Amersham Corp., and Appligène (Illkirch, France). Wurster's blue was synthesized as described by Michaelis (29).

Nucleotide sequence accession number. The accession number of the pTEK21 insert sequence in GenBank is M68488.

RESULTS

Isolation of Tn*5* insertion mutants of *P. testosteroni* defective in steroid degradation. *P. testosteroni* ATCC 17410 was mutagenized by Tn*5* as described in Materials and Methods, and mutants were selected on minimal medium plates supplemented with *p*-hydroxybenzoate and kanamycin. After 5,200 Km^r derivatives were isolated, they were streaked on minimal medium containing testosterone. A total of 41 mutants exhibited altered growth phenotypes on the testosterone minimal plates; 10 of them did not show any detectable growth while the other 31 produced tiny colonies on the testosterone plates. The 41 mutants were assayed for the conversion of testosterone into other intermediates such as 4-androstene-3,17-dione (4-ADO) and 1,4-androstadiene-3,17-dione (1,4-ADDO) (Fig. 1). Culture samples were taken at 5, 24, 48, 72, and 96 h after the start of the incubation and were analyzed by thin-layer chromatography. The majority of the mutants (24 of 41), like the wild-type strain, transformed testosterone without significant accumulation of 4-ADO and 1,4-ADDO. Fourteen other mutants accumulated 1,4-ADDO as the only intermediate detectable after 96 h of incubation. These 14 mutants are therefore defective in the subsequent step(s) to Δ^1-dehydrogenation. In three other mutants, 4-ADO was accumulated, although a small quantity of 1,4-ADDO was also produced. One of the three mutants was named mutant 06.



4-androstene-3,17-dione 1,4-androstadiene-3,17-dione

FIG. 1. Reaction catalyzed by 3-oxosteroid Δ^1-dehydrogenase.

Induced cell-free extracts of mutant 06 were assayed for several steroid-transforming enzymes. Comparison of these activities with the corresponding activities in the wild-type strain (Table 2) revealed that the Δ^1- and Δ^4-5α-dehydrogenase activities were dramatically reduced in mutant 06 while expression of other enzymes was not affected. The dehydrogenase activities in mutant 06 were only a small percent of the induced levels in the wild-type strain but about 500% of the noninduced levels in the wild-type strain. From these results, it was inferred that the Tn*5* insertion in mutant 06 is located in the same operon as, but upstream of, the structural genes for Δ^1- and Δ^4-5α-dehydrogenases.

Cloning of the *Eco*RI fragment containing Tn*5* from mutant 06. DNA was extracted from mutant 06 and digested by *Eco*RI, an endonuclease that does not cleave Tn*5* (20). Digested DNA larger than the size of Tn*5* (5.4 kbp) was selected on a sucrose gradient and then ligated to *Eco*RI-cleaved pUC19. *E. coli* DH5α was transformed by the ligated DNA, and Km^r clones were selected. All five Km^r clones thus isolated contained inserts of the same structure: a 13-kbp insert consisting of a 7.6-kbp *Eco*RI fragment originated from the *P. testosteroni* genome and 5.4-kbp-long Tn*5*. One of the plasmids was designated pTEO130 (Fig. 2). No Δ^1-dehydrogenase activity was detected in the *E. coli* cells harboring pTEO130.

Cloning of the gene encoding Δ^1-dehydrogenase. The 1-kbp *Eco*RI-*Bam*HI fragment of pTEO130 (Fig. 2) was used as a probe to screen DNA of *P. testosteroni*. Total DNA from *P. testosteroni* ATCC 17410 was cleaved by *Bam*HI, *Bgl*II,

TABLE 2. Comparison of specific activities of several steroid-degradative enzymes in *P. testosteroni* wild type and the Tn*5* mutant 06

Enzyme[a]	Sp act[b]	
	P. testosteroni ATCC 17410	Mutant 06
3α-Dehydrogenase	1,000	550
3(17)β-Dehydrogenase	590	440
Isomerase	8.9	16
Δ^1-Dehydrogenase	171[c]	3.5
Δ^4-5α-Dehydrogenase	109	1.5

[a] Cells sonicates were prepared from induced cultures (100 μg of testosterone per ml) in YE broth.

[b] All values are expressed in units per milligram of protein and are averages of duplicate assays.

[c] The noninduced activity was 0.7.

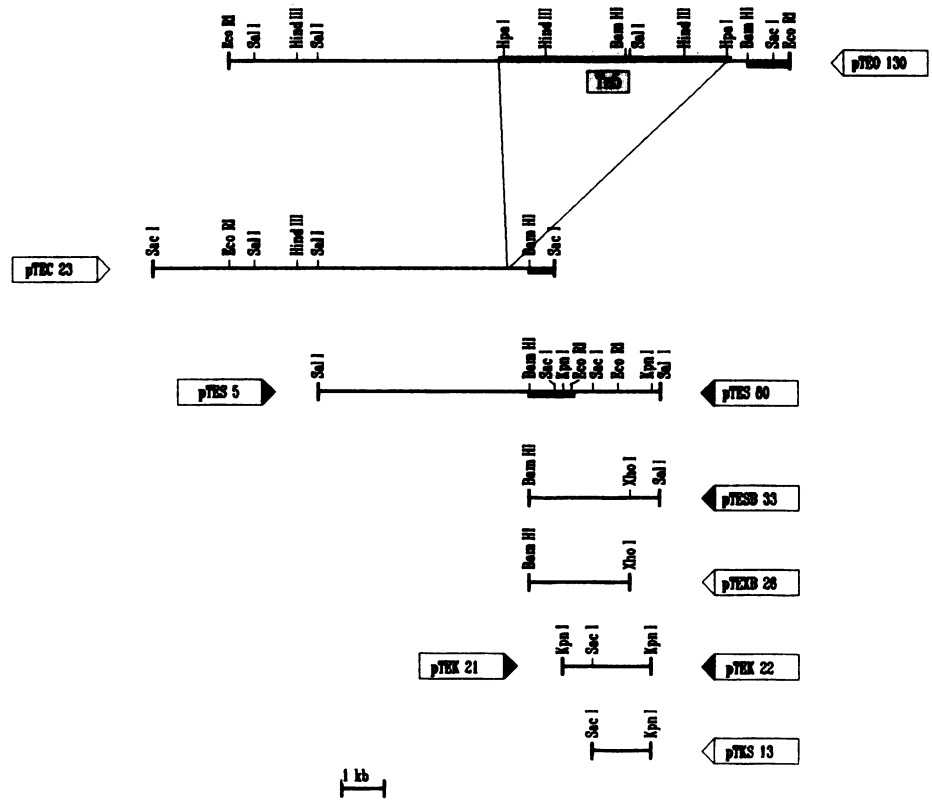


FIG. 2. Physical maps of plasmids containing the gene for Δ^1-dehydrogenase. Plasmid pTEO130 was constructed by cloning into pUC19 the 13-kbp *Eco*RI fragment containing Tn*5* from mutant 06. The heavy line and hatched box on plasmid pTEO130 indicate transposon Tn*5* and the fragment used as a probe, respectively. The direction of the transcription from the *lac* promoter is indicated by arrows. Filled and open tips of the arrows indicate detectable and nondetectable expression, respectively, of the Δ^1-dehydrogenase gene from these clones.

*Eco*RI, *Kpn*I, *Sac*I, *Sma*I, *Sph*I, *Xba*I, or *Xho*I and separated electrophoretically on agarose gels. The DNA was transferred onto nitrocellulose filters and hybridized to the radiolabeled *Eco*RI-*Bam*HI probe. The probe hybridized to a single fragment from each enzyme digest, except the *Kpn*I digest, from which the probe hybridized to two bands. To clone the *Sac*I (9.6 kbp) and *Sal*I (7.8 kbp) fragments hybridized to the probe, *P. testosteroni* DNA from the wild-type strain was cleaved by either *Sac*I or *Sal*I. The *Sac*I digests of about 9.6 kbp and the *Sal*I digests of about 7.8 kbp were isolated from a preparative agarose gel of electrophoresis and cloned into pUC19. Screening by the 1-kbp probe detected six transformants containing the 9.6-kbp *Sac*I insert and seven transformants containing the 7.8-kbp *Sal*I fragment. The *Sac*I fragment in these six transformants was inserted into pUC19 only in one orientation. The resultant recombinant plasmid was named pTEC23. The *Sal*I fragment was inserted into two orientations, as shown for pTES5 and pTES80 in Fig. 2. Tests for the 4-ADO conversion revealed that DH5α cells carrying pTES5 or pTES80 were capable of producing 1,4-ADDO from 4-ADO. In contrast, cells harboring pTEC23 did not exhibit any Δ^1-dehydrogenase activity. *E. coli* containing pTES80 also converted two other substrates of Δ^1-dehydrogenase, testosterone and norandrostene-dione, into the corresponding Δ^1-dehydrogenated products, Δ^1-testosterone and estrone, respectively. *E. coli* harboring pTEC23, pTES5, or pTES80 did not show any activities of other steroid degradative enzymes tested.

Subcloning of the Δ^1-dehydrogenase gene. The Δ^1-dehydrogenase activity was expressed from plasmid pTESB33 containing the 4.5-kbp *Sal*I-*Bam*HI fragment and from plasmids pTEK21 and pTEK22 containing the 2.2-kbp *Kpn*I-*Kpn*I fragment (Fig. 2). The Δ^1-dehydrogenase activity was not expressed from pTEXB26, which is a deletion derivative of pTESB33 lacking the 0.5-kbp *Xho*I-*Sal*I segment, and from plasmid pTKS13 derived from pTEK21 by deleting the *Kpn*I-*Sac*I segment. The Δ^1-dehydrogenase activity in *E. coli* cells containing pTEK21 was higher than those containing pTEK22, pTESB33, or pTES80 in semiquantitative tests. The quantitative assays showed that the activity conferred by pTEK21 (8,600 U/mg of protein) was much higher than that conferred by pTEK22 (100 U/mg of protein) or the activity in induced cells of *P. testosteroni* (170 U/mg of protein). These results suggested that the Δ^1-dehydrogenase gene in pTEK21 is expressed from the *lac* promoter on pUC18. The experiments described below confirmed this hypothesis. In order to localize the 5′ end of the Δ^1-dehydrogenase gene more precisely, nested deletions were introduced into pTEK22 by digestion with exonuclease III and S1 nuclease after cleavage of pTEK22 by *Sma*I and *Sph*I. A set of deletion derivatives thus constructed were tested for the 4-ADO conversion. A deletion of approximately 260 bp inactivated the Δ^1-dehydrogenase gene while the removal of 210 bp did not affect the expression of Δ^1-dehydrogenase. These results were confirmed by recloning the deleted inserts in a proper orientation within pUC19 after cleavage of the deletion derivatives by *Kpn*I and *Hin*dIII. Reconstructed plasmids were named pTEK213

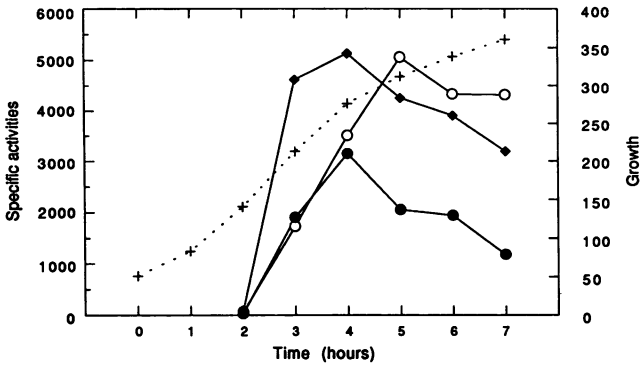


FIG. 3. Expression of the Δ^1-dehydrogenase gene in *E. coli*. Strain JM105 harboring the plasmids pTEK21 coding for Δ^1-dehydrogenase and pRG1 carrying the *lacI*^{q} gene was cultured in L broth at 30°C. After 2 h of incubation, the cells were induced by the addition of IPTG at final concentrations of 0.1 mM (○), 0.5 mM (◆), or 5 mM (●). Activities are expressed in units per milligram of protein. The dotted-line curve represents the growth curve of strain JM105 containing pTEK21 and pRG1 in Klett units. No significant growth retardation occurred even when *E. coli* cells were induced with a high concentration of IPTG.

(contains 260-bp deletion) and pTEK211 (contains 210-bp deletion). According to the results of the subcloning and deletion analysis, the size of the Δ^1-dehydrogenase gene was estimated to range between 1.6 and 2.0 kbp.

Expression of the Δ^1-dehydrogenase gene in *E. coli*. pTEK21 was introduced into strain JM105 containing the multicopy plasmid pRG1 which carries the *lacI*^{q} gene, and induction kinetics of the Δ^1-dehydrogenase activity in this strain were examined after addition of different concentrations of IPTG (Fig. 3). In the absence of IPTG, the expression of the Δ^1-dehydrogenase gene was very low. The addition of IPTG, however, resulted in a dramatic increase of the Δ^1-dehydrogenase activity, which reached a maximum level within 2 to 3 h. These data clearly demonstrated that the Δ^1-dehydrogenase gene on pTEK21 is transcribed from the *lac* promoter.

Cellular localization of Δ^1-dehydrogenase. Previous reports have suggested that Δ^1-dehydrogenase is associated with the inner membrane (43, 44). To determine the cellular location of this enzyme when synthesized in *E. coli* cells, the cell extract prepared from JM105 containing pTEK21 was fractionated, and the distribution of the Δ^1-dehydrogenase activity in different fractions was examined (Table 3). Of the total enzyme activity, 95% was sedimented by ultracentrifugation at 100,000 × *g*. Further fractionation showed that a major part (82.7%) of the activity resided in the inner membrane fraction, while 12.3% was present in the outer membrane fraction. However, NADH-dehydrogenase, a marker enzyme for the inner membrane (9), was distributed similarly between the inner and outer membrane fractions. This suggests that a cross-contamination of the two membrane fractions has occurred in this preparation. It therefore appears that, as has been shown for *P. testosteroni* (44), Δ^1-dehydrogenase is bound to the inner membrane in *E. coli*. To investigate the orientation of Δ^1-dehydrogenase in the inner membrane, inside-out vesicles were prepared from *E. coli* cells induced for the synthesis of Δ^1-dehydrogenase, and the vesicles were treated with trypsin. The Δ^1-dehydrogenase activity in the vesicles was constant in the absence of trypsin but continuously decreased with time in the presence

TABLE 3. Cellular distribution of the Δ^1-dehydrogenase activities in the recombinant strain JM105(pTEK21, pRG1)

Cellular fractions[a]	Enzyme activities (%)	
	Δ^1-Dehydrogenase[b]	NADH-dehydrogenase[c]
Periplasmic fraction	8.3 (1.7)	ND
Cytosolic soluble proteins	15.7 (3.3)	ND
Cytoplasmic membrane fraction	394.3 (82.7)	742 (84.7)
Outer membrane fraction	58.5 (12.3)	134.5 (15.3)

[a] The definition and the preparation of the different cell fractions are reported in Materials and Methods.
[b] Δ^1-Dehydrogenase activities induced with 0.5 mM IPTG are expressed in kilounits.
[c] NADH-dehydrogenase activities are expressed in nanomoles of $NADH_2$ oxidized per minute under the conditions of the assay (51). ND, not determined.

of trypsin: 7,700 U/mg of protein at 0 min, 5,000 at 10 min, 3,300 at 20 min, and 2,000 at 30 min. This result indicates that the enzyme was exposed to the outer surface of the vesicles and therefore that the enzyme is present at the inner surface of the cytoplasmic membrane in the intact bacteria.

Identification of the Δ^1-dehydrogenase polypeptide in minicells. Products from pTEK21 were analyzed in an *E. coli* minicell-producing strain, TH912, containing this hybrid plasmid. Autoradiograms revealed the synthesis of a polypeptide of an apparent molecular mass of 59 kDa (Fig. 4). This protein was not synthesized from cells harboring the plasmid vector pUC18. The synthesis of the 59-kDa polypeptide was inducible in JM105 containing pTEK21 and pRG1 and found in the inner membrane fraction (data not shown). These characteristics of the 59-kDa polypeptide strongly suggest that this polypeptide corresponds to Δ^1-dehydrogenase. Interestingly, the Δ^1-steroid dehydrogena-

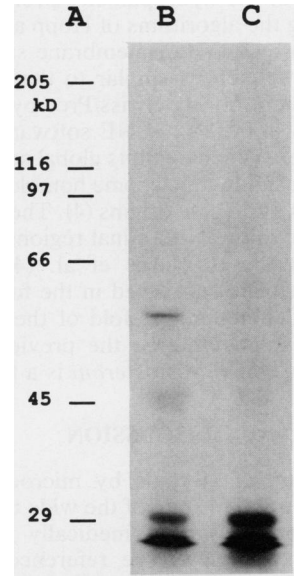


FIG. 4. Autoradiogram of [^{35}S]methionine-labeled peptides produced in minicells of *E. coli* TH912. The positions of the migration of protein standards are indicated in lane A. The products from minicells containing pTEK21 are in lane B and from those containing pUC18 are in lane C. Note that a polypeptide of 59 kDa was synthesized from pTEK21.

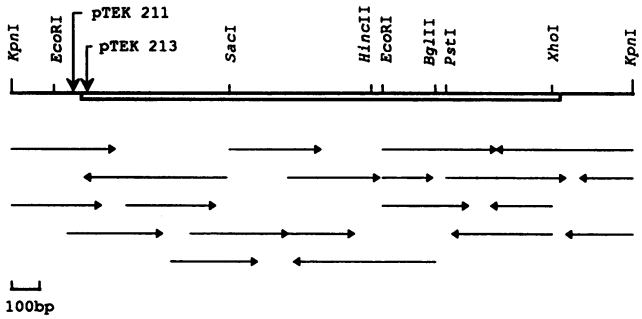


FIG. 5. Sequencing strategy of the 2.2-kbp insert of pTEK21. Locations of deleted fragments of plasmids pTEK211 and pTEK213 are indicated. The open box represents the coding sequence of the Δ^1-dehydrogenase gene.

ses of nocardioform bacteria have similar molecular masses (56 and 58 kDa) (21).

Nucleotide sequence determination of the Δ^1-dehydrogenase gene. The DNA sequence of the 2.2-kbp *Kpn*I-*Kpn*I insert on pTEK21 was determined for both strands by the strategy presented in Fig. 5. The overall base content of the pTEK21 insert (G+C = 61.7%) was very similar to that (61.8%) reported previously for the *P. testosteroni* genome (Fig. 6) (42). A potential translational start codon which is preceded by a potential ribosome binding site (A-GGAGA) was found at position 248 (12). An open reading frame consisting of 1,722 nucleotides was downstream of this putative start codon. These data are in agreement with those of the deletion analysis which indicated that the Δ^1-dehydrogenase gene starts between nucleotides 210 and 260. Moreover, the molecular mass of Δ^1-dehydrogenase predicted from the DNA sequence (62.4 kDa) is consistent with the size of the product synthesized in minicells (59 kDa). The smaller size observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis is a general tendency of membrane proteins.

Examination of the hydrophilicity profile of the deduced protein by using the algorithms of Hopp and Woods (17) did not detect any obvious transmembrane stretches. A search for amino acid sequences similar to that of Δ^1-dehydrogenase in the protein library Swiss/Prot by using the FASTSCAN program in the PC/GENE software package did not detect any protein which exhibits global sequence similarity. However, the scan identified some homology with lipoamide dehydrogenases of diverse origins (4). The sequence similarity was confined in the N-terminal regions of these proteins (Fig. 7). According to Burns et al. (4), the N-terminal glycine-rich stretches conserved in the four proteins correspond to the adenine-binding fold of the FAD-binding domain. These results reinforce the previous inference that Δ^1-dehydrogenase of *P. testosteroni* is a flavoprotein (43).

DISCUSSION

The catabolism of steroids by microorganisms has received little attention in spite of the wide use of bacteria and fungi for the production of medically important steroid derivatives (for a review, see reference 5). Only a few studies about the genes involved in the catabolism of steroids exist (6, 7, 22, 24). In this work, we characterized the Δ^1-dehydrogenase gene of *P. testosteroni*.

In order to study the Δ^1-dehydrogenase gene, we developed a new assay method for Δ^1-dehydrogenase based on the reduction of Wurster's blue. This method was more sensitive and reproducible than the previous methods based on the anaerobic reduction of phenazine methosulfate (26) or the use of radiolabelled substrates (44). In this work, we obtained 41 mutants of *P. testosteroni* after Tn*5* insertion mutagenesis. One of these mutants, called 06, expressed Δ^1- and Δ^4-5α-dehydrogenases at very low levels but expressed other steroid catabolic enzymes at the same levels as the wild-type strain. These observations suggest that the transposon Tn*5* in mutant 06 was inserted in the operon containing the Δ^1- and Δ^4-5α-dehydrogenase genes but upstream of these genes. Although Tn*5* usually exerts a polar effect on distal genes when inserted in an operon, genes located downstream of Tn*5* can be expressed at low levels because of a promoter associated with this transposon (2). This promoter may also be responsible for the higher expression of the Δ^1- and Δ^4-5α-dehydrogenase genes; the expression of these genes was five times higher in mutant 06 than in the uninduced wild-type strain.

The structural gene for Δ^4-5α-dehydrogenase was not cloned in this study. However, the present results suggest that the gene is located on the right side of the pTEK21 DNA (Fig. 2) and that the steroid operon promoter is located on the left side of the Tn*5* insertion. Therefore, the partial structure of the steroid operon revealed in this study is the following: promoter–Δ^1-dehydrogenase gene–Δ^4-5α-dehydrogenase gene.

E. coli cells harboring pTEK21 expressed the Δ^1-dehydrogenase activity 40 to 50 times higher than that in induced cells of *P. testosteroni*. The hyperproduction of the enzyme should facilitate its current purification. However, the induction of *E. coli* JM105 containing pTEK21 by high concentrations of IPTG (5 mM) reduced the yield of Δ^1-dehydrogenase activity (Fig. 3). The overproduction of foreign proteins by *E. coli* sometimes results in the formation of inactive molecules (36), and this phenomenon may be responsible for the reduced activity of Δ^1-dehydrogenase in highly induced *E. coli* cells.

The amino acid sequences of several dehydrogenases involved in the metabolism of steroids have been determined. They are 3β-hydroxysteroid dehydrogenase from *P. testosteroni* (50), estradiol 17β-dehydrogenase from humans (32), 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* (27), and corticosteroid 11β-dehydrogenase from rats (1). These enzymes are soluble proteins and members of a short-chain alcohol dehydrogenase family. They did not exhibit any sequence similarity to Δ^1-dehydrogenase. We also examined the sequence similarity of Δ^1-dehydrogenase to other dehydrogenases. The alignment between lactate dehydrogenase (48) and Δ^1-dehydrogenase, for example, exhibits 9.3% identity and 43% similarity (data not shown). Although the sizes of these proteins are similar (573 amino acid residues for Δ^1-dehydrogenase versus 591 for lactate dehydrogenase), we could not conclude from this result whether these two enzymes share a common ancestor. Further biochemical studies will be necessary to clarify the relationship between Δ^1-dehydrogenase and other dehydrogenases.

As for many other dehydrogenases bound to the inner membrane, Δ^1-dehydrogenase from *P. testosteroni* seems to contain neither typical signal-anchor sequence nor transmembrane hydrophobic stretches (9).

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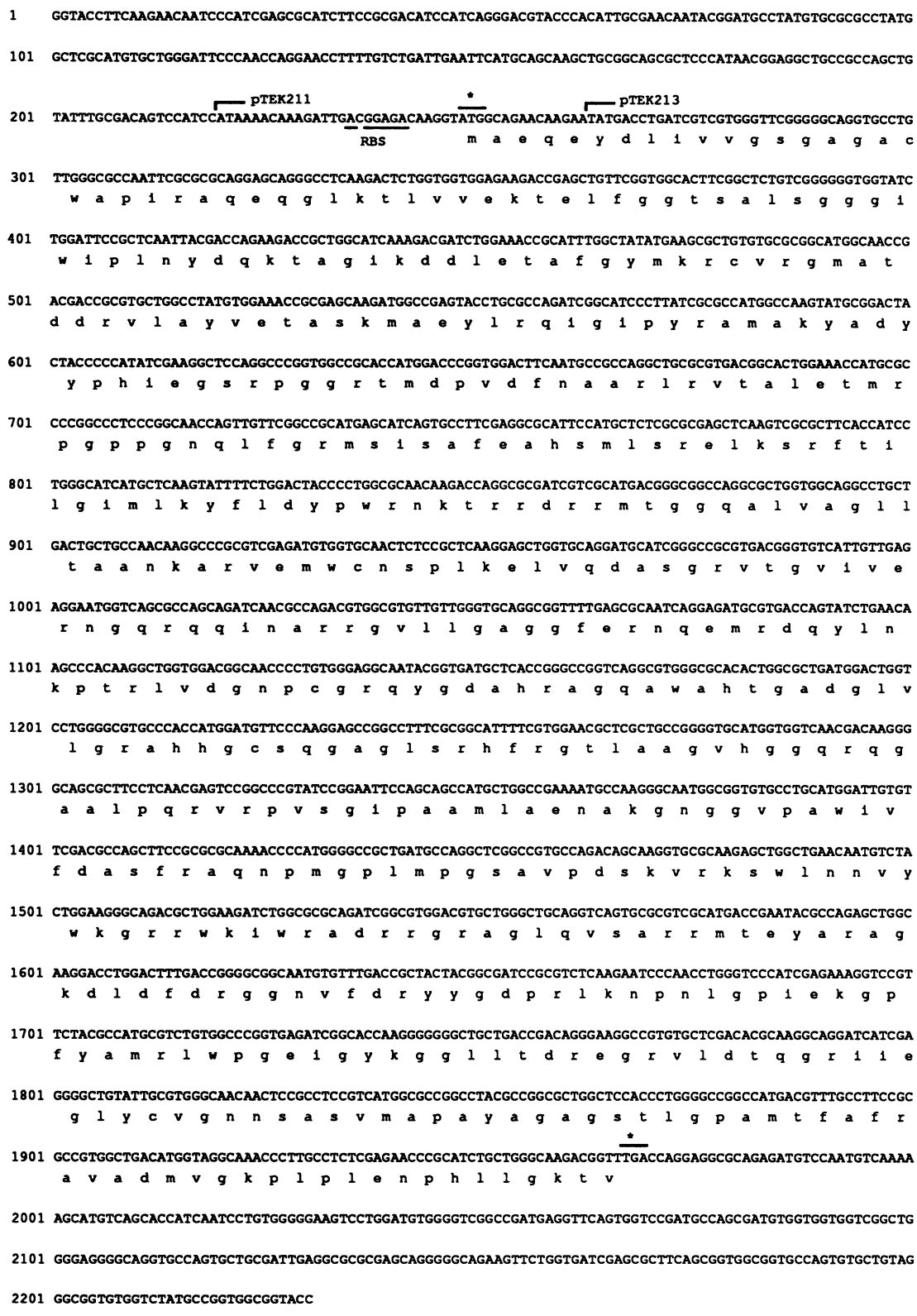

FIG. 6. Nucleotide sequence of the Δ^1-dehydrogenase gene and predicted amino acid sequence of the enzyme. The sequence of the insert of plasmid pTEK21 is shown. The potential ribosome-binding site (RBS) is underlined. The 5′ ends of the fragments which are contained on plasmids pTEK211 and pTEK213 and which confine the start point of the Δ^1-dehydrogenase gene are also indicated. The initiation codon ATG and the termination codon TGA are labeled with asterisks. The translation of the coding segment into amino acids (573 residues) is given under the nucleotide sequence.

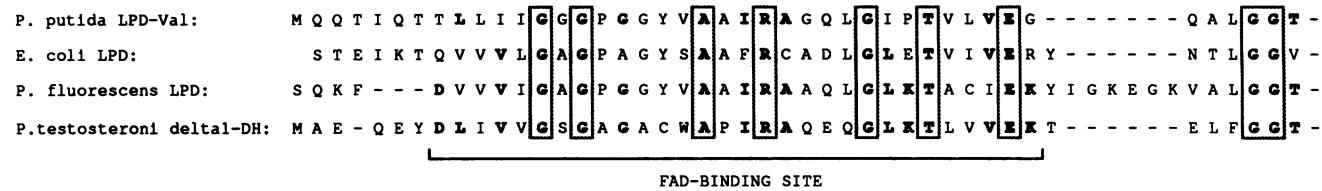

FIG. 7. Alignments of the N-terminal sequences of Δ^1- and lipoamide dehydrogenases. Conserved residues are in boldface. Boxes represent complete homologies among the four proteins.

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