Steroid catabolism in bacteria: Genetic and functional analyses of stdH and stdJ in Pseudomonas putida DOC21

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Abstract

Pseudomonas putida DOC21 assimilates a large variety of steroids, including bile acids, via a single 9, 10-seco pathway. Two specific mutants knocked down in stdH and stdJ were obtained by deletion (strains P. putida DOC21ΔstdH and P. putida DOC21ΔstdJ). Analysis of these mutants revealed that both had lost the ability to fully degrade bile acids and that the genes stdH and stdJ are involved in oxidation of the A and B rings of the polycyclic steroid structure. Moreover, whereas P. putida DOC21ΔstdH and P. putida DOC21ΔstdJ were unable to degrade testosterone or 4-androstene-3,17-dione (AD), P. putida DOC21ΔstdJ was also unable to assimilate androsta-1,4-diene-3,17-dione (ADD). When cultured in medium containing lithocholate and succinate, P. putida DOC21ΔstdH and P. putida DOC21ΔstdJ accumulated AD and ADD, respectively. Genetic and bioinformatic analyses revealed that: (i) stdH encodes a 3-ketosteroid-Δ1-dehydrogenase; (ii) StdJ is the reductase component of a 3-ketosteroid 9α-hydroxylase; (iii) the trans-expression of stdH and stdJ in the corresponding mutant restored the lost catabolic function(s), and (iv) full steroid metabolism by P. putida DOC21ΔstdH was restored by its expression of kstD2, but not kstD1 or kstD3, of Rhodococcus ruber Chol-4. Our results shed light on the systems used by bacteria to oxidize the A and B rings of steroid compounds. In addition, as the mutants described herein were able to synthesize two pharmaceutically important synths, AD and ADD, they may be of value in industrial applications.

Keywords: Steroids, Ketosteroid dehydrogenases, KstD isozymes, Bile acids

Introduction

Microbial degradation of cholesterol, bile acids, and other steroids containing a hydroxyl group at the C3 position (ring A in Fig. 1) requires the actions of 3α/3β-hydroxysteroid dehydrogenases or cholesterol oxidases. These enzymes catalyze oxidation of the hydroxyl group, resulting in its conversion to a keto group [1-4]. The reaction is followed by progressive shortening of the aliphatic side chain at C17 (if present) and further oxidation of the A ring [4-7]. Two ketosteroid dehydrogenases play key roles in steroid degradation. Desaturation of the bond between carbon atoms 1 and 2, with concomitant trans axial removal of the hydrogen atoms C1 (α) and C2 (β), is catalyzed by a 3-ketosteroid-Δ1-dehydrogenase [8-10]. The removal of hydrogen atoms C4 (β) and C5 (α) is mediated by 3-ketosteroid-Δ4-dehydrogenase [11, 12]. Depending on the structure of the initial steroid, the end products consist of different keto-diene-containing molecules which are either androsta-1,4-diene-3,17-dione (ADD) or its C7 and/or C12 hydroxyl derivatives (Fig. 1) [4, 13]. Further degradation of these compounds proceeds via a 3-ketosteroid 9α-hydroxylase that introduces a hydroxyl group at C9, leading to 9α-hydroxy-1,4-androstadiene-3,17-dione (9-OH-ADD) or its C7 and/or C12 hydroxylated derivatives (Fig. 1). As the 9α-hydroxy-1,4-androstadiene-3,17-dione compounds are chemically unstable, they are hydrolyzed spontaneously to yield the phenol 3-hydroxy-9,10-secoandrost-1,3,5(10)-...
triene-9,17-dione or the corresponding derivatives with hydroxylations of the specific carbons (Fig. 1) [5, 13-18].

Both ketosteroid dehydrogenases (Δ1 and Δ1-dehydrogenases) are flavin adenine dinucleotide (FAD)-containing flavoproteins featuring a single-domain Rossmann-related fold [9, 12, 19]. However, the percentage identity between the amino acid sequences of the 3-ketosteroid Δ1- and 3-ketosteroid-Δ1-dehydrogenases belonging to different species does not usually exceed 35%. Thus, researchers have sought to develop a model that allows the identification of putative orthologues present in different bacterial taxonomic groups [20, 21].

Several species of bacteria contain 3-ketosteroid-Δ1-dehydrogenase. In Comamonas testosteroni, a gram-negative bacterium used as a model to study steroid catabolism, the enzyme is encoded by the gene tesH [22], whereas Rhodococcus ruber Chol-4, R. erythropolis SQ1, and other actinobacteria (species belonging to the genera Nocardia, Mycobacterium, Arthrobacter) express different isoforms of the enzyme. The respective isoforms are responsible for introducing an unsaturated bond between C1 and C2 and include KstD1, KstD2 and KstD3 of R. ruber Chol-4 [9-11, 23, 24]. In the experiments using cell-free extracts from this strain, the three R. ruber KstD enzymes differed in their substrate profiles, with KstD1 showing a preference for 9α-hydroxy-4-androstene-3,17-dione (9-OH-AD) and testosterone; KstD2 for progesterone, androstanolone, AD, and testosterone; and KstD3 for saturated steroid substrates, including androstanolone [25].

C9α-hydroxylation catalyzed by 3-ketosteroid 9α-hydroxylase is a key step in the degradation of bacterial steroids, since it results in the opening of the polycyclic steroid structure [14, 15]. All bacterial 3-ketosteroid 9α-hydroxylases described thus far are two-component Rieske type non-heme monoxygenases consisting of a terminal oxygenase and a ferredoxin reductase [15].

Pseudomonas putida DOC21 is an environmental isolate that is able to use certain bile acids, testosterone, and androstanolone for growth. In this study, we analyzed the functions of stdH and stdJ from P. putida DOC21 and then, the 9, 10-seco pathway used by this strain to completely degrade steroids. Through specific deletions, we obtained two different mutants, one of them knocked-out in stdH, resulting in the accumulation of AD when the mutant is cultured in lithocholate or testosterone, and the other in stdJ, resulting in the secretion of ADD into the culture broth when the mutant is cultured with either of these steroids. As both AD and

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**Fig. 1:** (A) General structures of bile acids. (B) The initial steps of the lithocholate catabolic pathway from Pseudomonas putida DOC21 showing the intermediates 4-androstene-3,17-dione (AD), androsta-1,4-diene-3,17-dione (ADD), 9α-hydroxy-4-androstatriene-3,17-dione (9-OH-ADD), 9α-hydroxy-4-androst-14-en-3,17-dione (9-OH-AD), and 3-hydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione (3-HSA). (C) The location of the steroid catabolic genes stdH and stdJ in clusters std2 and std3 of P. putida DOC21. StdH and KstD are 3-ketosteroid-Δ1-dehydrogenases, KshAB is a 3-ketosteroid-9α-hydroxylase, and StdJ is the reductase component of a 3-ketosteroid-9α-hydroxylase in which the terminal oxygenase component is still unknown.
ADD are important synthons widely used in the pharmaceutical industry, these mutants may be of biotechnological relevance. In addition, we tested the three KstD isoforms present in *R. ruber* Chol-4 for their ability to substitute for StdH in a *P. putida* DOC21×stdH deletion mutant.

**Materials and Methods**

**Materials**

Bile acids, testosterone, and succinic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-Androstene-3,17-dione (AD, purity 94%) was kindly provided by Prof. F. Bermejo (Dept. Química Orgánica, Universidad de Salamanca, Spain). Androsta-1,4-diene-3,17-dione (ADD, purity 98%) was supplied by Gadea Biopharma (León, Spain). Molecular biology products were from Invitrogen (Carlsbad, CA, USA), Amersham Pharmacia Biotech Gmbh (Barcelona, Spain), Stratagene (San Diego, CA, USA), Agilent Technologies (Santa Clara, CA, USA), and Promega (Madison, WI, USA). Other chemicals and reagents were of analytical or HPLC grade.

**Bacterial strains, plasmids, genes, and vectors**

*Pseudomonas putida* DOC21 (Colección Española de Cultivos Tipo, CECT 8043) was isolated from a soil sample (León, Spain) [26]. *Escherichia coli* DH10B [27] was used for plasmid propagation, and *E. coli* HB101 (pRK600) was used as the helper strain in triparental filter mating [28]. Genomic fragments were subcloned using the commercial plasmids pGEM-T Easy (Promega) and pTZ21R/T (Thermo Scientific, Waltham, MA, USA), pJQ200KS [29] was used to induce specific gene deletions; and pBBR1MCS-3 (Tc²), a broad-host-range cloning and expression vector [30], was used to express the genes in strain DOC21. The kstD genes from *Rhodococcus ruber* Chol-4 were supplied by Prof. J. Perera (UCM, Madrid, Spain).

**Culture media and growth conditions**

Strain DOC21 and its mutants were maintained on trypticase soy agar (Difco, Detroit, MI, USA). Growth slants (12 h at 30°C) were used to inoculate chemically defined medium (referred to herein as MM) of different composition depending on the experiment. Mutants blocked in the degradation of bile acids and testosterone were identified in MM medium containing (g L⁻¹): KH₂PO₄ (13.6), (NH₄)₂SO₄ (2.0), MgSO₄·7H₂O (0.25), and FeSO₄·7H₂O (0.0005) [31]. The carbon sources used for culture were succinate (10 mM), bile acids (5 mM), testosterone, or either of its structural analogues (AD or ADD) (5 mM). Erlenmeyer flasks (500 ml) containing 100 ml of MM were inoculated with 2 ml of a bacterial suspension (Abs₅₇₀ = 0.5). These cultures were incubated on a rotary shaker (250 rpm) at 30°C (or 37°C) for the stated times. The following antibiotics in the indicated concentrations were added to the medium as needed: 100 μg ampicillin/ml, 10 μg streptomycin/ml, 30 μg chloramphenicol/ml, 37.5 μg tetracycline/ml, and 30 μg gentamicin/ml. All the experiments were performed in triplicate. *E. coli* strains were maintained on Luria-Bertani (LB) agar plates and cultured at 37°C overnight. When solid medium was required, 25 g of Difco agar L⁻¹ was added.

**DNA manipulation and bioinformatics analyses**

DNA manipulations and sequence analyses were performed as indicated elsewhere [32, 33]. Putative open reading frames were identified by combining the prediction results of the Glimmer 3.02 [34], GeneMark.hmm [35], and BLAST programs [36]. Databases available from NCBI were used for gene annotations. The sequences reported herein were deposited in the GenBank database (accession numbers: KP998047 and KF548089). Multiple sequence alignments were performed using MUSCLE [37].

A putative three-dimensional structure was determined for StdH by homology modeling through the PHYRE2 server (http://www.sbg.bio.ic.ac.uk/phyre2/) [38], using the crystal structure of 3-ketosteroid Δ¹-dehydrogenase from *Rhodococcus erythropolis* SQ1 as template [9].

Phylogenetic analyses were conducted using MEGA7 [39] and the neighbor-joining method [40], generating an optimal tree drawn to scale. The branch lengths of the tree were in the same units as the evolutionary distances used to infer the phylogenetic tree. Both were computed using the Poisson correction method [41]. Other analyses were performed by applying the maximum-likelihood method based on the JTT model [42], which represents the scale tree with branch lengths indicating the number of substitutions per site. The strategy followed to select the sequences used for these analyses can be found in Supplementary Material.

In comparisons of multiple aligned proteins, sequence logos showing the frequency of appearance of residues in the consensus sequences were constructed using WebLogo3 [43].

**Generation of gene deletion mutants**

Specific gene deletions were accomplished according to a previously described procedure [29, 44] involving a double recombinant event and selection of the required mutant by expression of the lethal sacB gene. In brief, the sequences flanking the DNA to be deleted were PCR-amplified and cloned together into pJQ200SK [29], a conjugative plasmid containing both a gentamicin resistance gene and a sacB gene and unable to replicate in *Pseudomonas*. This plasmid was transferred to *P. putida* DOC21 by triparental mating [28]. Under gentamicin selection, surviving cells were those in which homologous recombination of one of the cloned fragments had occurred. These recombinant strains were cultured in a medium containing sucrose which, in the presence of plasmid-encoded sacB, was lethal to the cells.
The clones that survived after homologous recombination using the second cloned fragment were those that lost the desired fragment from the genome. All mutants identified as Δgene were analyzed by PCR to confirm deletion of the gene of interest.

**PCR conditions**

PCRs were carried out in a PerkinElmer DNA thermal cycler 2400 (Waltham, MA, USA). Each reaction (50 µl) contained 75 mM of Tris-HCl buffer (pH 9), 50 mM of KCl, 20 mM of (NH₄)₂SO₄, 100 ng of genomic DNA, 0.4 µM of each primer, 2 mM of MgCl₂, 0.4 mM of dNTPs, and a mixture of the thermostable DNA polymerase (2 U) from *Thermus thermophilus* (Biotools, Madrid, Spain) and the PfU DNA polymerase (1 U) from *Pyrococcus furiosus* (Promega). The annealing temperature was 60°C and the extension time was 1 min. The oligonucleotides used in the reaction are detailed in Supplementary Table 1. Modified oligonucleotides containing specific restriction sites were designed for use in the study.

**Thin-layer chromatography**

AD, ADD, and the products accumulated by the mutants when cultured in MM containing succinic acid (10 mM) as the carbon and energy source, and lithocholic acid (5 mM) as the precursor of the intermediates were purified by thin-layer chromatography (TLC). Cells were eliminated from the culture samples by centrifugation. After filtration of the culture broth (0.22 μm pore size filters), 10 μl of the filtrate was directly spotted onto the TLC plate. The mobile phase was benzene:acetone (5:3, by vol.). The chromatographic spots were revealed by spraying the plate with a solution of H₂SO₄ (30% by vol.) and then heating it at 100°C for 10 min.

**Results and Discussion**

**Functional analysis of StdH**

*Pseudomonas putida* DOC21, a natural soil-isolated strain able to assimilate bile acids, testosterone, androstanolone, and AD, was used as a model organism to study the catabolism of these compounds [26]. Genetic studies based on insertional mutagenesis with the transposon Tn5 resulted in the identification of four different clusters (std1–std4) containing genes involved in steroid catabolism [4]. One of the genes, belonging to cluster std3, stdH (Fig. 1C), encodes a protein showing a 50.5% sequence conservation of the residues at equivalent positions with TesH from *C. testosteroni* [22, 45] (Fig. 2A).

To analyze the function of StdH, the stdH gene was deleted from *P. putida* DOC21 using a double-recombination strategy and plasmid pJQ200SK [29]. The knocked-out mutant, *P. putida* DOC21 ΔstdH (abbreviated as DOC21ΔstdH), was unable to grow in chemically defined medium containing either testosterone or AD (5 mM) as the sole carbon source and grew poorly when lithocholate, or other bile acid, was supplied to the same medium. The limited growth was presumably due to degradation of the C17 side-chain of bile acids but an inability to use the resulting steroid nucleus (Fig. 3). When grown in medium containing succinate (10 mM) as the carbon and energy source and supplemented with lithocholate (5 mM), the mutant accumulated AD, which supported the presence of a metabolic pathway involving degradation of the lateral chain (Fig. 4) and the involvement

![Fig. 2](image_url)

**Fig. 2:** Amino acid sequence alignments of (A) StdH from *P. putida* DOC21 (PpDOC21) and TesH from *Comamonas testosteroni* (Ctestos), and (B) StdH from *P. putida* DOC21 and KstD2 from *Rhodococcus ruber* Chol-4 (Rruber). StdH has 50.3% identity with TesH and 37.59% identity with KstD2. Amino-acids indicated in gray correspond to the FAD-binding site and the conserved catalytic residues.
of StdH in the degradation of AD in *P. putida* DOC21.

Further support for this hypothesis was obtained by transforming strain DOC21ΔstdH with plasmid pBBR1MCS-3 (referred to hereon as pMC constructs) [30] containing the gene *stdH* (DOC21ΔstdH pMCstdH). The expression of *stdH* restored the mutant’s ability to grow in MM containing AD as the sole carbon source (Fig. 3A, 5). Moreover, the absence of AD accumulation when the mutant was cultured in medium containing lithocholate demonstrated that the catabolic route responsible for steroid assimilation had been restored (data not shown).

The above results indicated that StdH has 3-ketosteroid-Δ¹-dehydrogenase activity. The catabolic behavior of DOC21ΔstdH (Fig. 3A), together with the fact that a database search did not lead to the identification of any other gene in the genomic DNA of *P. putida* DOC21 (unpublished draft genome) with significant similarity to *stdH*, suggested that this enzyme is the only protein involved in the elimination of the hydrogen atoms C1 (α) and C2 (β) of the steroid ring A in *P. putida* DOC21.

When the three genes encoding the Δ¹-ketosteroid dehydrogenase isozymes in *Rhodococcus ruber* Chol-4 (KstD1, KstD2 and KstD3) were cloned independently into pBBR1MCS-3 and expressed in the mutant DOC21ΔstdH, only one of the recombinant strains (DOC21ΔstdH pMCKstD2) was able to efficiently grow in MM containing AD or testosterone as the carbon source (Fig. 5). Expression of the cloned genes was confirmed by RT-PCR (data not shown) which demonstrated: (i) that *kstD2* from *R. ruber* was correctly expressed in *P. putida* DOC21 and (ii) the shared physiological function of KstD2 (but neither KstD1 nor KstD3) and StdH despite their different amino acid sequences (Fig. 2B). These observations were in agreement with the results reported by Fernández de las Heras and colleagues, who examined the involvement of KstD2 in AD degradation in *R. ruber* [10, 25]. The presence of several isozymes in the same microbe, as has been described in model actinobacteria [9-11, 23, 24], showing preference for different, but closely related substrates, explained the catabolic behaviors of the strains derived from the DOC21ΔstdH mutant and expressing each of the paralog Δ¹-ketosteroid dehydrogenase genes from *R. ruber*. Thus, in *Mycobacterium neoaurum* ATCC25795, a microbe that also contains three putative Δ¹-ketosteroid dehydrogenases, KstD1 had a higher affinity for 9-OH-AD while KstD3 preferred AD. These results suggested that the configuration of steroid rings A and B influences the substrates preferred by these isozymes [46]. Furthermore, Δ¹-ketosteroid dehydrogenase isozymes from other actinobacteria (*M. smegmatis*, *M. tuberculosis*, and *R. erythropolis* SQ1) also showed different

![Fig. 3: (A) Growth of *P. putida* DOC21 (●), *P. putida* DOC21 ΔstdH (●, ▲), and *P. putida* DOC21 ΔstdH pMCstdH (●, △) when cultured in minimal medium (MM) containing 5 mM lithocholate (●, ▼, ▼) or 5 mM testosterone (▲, △). (B) Growth of *P. putida* DOC21 (●), *P. putida* DOC21 ΔstdJ (●, ▲), and *P. putida* DOC21 ΔstdJ pMCstdJ (●, △) when cultured in MM containing 5 mM lithocholate (●, ▼, ▼) or 5 mM testosterone (▲, △). In both, similar results were obtained when testosterone was replaced by 4-androstene-3,17-dione (AD), and when cholate or deoxycholate was used instead of lithocholate.](image-url)
substrate specificities [23, 47]. Conversely, proteobacteria use to have a single copy of the stdH gene (encoding a Δ1-ketosteroid dehydrogenase) suggesting that this could be one of the reasons to explain the scarce ability to catabolise some steroids (i.e. sterols) by these bacteria.

Three-dimensional structural analyses based on homology modeling, using the 3-keto-Δ1-steroid dehydrogenase 2 from *R. erythropolis* SQ1 (PDB 4C3Y; [9]) as the template, revealed further details of the homologous nature of StdH and KstD. Although only 39% of the amino acids of these proteins are identical in equivalent positions, the crystal structure of 3-keto-Δ1-steroid dehydrogenase 2 from *R. erythropolis* SQ1 was a suitable template. The model covered 561 amino acids (98% of the whole sequence) with an estimated precision > 99%. These studies suggested that StdH has two domains, FAD-binding domain, and a catalytic domain, connected by a two-stranded antiparallel β-sheet (Fig. 6). The FAD-binding domain is arranged according to a Rossmann fold, as reported in other flavoproteins (FAD-containing enzymes) [48], and contains a consensus dinucleotide-binding motif (\(\text{VIVVGSAGAMTSAVFLADHGLRLVE}\)) with residues from the proposed conserved sequence indicated in bold as part of the fold [48].

The catalytic domain includes the conserved residues Y544 and G548 proposed to be involved in keto-enol tautomerization of the substrate in the *R. erythropolis* model [9]. Residues Y367 and Y124, together with the FAD-binding domain (Fig. 2, 6), likely participate in the catalytic mechanism in which a proton and a hydride ion are abstracted [21].

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**Fig. 4:** Thin-layer chromatogram of AD (lane a), ADD (lane b), and the products accumulated by the mutants *P. putida* DOC21 ΔstdH (lane c) and *P. putida* DOC21 ΔstdJ (lane d) when cultured in MM containing succinic acid (10 mM) as the carbon and energy source, and lithocholic acid (5 mM) as the precursor of the intermediates. The mobile phase was benzene:acetone (5:3, by vol.). The chromatographic spots were revealed by spraying the plate with a solution of H2SO4 (30% by vol.) and then heating it at 100°C for 10 min.

**Fig. 5:** Growth of *P. putida* DOC21 ΔstdH pBRR1MCS-3 (A), *P. putida* DOC21 ΔstdH pMCstdH (B), and *P. putida* DOC21 ΔstdH pMCkstD2 (C) when cultured in MM containing AD (5 mM) as the sole carbon source.

**Fig. 6:** Putative three-dimensional structure of StdH from *P. putida* DOC21 as determined by homology modeling using the PHYRE2 server (http://www.sbg.bio.ic.ac.uk/~phyre2/) and, as template, the crystal structure of 3-keto-Δ1-dehydrogenase from *Rhodococcus erythropolis* SQ1 at a resolution of 2.30 Å (Protein Data Bank accession number 4C3YB). Lateral chains of the proposed catalytic residues (Y124, Y367, Y544 and G548) are depicted as sticks.
**Functional analysis of StdJ**

Within the std2 cluster of *P. putida* DOC21 (GenBank KF548089), we also identified the gene stdJ, transcriptionally related to the other genes involved in steroid catabolism such as stdA3, encoding a 3α-H-4α (3’propanoyl)-7αβ-methylhexahydro-1,5-indanedione CoA synthetase involved in the degradation of the C and D rings [4]. The encoded protein displayed homology with flavoprotein reductases, including a 56% identity with the ferredoxin reductase component of a two-component Rieske-type class IA monooxygenase which is involved in testosterone catabolism in *C. testosteroni* [45]. Similar monooxygenases have been reported in actinobacteria that use the 9, 10-seco pathway for steroid degradation [5, 15-17, 24, 49-51]. These enzymes have been annotated as the reductase component of 3-ketosteroid 9α-hydroxylases which catalyze the C9 hydroxylation of ADD to yield 9α-hydroxy-1,4-androstadiene-3,17-dione (an unstable compound that spontaneously forms a 9, 10-seco phenol derivative) [13, 52].

To analyze the role played by StdJ in the catabolism of steroids, its gene (*stdJ*) was deleted. The resulting mutant, *P. putida* DOC21 ΔstdJ (abbreviated as DOC21ΔstdJ), was unable to grow in a MM containing testosterone or AD (5 mM) as carbon source; however, when these compounds were replaced by lithocholate or other bile acid (5 mM), the mutant grew, albeit poorly (Fig. 3B). Transformation of DOC21ΔstdJ with plasmid pMCstdJ restored the catabolic potential of this mutant. Thus, the recombinant strain, *P. putida* DOC21ΔstdJ pMCstdJ, regained the ability to grow in medium containing testosterone or AD as the carbon source (Fig. 3B). When mutant DOC21ΔstdJ was cultured in MM containing 10 mM succinate (as carbon and energy source) and 5 mM lithocholate (as a source of catabolites), ADD (but not AD) accumulated in the culture broth (Fig. 4).

Comparative analyses of StdJ with the putative reductase subunit of a 3-ketosteroid 9α-hydroxylase sequences from actinobacteria, β-proteobacteria, and γ-proteobacteria included in the Protein Sequence Database (GenBank) allowed us to establish the phylogenetic distribution of these proteins by using the neighbor-joining (Fig. 7) and maximum-likelihood (Suppl. Fig. 2) methods. The two methods generated trees with similar topologies and established a clear cladistic correspondence between the proteins and the taxonomic group to which they belonged. Thus, the sequences corresponding to β- and γ-proteobacterial proteins constituted a cluster that clearly differed from clusters of actinobacterial origin. It is worth noting that within the distribution of actinobacterial sequences, there was a clade that included all the sequences present in *Mycobacterium* and which branched out to generate two different subclades (Fig. 7).

Another clade included the reductases of *Tsukamurella, Gordonia, Rhodococcus,* and *Nocardia.* However, the *Tsukamurella* subclade was clearly different from the *Gordonia-Rhodococcus-Nocardia* group. Moreover, in the latter, there was a close correspondence between the homology groups and the genera to which these bacteria belong.

Phylogenetic analyses of these reductase subunit sequences from gram-negative bacteria revealed that, although there was less homology between the proteins, two different subclades could nonetheless be distinguished. One consisted of the proteins found in the *Comamonadaceae,* and the other of the reductases identified in *Pseudomonas* species, which showed a moderate degree of variability between them. Thus, based on the phylogenetic distribution of those sequences, 3-ketosteroid 9α-hydroxylase reductase components may be a useful taxonomic tool to classify new steroid-degrading strains. However, in strains that possess multiple copies of the gene encoding the reductase component [24], the possibility of horizontal transfer or duplication and subsequent divergence via the accumulation of spontaneous mutations cannot be ruled out.

Structural analyses also revealed that the reductase component of the 3-ketosteroid 9α-hydroxylase from *P. putida* DOC21 contains the typical class IA monooxygenase reductase domains: a flavin-binding domain (WLPRCYLSLSTP, corresponding to residues 52–63), a NADH-binding domain (LLLFGGGSGVTPVLSILRLSAL, residues 113–133), and a C-terminal plant-type iron-sulfur cluster [Fe2S2Cys4] domain (GLNPPSACRVGGCASCMCTLESGEVELLHNDALDG ELAEGWILACQAV, resi-dues 280–328) (Suppl. Fig. 1). Supplementary Fig. 3 depicts the consensus sequences established according to the individual frequency of appearance of specific residues from the 3-keto-9α-hydroxylase reductases from selected actinobacteria and gram-negative bacteria.

**Conclusion**

The above results led to the following conclusions: (i) StdH from *Pseudomonas putida* DOC21 is a 3-ketosteroid-Δ¹-dehydrogenase, (ii) StdH and KstD2 (from *Rhodococcus ruber* Chol-4) are analogous enzymes, (iii) *P. putida* DOC21, unlike *R. ruber* Chol-4 and other actinobacteria, contains a single pathway for steroid degradation, and (iv) StdJ is the reductase component of the 3-keto-9α-hydroxylase involved in the degradation of the ADD generated by StdH during steroid catabolism.
Fig. 7: Consensus tree showing the phylogeny, according to the neighbor-joining method, of the reductase components from 3-ketosteroid 9α-hydroxylases present in the genome of actinobacteria, β-proteobacteria, and γ-proteobacteria. The scale bar indicates the amino acid substitutions per site. DOC21_StdJ (Pseudomonas putida DOC21), Ps_Choll (Pseudomonas sp. Choll, WP_008568639), Ps_GM33 (Pseudomonas sp. GM33, WP_007976914), Ps_NBRC111124 (Pseudomonas sp. NBRC 111124, WP_060511844), Ps_ES3-33 (Pseudomonas sp. ES3-33, WP_045058525), Ps_GM55 (Pseudomonas sp. GM55, WP_008021057), Psput_CBB5 (P. putida CBB5, KKX63417), PsknaC (P. knackmussii, WP_052355350), Pnbres_NBRC106553 (P. resinovorans NBRC 106553, BAN50097), Psbale (P. balearica, WP_061338414), Ptae (P. taetrolens, WP_048380255), Psvran (P. vranovensis, WP_028944916), Pjapo (P. japonica, WP_042130308), Ctest_TK102 (Comamonas testosteroni TK102, AJJ45702), Ctest_ATCC11996 (C. testosteroni ATCC 11996, EHN64411), Ctest_S44 (C. testosteroni S44, EF158782), Ctest_CN2 (C. testosteroni CN2, ACY32105), Ctest_TA441 (C. testosteroni TA441, BAC81694), Poglac (Polaromonas glacialis, WP_029527755), Sipsy (Simplicispira psychrophila, WP_027996465), Tpsau_DS20162 (Tsukamurella paurometabola DSM 20162, ADG80030), Tpspe_JCM13375 (T. pseudomurensis JCM 13375, KXO96246), Tstyr_CCUG38499 (T. tyrossinosolvens CCUG 38499, KXO95517), Tsspul_CCUG35732 (T. pulmonis CCUG 35732, KXO89939), Tsspo_DSM44990 (T. pulmonis DSM 44990, KXP11195), Mkyor (Mycobacterium kyorinense, WP_04538150), Mint_MOTT-02 (M. intracellularare MOTT-02, AFC46862), Mpar_ATCCBAA-614 (M. parascrophulaceum ATCC BAA-614, EFG77601), Mhae_DSM44634 (M. haemophilum DSM 44634, AKN15982), Mafr_MAL010081 (M. africanum MAL010081, KBG29755), Mbov_AF212297 (M. bovis AF212297, CDO44872), Mtub_H37Rv (M. tuberculosis H37Rv, CCP46394), Morv_112400015 (M. oryctes 112400015, EMT34083), Mmar_M (M. marinum M, ACC43470), Mule_Harvey (M. ulcerans str. Harvey, EUA86886), Mneo_DSM44074 (M. neoaurum DSM44074, CDQ47131), Mneo_JC-12 (M. neoaurum JC-12, AKH14758), Mphi_DSM43072 (M. phlei DSM 43072, KXW59844), MgoO_X7B (M. goodii X7B, AKS32245), Msme_MC2-155 (M. smegmatis str. MC2 155, YP_890263), Msme_NCTC8159 (M. smegmatis NCTC 8159, CKJ34632), Mmag_DSM44476 (M. mageritense DSM 44476, CDO22511), Mwol_CDC01 (M. wolinii CDC 01, KWX223398), Mset_DSM45070 (M. setense DSM 45070, KHO17935), Mper_ACS819 (M. percreumin ACS 819, OBF42413), Mcon_ACS273 (M. conceptionense ACS 273, OBB15633), Mfor_E3377 (M. fortuitum, OBB02630), Mnuv_ACS3670 (M. vulnefer ACS 3670, OCB13127), Mnuv_ACS4093 (M. vulnefer ACS 4093, OCB61730), Mbri_JCM15654 (M. brishanense JCM15654, GAS8867), Mvac_95051 (M. vaccae 95051, ANI38868), Nfauc (Nocardia paucivorans, WP_040792682), Nfus (N. fusca, WP_063131162), Nsiene (N. sienata, WP_063061495), Nser_N-2927 (N. seriolae N-2927, GAM51280), Nsal (N. salmonicida, WP_062987070),
Rhodococcus pyridinivorans
G. polyisoprenivor
G. rubripertincta
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G.
180), Rery_SQ1 (R. triatomae
LE029P17
R. defluvii
N. brasiliensis
R. opacus
R. jostii
R.
N. farcinica
, WP_029901615), Ntene (R. erythropolis

hain
G. soli
R. equi
96
[4]
[3]
[2]
[1]
72x60
72x132
72x180
72x227
72x343
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Conflict of Interest
None of the authors has any conflict of interest to declare.
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