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Identification of a novel steroid inducible gene associated with the *βhsd* locus of *Comamonas testosteroni*☆

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Abstract

Comamonas testosteroni is a soil bacterium, which can use a variety of steroids as carbon and energy source. Even if it can be estimated that the complete degradation of the steroid nucleus requires more than 20 enzymatic reactions, the complete molecular characterization of the genes encoding these steroid degradative enzymes as well as the genetic organization of them remain to be elucidated. We have previously reported the cloning and nucleotide sequence of two steroid-inducible genes, *βhsd* and *stdC* encoding 3β-17β-hydroxysteroid dehydrogenase and a hypothetical protein respectively, located in both ends of a 3.2 kb *Hin*dIII fragment. Herein, we report the cloning and characterization of another steroid-inducible gene, called *sip48* (steroid inducible protein), located between these two genes. The analysis of Sip48 amino acid sequence predicts a protein of 438 amino acids with a molecular mass of 48.5 kDa. This protein bears high homology with conserved hypothetical proteins of unknown function described in *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Pseudomonas putida*, *Burkholderia fungorum*, *Shewanella oneidensis*, *Pseudomonas fluorescens* and *Thauera aromatica*. The predicted protein shows a typical structure of a leader peptide at its N-terminus. A 48.5 kDa protein encoded by the recombinant plasmid was detected by SDS–PAGE analysis of in vitro [^{35}S]-methionine labeled polypeptides. Analysis of gene expression indicates that Sip48 is tightly controlled at the transcriptional level by several steroid compounds. In addition, transcriptional analysis of *sip48* and *βhsd* in a *sip48* mutant strain, indicates that both genes are transcribed as a polycistronic mRNA. *lacZ* transcriptional fusions integrated into the chromosome of *C. testosteroni* demonstrate that a steroid-inducible promoter located upstream of *sip48* regulates the expression of both genes.

Keywords: Steroid; Degradation; *Comamonas testosteroni*

1. Introduction

Steroids belong to a group of molecules that is widespread in the environment as breakdown products of lignin and other plant-derived molecules. It has been described that this group of compounds can interfere with the mechanisms governing vertebrate reproductive function and development, acting as endocrine disruptors [1]. As a result of this, a growing interest in the study of microorganisms that can degrade these compounds has emerged in the biotechnology field.

Comamonas testosteroni is a soil bacterium that can grow on a variety of steroid compounds as the sole carbon and energy source [2,3]. These compounds can be used as carbon sources after conversion to common intermediates of the conventional central metabolic pathways [4]. *C. testosteroni* is able to transform steroids to CO_2 and H_2O, through a complex catabolic pathway that involves a set of steroid-inducible enzymes. [5,6]. In addition to these steroid catabolic features, several other non-steroid metabolizing activities involving different xenobiotic compounds such as phenylalkanoic acids [7], polycyclic aromatic hydrocarbons [8,9] and resin acids [3] have been reported. It has been suggested that a testosterone-inducible gene encoding an extradiol dioxygenase could be involved in both steroid and aromatic hydrocarbon degradation pathways. The isolation and characterization of genes encoding steroid-induced and steroid-degradative proteins is a research avenue that is unveiling a wide range of potential uses of *C. testosteroni* in bioremediation.

Even though several genes encoding steroid-degradative proteins of *C. testosteroni* have been cloned, the genetic

Abbreviations: *βhsd*, 3β-17β-hydroxysteroid dehydrogenase; *sip48*, steroid inducible protein; Sp^r, spectinomycin resistance; *stdC*, steroid degradation gene

☆ The DDBJ/EMBL/GenBank accession number for the DNA sequence of *sip48* is U41265.2.

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organization of most of them is still unknown [10–21]. There is evidence that 3-oxo-steroid Δ^1-dehydrogenase and 3-oxo-steroid Δ^4-(5α)-dehydrogenase genes are located in one operon [17]. In addition, two clusters of steroid-regulated genes were recently described. One of them corresponds to genes encoding Δ^5-3-ketoisomerase, 3α-hydroxysteroid dehydrogenase and a repressor protein A [21] and the other comprises *tesB* encoding a steroid-inducible extradiol dioxygenase and three uncharacterized open reading frames (ORFs) [10,11].

In the present work, we report the identification of a new steroid-inducible gene located between the two previously reported *stdC* and *βhsd* genes [14,22], called *sip48*. This gene encodes a protein that bears high homology with conserved hypothetical proteins of unknown function described in *P. aeruginosa* [23,24], *P. syringae*, *P. putida* [25], *Burkholderia fungorum*, *Shewanella oneidensis* [26], *Pseudomonas fluorescens* [27] and *Thauera aromatica* [28]. Transcriptional analysis of *sip48* and *βhsd* in a *sip48*-mutant strain allowed us to conclude that both genes are expressed together as a polycistronic mRNA. In addition, by the use of transcriptional fusions we identified the *sip48-βhsd* steroid-inducible promoter in *sip48* 5′ untranslated region.

2. Material and methods

2.1. Chemicals and reagents

Restriction endonucleases were obtained from US Biochemical Corp. (Cleveland, OH). All other chemicals were of the highest available purity and were purchased from Sigma Chemical Co. [α-^{32}P]dATP was purchased from New England Nuclear (Boston, MA).

Table 1
Bacterial Strains and Plasmids used

Strains or plasmids	Relevant genotype	Source or reference
Strains		
Escherichia coli		
DH5α	F$^-$ recA1 endA1 gyr96 thi-1 hsdR17 (rk$^-$ mk$^+$) supE44 relA1 ΔlacZYA-argF U169 ϕ80dlacZΔM15. Host strain for DNA manipulation	Stratagene
CC118 λpir	Rif^r; λ-pir lysogen. Host strain to propagate plasmids with a R6K origin of replication	[34]
S17.1λpir	Tp^r Sm^r *hsd*R *pro rec*A RP4-2-T :: Mu :: Tn7 in chromosome. Strain for conjugative transfer of plasmids into *C. testosteroni*	[34]
Comamonas testosteroni		
Wild type		ATCC 11996
βhsd :: Ω	Gm^r, Sp^r, βhsd mutant of ATCC 11996 carrying *Sma*I 2 kb cassette from pHP45Ω	This study
sip48 :: Ω	Gm^r, Sp^r, sip48 mutant of ATCC 11996 carrying *Sma*I 2 kb cassette from pHP45Ω	This study
UT2.5	Gm^r, Cm^r, transconjugant cell carrying *Hin*dIII–*Eco*RV 2.5 kb *lacZ* fusion	This study
UTΔH980	Gm^r, Cm^r, transconjugant cell carrying *Hin*cII–*Eco*RV 860 bp *lacZ* fusion	This study
UTHH1.5	Gm^r, Cm^r, transconjugant cell carrying *Hin*dIII–*Hin*cII 1.5 kb *lacZ* fusion	This study
UTHHΔP	Gm^r, Cm^r, transconjugant cell carrying *Hin*dIII–*Hin*cII 1.5 kb Δ*Pst*I 0.6 kb *lacZ* fusion	This study
UT401	Gm^r, Cm^r, transconjugant cell carrying 401 bp *lacZ* fusion	This study
Plasmids		
pSL9	pGEM3 containing 3.2 kb *Hin*dIII fragment of *βhsd* gene and contiguous genes, Ap^r	[18]
pRK2013	Km^r Mob$^+$ Tra$^+$: donor of transfer functions	[41]
pUC19	Multipurpose cloning vector, Ap^r	[42]
pSL9ΔP	pGEM3 containing 2.9 kb *Pst*I–*Hin*dIII fragment from pSL9	This study
pGEM T-easy	PCR cloning vector, Ap^r	Promega
pG401	pGEM T-easy carrying 401 bp fragment 5′ upstream of *sip48* gene	This study
pUJ18 NotI	pUC18 derivative	[34]
pUJ2.5	Ap^r carrying *Hin*dIII–*Eco*RV 2.5 kb fragment	This study
pUJΔH980	Ap^r carrying *Hin*cII–*Eco*RV 860 bp fragment	This study
pUJHH1.5	Ap^r carrying *Hin*dIII–*Hin*cII 1.5 kb fragment	This study
pUJHHΔP	Ap^r carrying *Hin*dIII–*Hin*cII 1.5 kb Δ*Pst*I 0,6 kb fragment	This study
pUJ401	Ap^r carrying 401bp DNA fragment 5′upstream of *sip48* gene	This study
pUTminiTn5	Ap^r, Cm^r	[34]
pUT2.5	Ap^r, Cm^r carrying *Hin*dIII–*Eco*RV 2.5 kb *lacZ* fusion	This study
pUTΔH980	Ap^r, Cm^r, carrying *Hin*cII–*Eco*RV 860 bp *lacZ* fusion	This study
pUTHH1.5	Ap^r, Cm^r, carrying *Hin*dIII–*Hin*cII 1.5 kb *lacZ* fusion	This study
pUTHHΔP	Ap^r, Cm^r, carrying *Hin*dIII–*Hin*cII 1.5 kb Δ*Pst*I 0.6 kb *lacZ* fusion	This study
pUT401	Ap^r, Cm^r, carrying 401 bp *lacZ* fusion	This study
pHP45:Ω	Ap^r, Sm^r/Sp^r	[36]
pG2.5	pGEM3 containing 2.5 kb *Hin*dIII–*Eco*RV fragment	This study
pG3.2βhsd :: Ω	pSL9 derivatives containing Ω cassette insertion	This study
pG2.5 sip48 :: Ω	pG2.5 derivatives containing Ω cassette insertion	This study

Ap^r, Cm^r, Sm^r, Sp^r, Gm^r, Km^r indicate resistance to ampicillin, chloramphenicol, streptomycin, spectinomycin, gentamycin and kanamycin, respectively.

2.2. Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown at 37 °C in Luria–Bertani (LB) medium [29]. *C. testosteroni* was grown at 30 °C in LB medium or in M9 minimal medium plus acetate (0.2%, w/v) or testosterone [29]. The ON cultures were diluted 1/100 in fresh medium, incubated 2 h in LB or 12 h in M9, diluted 1/50 in fresh medium and incubated as indicated in each experiment. When indicated testosterone was added to reach a concentration of 0.29 mg/ml (1 mM).

Growth of *C. testosteroni* was monitored by counting colonies that appeared on LB plates, on which appropriately diluted cultures have been spread, after incubation at 30 °C. Alternatively, growth was monitored by measuring OD_{600}. When needed, antibiotics were added at the following concentrations (in μg/ml): ampicillin, 100; chloramphenicol, 20; gentamycin, 10; kanamycin, 20; and spectinomycin, 600.

2.3. DNA manipulations and sequence determinations

Standard protocols or manufacturer's instructions were followed for DNA isolation and recombinant DNA procedures [29]. DNA sequencing was performed on double-stranded templates derived from different clones in pUC19 using the dideoxy chain termination method [30] with the sequenase kit, version 2, using [α^{32}S-dATP]. The Blast program was used to screen DNA and protein databases for similar protein [31]. Multiple sequence alignments were made in ClustalW v 1.7 [32].

2.4. RNA isolation and analysis

C. testosteroni was grown in LB or M9 medium plus acetate during the indicated periods of culture either in the absence or presence of testosterone. Total RNA was extracted as described previously [14]. RNA samples (20 μg per lane) were electrophoresed on a 1.2% (w/v) agarose gel containing 18% (v/v) formaldehyde and transferred to nitrocellulose membranes [14]. Equal loading and transfer were assessed by methylene blue staining of membranes. Prehybridization and hybridization reactions were performed as described previously [14]. The 300 bp *Hin*dIII–*Pst*I (probe a), 600 bp *Hin*cII–*Pst*I (probe b) and 650 bp *Eco*V–*Hin*dIII (probe c) restriction fragments (Fig. 1B) were labeled with [α^{32}P]-dATP (3000 Ci/mmol) by the random priming method [33].

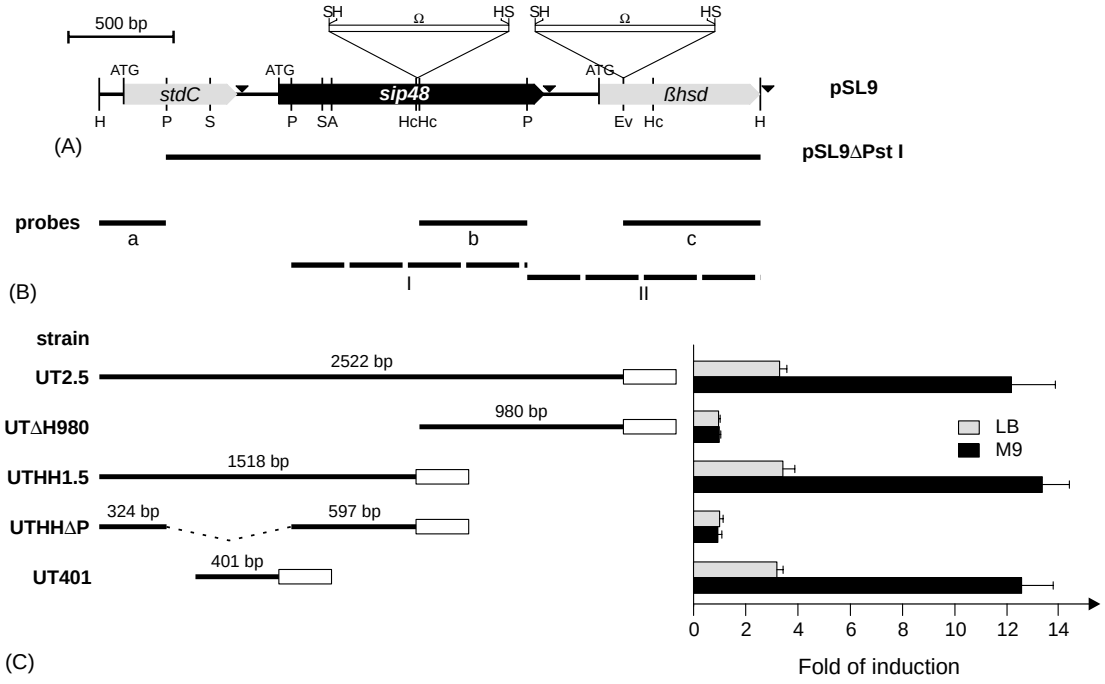

Fig. 1. Restriction map of the 3.2 kb *Hin*dIII fragment cloned into pSL9 plasmid. (A) The regions encoding the *stdC*, *sip48* and *βhsd* genes are indicated by boxes. H, *Hin*dIII; P, *Pst*I; A, *Acc*I; Hc, *Hin*cII; S, *Sma*I; and Ev, *Eco*RV. Inverted repeat sequences are denoted (▼). The restriction sites used for mutagenesis by gene disruption are indicated. pSL9 was partially digested with *Pst*I and the 2.9 kb *Pst*I–*Hin*dIII fragment was isolated and religated to obtain pSL9ΔP plasmid. (B) DNA probes used in Southern (broken lines) and Northern blot hybridization experiments (thick lines) are indicated. (C) Promoter analysis of the *stdC*, *sip48* and *βhsd* 5′ untranslated regions. *C. testosteroni* transconjugant strains carrying transcriptional fusions of different fragments of the *βhsd* upstream region (thick lines) to a promoterless *lacZ* gene (open boxes) were grown in LB medium (gray bars) and M9 minimal medium (black bars) in absence or presence of 1 mM testosterone. The promoter activity of each transcriptional fusion was determined as folds of induction (β-galactosidase activity in the presence of testosterone/β-galactosidase activity in the absence of testosterone) and each value is the average of results from three independent experiments (error bars indicate standard deviations).

2.5. Southern blot analysis

The genomic DNAs were prepared essentially as described in Sambrook et al. [29]. Southern blot analysis was performed as described previously [14]. The 1.2 kb *Pst*I (probe I) and 1.2 kb *Pst*I–*Hin*dIII (probe II) restriction fragments (Fig. 1B) were labeled with [α^{32}P]-dATP (3000 Ci/mmol) by the random priming method [33].

2.6. Testosterone degradation

It was analyzed as described previously [18]. Briefly, bacterial cells, grown on LB medium plus testosterone during 12 h of culture were harvested by centrifugation at 4 °C. Aliquots of culture supernatants were extracted three times with 5 vol. of ethyl ether and submitted to TLC on silica gel GF254 plates using benzene-ethanol (95:5, v/v) as solvent system. The pattern of testosterone degradation was visualized using UV 254 light. Testosterone, 4-androstene-3,17-dione and 1,4-androstadiene-3,17-dione were used as standards.

2.7. In vitro transcription–translation assay

Plasmid DNA (5 μg) and [^{35}S]methionine (specific activity 1139 Ci/mmol, New England Nuclear) were used in a prokaryotic-coupled transcription–translation system as by the supplier's instructions (Amersham). Templates consisted of pSL9ΔP or pGEM3 plasmids that had been linearized with *Eco*RI enzyme.

2.8. Construction of the transcriptional fusions

The 2.5 kb *Hin*dIII–*Eco*RV fragment of pSL9 bearing *stdC*, *sip48* and the intergenic region between *sip48* and *βhsd* genes, and different deletions of this fragment were cloned into the *Sma*I site of the pUJ8 plasmid, upstream of the promoterless *lacZ* gene (Fig. 1C and Table 1) [34]. Also, pSL9 plasmid was used as a template to amplify a 401 bp DNA fragment corresponding to the 5′ upstream region of *sip48* gene. PCR was performed with primer 1 (5′-CATTAcccgggCCAGCCCCAGGGGATGAA-3′) and primer 2 (5′-CGGACggatccGCCTAGTCTCCTTGGATG-CA-3′) to generate a *Sma*I site and a *Bam*HI site at the 5′ and 3′ ends of the 401 bp fragment. The amplified product was cloned into the pGEM T-easy vector (pG401), the recombinant plasmid was digested with *Sma*I and *Bam*HI restriction enzymes and the released fragment was cloned into the pUJ8 plasmid to generate pUJ401. The different *Not*I fragments from the resultant recombinant pUJ8 plasmids were excised and ligated into the single *Not*I site of the transposon delivery plasmid pUT/mini-Tn5Cm^r to generate different Cm^r hybrid mini-transposons (Table 1). Insertions of hybrid mini-transposons into the chromosome of the *C. testosteroni* cells were done following the procedure described in detail by De Lorenzo and Timmis [34]. Matings between the donor strain *E. coli* S17.1λ*pir*, transformed with the delivery plasmid indicated in each case, and the recipient strain were run at 30 °C for 4–6 h on the surface of a filter placed on an LB plate. The cells were then resuspended in 1% NaCl and plated on LB plates containing the appropriate antibiotics for selection of chromosomal transposon insertions and counter selection of the donor strain (Cm^r and Gm^r). Southern blot analysis (total DNA from the selected transconjugant strains was digested with *Hin*dIII and the membranes were hybridized with probe II – Fig 1B) and βHsd enzyme activities were performed to rule out that the insertions of the transposon do not affect *βhsd* locus (data not shown). The selected transconjugant strains showed the same growth rates and doubling times in LB and M9 minimal medium plus acetate or testosterone, when they were compared to *C. testosteroni* wild type *strain*.

2.9. β-Galactosidase assays

The standard procedures described by Miller [35] were used for quantitative measurements of β-galactosidase activity. Samples were collected after 12 h (LB) or 17 h (M9) of incubation. Induction of *sip48* promoter in *C. testosteroni* UT401 was made by growing the corresponding cultures in LB medium in presence of inducers added at the concentrations indicated in Table 2. Activity of the transcriptional fusion was calculated as the ratio between the β-galactosidase activity in the presence of the steroid compound and the ß-galactosidase activity in the absence of it (fold of induction). The values given throughout this paper represent the average of three independent experiments, each of which was conducted in duplicated samples.

2.10. Construction of plasmids and alleles replacement

Introduction of the Ω cassette in the *sip48* and *βhsd* genes involved cloning the Ω cassette (2 kb *Sma*I fragment) from pHP45:Ω [36] into the *sip48 Hin*cII site of pG2.5 and into the *βhsd Eco*RV site of pSL9, respectively. The recombinant plasmids, named pG2.5*sip48*Ω and pSL9*βhsd*Ω were transferred into *C. testosteroni* wild type by triparental mating using the mobilizing plasmid pRK2013. Donor, helper and recipient cells were grown overnight in LB medium. The cell suspensions (0.2 ml each) were mixed, filtered on a 0.4 μm nitrocellulose membrane and incubated on an LB agar plate for 24 h. The cells on the filter were suspended in 1% NaCl and plated onto LB-agar containing 10 μg gentamycin and 600 μg spectinomycin/ml to select transconjugants. Southern blot analysis was performed to confirm the genomic structure of the mutants.

2.11. Nucleotide sequence accession number

The nucleotide sequence determined in this study has the GenBank accession no. U41265.2.

Table 2
Transcriptional activity of the *sip48* promoter in *C. testosteroni* UT401 in LB

Steroid compound		Concentration (mM)[a]	Fold of induction[b]
Trivial name	IUPAC name		
Cholesterol	5-Cholesten-3β-ol	1	6.0
Dehydroepiandrosterone	5-Androsten-3β-ol-17-one	1	4.2
Androstenedione	4-Androsten-3,17-dione	0.5	3.9
Testosterone	4-Androsten-17β-ol-3-one	1.0	3.1
Testosterone	4-Androsten-17β-ol-3-one	0.5	3.2
17α-Hydroxyprogesterone	4-Pregnen-17-ol-3,20-dione	0.5	3.3
Progesterone	4-Pregnen-3,20-dione	1	3.1
5α-Dyhydrotestosterone	5α-Androstan-17β-ol-3-one	1	3.0
Estrone	Estra-1,3,5(10)-trien-3-ol-17-one	1	1.1
Estradiol	Estra-1,3,5(10)-trien-3,17-diol	1	0.9

[a] Final concentration of the steroid in the culture media.
[b] β-Galactosidase activity in the presence of the steroid compound/β-galactosidase in the absence of the steroid compound. The results are representative of three independent experiments.

3. Results

3.1. Identification of a DNA region with steroid-inducible promoter activity

In order to localize the testosterone-inducible promoter activity responsible of *βhsd* gene expression we investigated for promoter activity several DNA regions located upstream of the *βhsd* gene. We constructed transcriptional fusions between different DNA fragments of interest and the *lacZ* gene (see materials and methods). Transcriptional fusions were inserted as monocopy into *C. testosteroni* chromosome and levels of β-galactosidase were determined after growing several independent isolates in LB or M9 medium plus acetate in the absence or presence of inducing levels of testosterone (0.5–1.0 mM). *C. testosteroni* transconjugant strains carrying 2.5 and 1.5 *lacZ* fusions (UT2.5 and UTHH1.5, respectively) produced higher levels of β-galactosidase activity in the presence than in the absence of testosterone, thus indicating the existence of a testosterone-inducible promoter in the 1.5 kb *Hin*dIII–*Hin*cII fragment (Fig. 1C). Furthermore, the low level of β-galactosidase activity measured in UTHHΔP transconjugant bacteria suggests that the steroid-inducible promoter is located in the deleted 0.6 kb *Pst*I fragment. Moreover, the inducible β-galactosidase activity was recovered when the transcriptional fusion carrying part of the *stdC-βhsd* intergenic region (*C. testosteroni* UT401) was analyzed. In addition, no testosterone inducible β-galactosidase activity was observed when *C. testosteroni* UTΔ980 carrying 980 bp upstream the *βhsd* gene was analyzed. Altogether, these results indicate that there is only one steroid-inducible promoter located approximately 1.4 kb upstream from the *βhsd* ATG codon governing the steroid-inducible *βhsd* transcription.

3.2. A new gene located between the steroid-inducible promoter region and βhsd gene

The relatively high distance between the steroid-inducible promoter region of *βhsd* gene and its ATG codon suggests that an additional gene may be encoded in the region between them. We sequenced the 1 kb *Acc*I–*Pst*I fragment located between the two previously reported genes, *βhsd* and *stdC* encoding 3β-17β-hydroxysteroid dehydrogenase and a hypothetical protein respectively (Fig. 1A). Analysis of the complete nucleotide sequence allowed us to identify a new ORF encoding a 438 amino acid putative protein with a predicted molecular weight of 48.5 kDa and pI of 9.29. The predicted protein, Sip48 (for steroid inducible protein), showed a typical structure of a leader peptide at its *N*-terminus with predicted leader peptidase cleavage site C-terminal of Ala-25. This conclusion was further supported by prediction using the Dense Alignment Surface program according to Cserzo et al. [37]. The hydrophobicity plot of the Sip48 protein is shown in Fig. 2. Comparison of this

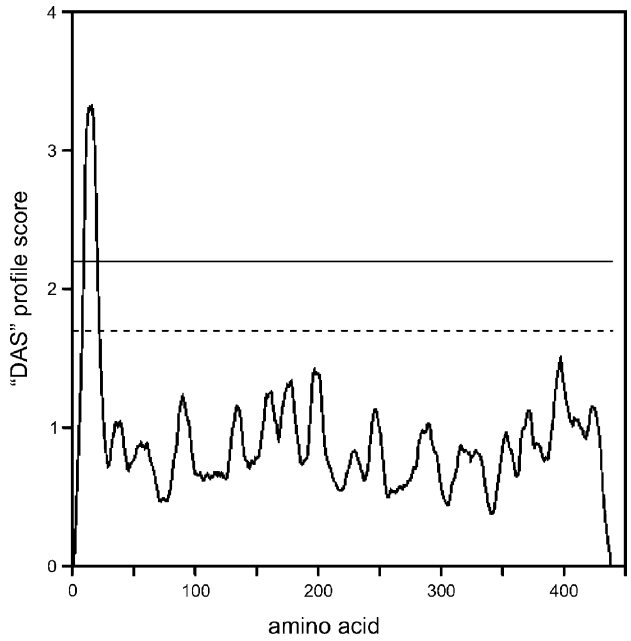

Fig. 2. Hydrophobicity plot of the Sip48 protein calculated by prediction using the Dense Alignment Surface program according to Cserzo et al. [37].

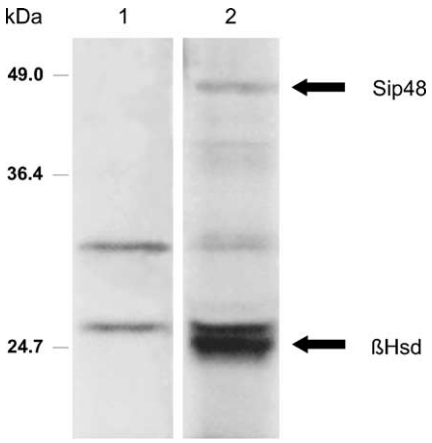

Fig. 3. Analysis of the in vitro transcription–translation products encoded by pGEM3 (lane 1) and pSL9ΔP (lane 2). Plasmid DNA (5 μg) and [^{35}S]methionine (specific activity 1139 Ci/mmol, New England Nuclear) were used in a prokaryotic coupled transcription–translation system as by the supplier's instructions (Amersham). The sizes of the molecular mass markers (pre-stained protein ladder, GIBCO) are shown in kDa. The arrows indicate the Sip48 (48.5 kDa) and β-Hsd (26.5 kDa) proteins (lane 2). The labeled polypeptides were separated 0.1% SDS–12% PAGE.

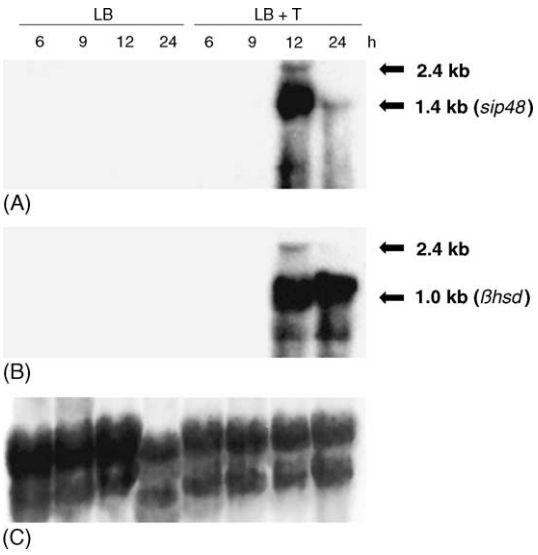

Fig. 4. Northern blot analysis of the *C. testosteroni sip48* gene. Total RNA samples (20 μg per lane) prepared from *C. testosteroni* grown on LB medium in absence or presence of testosterone during 6, 9, 12 and 24 h of culture. Samples were analyzed by electrophoresis on formaldehyde-agarose gels, transferred to a nylon membrane and stained with methylene blue (C). The membrane was hybridized with 600 bp *Hin*cII–*Pst*I fragment of C-terminus of *sip48* gene (probe b) (A) and 650 bp *Eco*RV–*Hin*dIII fragment encoding the C-terminal region of *βhsd* gene (probe c) (B).

putative protein with sequences in the GenBank database (BLASTP) showed sequence similarity with seventeen conserved hypothetical proteins of unknown function, five of them described in *P. aeruginosa* (GenBank accession nos. NP 252611.1, NP 251771.1, NP 252111.1, NP 249074.1, AAK01515.1); two in *P. syringae* (GenBank accession nos. ZP 00124548.1, ZP 00125503.1); four in *P. fluorescens* (GenBank accession nos. ZP 00086062.1, ZP 00084659.1, 00088041.1, U10470); three in *P. putida* (GenBank accession nos. NP 74297.1, NP 744193.1, NP 744954.1); one in *S. oneidensis* (GenBank accession no: NP 718182.1); one in *B. fungorum* (GenBank accession no. ZP 00033085.1) and one truncated protein in *T. aromatica* (GenBank accession no. AJ001848) with 32–47% identity and 45–57% similarity.

In vitro transcription–translation analysis showed that several labeled polypeptides were synthesized upon the addition of pSL9ΔP or pGEM3 DNA to the reaction mixture (Fig. 3). However, there are to distinct 48.5 and 26.5 kDa proteins encoded by pSL9ΔP that could not be detected in the transcription-translation reaction of the cloning vector pGEM3. The molecular weights of these in vitro labeled proteins are in agreement with those predicted from the ORFs encoding Sip48 and β-Hsd.

3.3. Transcriptional induction of the sip48 and βhsd genes

In order to characterize this new ORF at the transcriptional level, we isolated RNA from *C. testosteroni* growing in different experimental conditions and performed Northern blot assays. Labeled DNA fragment with sequence complementary to the C-terminal region of *sip48* (600 bp *Hin*cII–*Pst*I fragment, probe b) recognized a strong signal corresponding to 1400 nt transcript (Fig. 4A). The *sip48* mRNA was present at high levels at 12 h of culture when *C. testosteroni* was grown on LB medium plus testosterone and barely detected at 24 h of culture period. The transcript was not detected when the *C. testosteroni* was grown on LB medium in absence of testosterone at any time of culture. A second *sip48* mRNA fragment of 2400 nt was detected at 12 h of culture suggesting that this RNA may be part of a polycistronic transcript. In order to confirm this hypothesis the membrane was rehybridized with the 300 bp *Hin*dIII–*Pst*I fragment (probe a) encoding the N-terminal region of *stdC* gene (data not shown) and 650 bp *Eco*RV–*Hin*dIII fragment (probe c) encoding the C-terminal region of *βhsd* gene (Fig. 4B). The results indicate that only *βhsd* probe was able to recognize the 2400 nt transcript at 12 h of culture. This probe also detected the previously reported strong signal of 1 kb, which remains constant at 24 h of culture [22]. Identical results were obtained when RNA was extracted from *C. testosteroni* cells grown in M9 in the presence of testosterone (data not shown). These data suggest that *sip48* and *βhsd* steroid-inducible genes are transcribed as a polycistronic mRNA.

3.4. The sip48 and βhsd genes constitute an operon

In order to determine if *sip48* and *βhsd* are transcribed as a polycistronic mRNA, *C. testosteroni sip48* and *βhsd* mutant strains were constructed. Inactivated versions of the cloned genes were constructed by insertion of a spectinomycin resistance cassette (Sp^r). The plasmids containing the

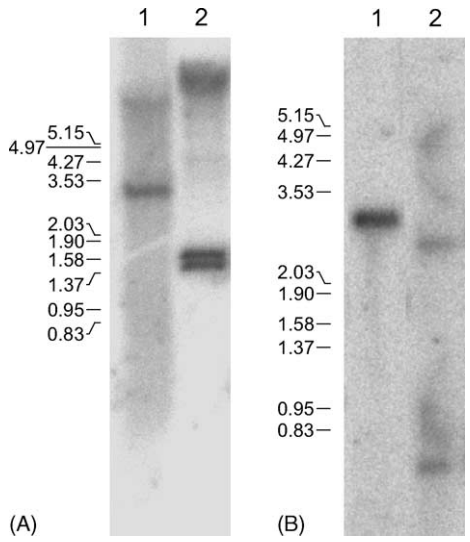

Fig. 5. Southern blot analysis of the genomic DNA of *C. testosteroni* *βhsd*::Ω and *sip48*::Ω strains. DNAs of *C. testosteroni* wild type and mutant strains were digested with *Hin*dIII. Each lane was loaded with 1 μg of DNA and probed with a 1.2 kb *Pst*I fragment encompassing *sip48* gene (A) or 1.2 kb *Pst*I–*Hin*dIII fragment encompassing *βhsd* gene (B). The wild type fragment is the expected 3.2 kb size (lane 1A and 1B), whereas the *sip48*::Ω mutant has two *Hin*dIII fragments of 1.7 and 1.5 kb generated by the insertion of 2 kb *Hin*dIII Sp^r cassette (lane 2A). The *βhsd*::Ω mutant has two *Hin*dIII fragments of 2.5 and 0.7 kb generated by the insertion of 2 kb *Hin*dIII Sp^r cassette (lane 2B).

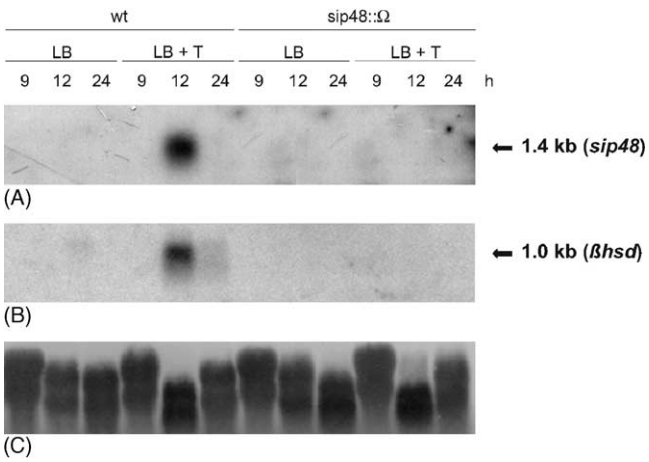

Fig. 6. Expression of the *sip48* and *βhsd* genes in *C. testosteroni* wt and sip48::Ω. Total RNA samples (20 μg per lane) prepared from *C. testosteroni* wt and sip48::Ω were grown on LB medium in absence or presence of testosterone during 9, 12 and 24 h of culture. Samples were analyzed by electrophoresis on formaldehyde-agarose gels, transferred to a nylon membrane and stained with methylene blue (C). The membrane was hybridized with 600 bp *Hin*cII–*Pst*I fragment of C-terminus of *sip48* gene (probe b) (A) and 650 bp *Eco*RV–*Hin*dIII fragment encoding the C-terminal region of *βhsd* gene (probe c) (B).

interrupted genes were introduced into the *C. testosteroni* wild type strain by triparental mating, and the Sp^r colonies were obtained. Analysis by Southern blot hybridization of Sp^r mutant colonies showed one 3.2 kb *Hin*dIII band in *C. testosteroni* wild type (Fig. 5A and B, lane 1); two *Hin*dIII bands of 1.7 and 1.5 kb in *sip48* mutant (Fig. 5A, lane 2); and two *Hin*dIII bands of 2.5 and 0.7 kb in *βhsd* mutant strain (Fig. 5B, lane 2) generated by the insertion of 2 kb *Hin*dIII Sp^r cassette. Having obtained evidence for the successful construction of *sip48*::Ω and *βhsd*::Ω mutants, we next confirmed by Northern blot assays that these strains are unable to express their respective transcripts (Fig. 6). In addition, *sip48*::Ω mutant strain is unable to synthesize *βhsd* mRNA indicating that both genes are transcribed as a polycistronic mRNA.

3.5. Phenotypic analysis of the *C. testosteroni sip48* and βhsd mutant strains

In order to test the phenotype of the mutants, *C. testosteroni* wild type, *sip48*::Ω and *βhsd*::Ω strains were grown under different culture conditions. As it is shown in Fig. 7, both bacteria grew with equal growth rates on LB medium or M9 minimal medium plus acetate. The mutant bacteria grew significantly slower than the wild type strain at the beginning of the exponential phase of growth in the minimal media plus testosterone. After this lag growth during the early exponential phase, the mutant strains were able to grow in minimal media plus testosterone at the same rate as the wild type. The measurement of testosterone degradation in *C. testosteroni* mutant strains indicated that they were able to transform testosterone into androstenedione only after prolonged culture times in comparison to the wild type bacteria (data not shown). This suggests that an alternative and less efficient initial metabolic pathway of testosterone degradation is present in *C. testosteroni*.

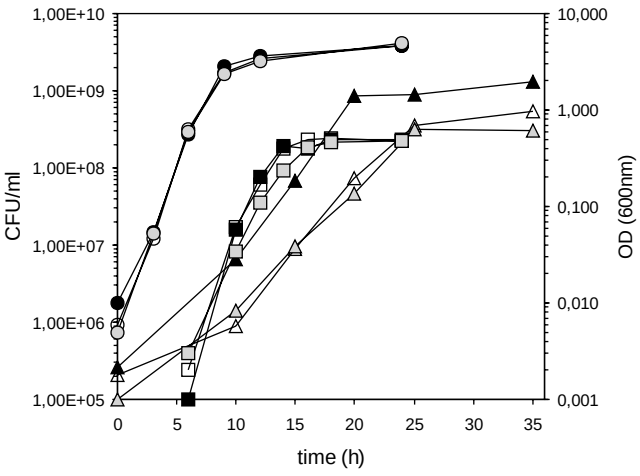

Fig. 7. Growth of *C. testosteroni* wild type (black), *βhsd*::Ω (grey) and *sip48*::Ω (white) strains in LB medium (circle), M9 medium plus acetate (square) and M9 medium plus testosterone (triangle). Growth of bacteria cultured in LB medium was monitored by measuring OD_{600}. Growth of bacteria cultured in M9 medium plus testosterone was monitored by counting colonies that appeared on LB plates, on which appropriately diluted cultures have been spread, after incubation at 30 °C. The data are representative of three independent cultures.

3.6. Several steroid compounds induce the sip48-βhsd transcript

In order to determine if other steroid compounds are able to activate the identified testosterone-inducible promoter, *C. testosteroni* UT401 was grown in LB medium supplemented with different steroids and β-galactosidase activity induction was quantified in each experimental condition. Results, shown in Table 2, indicate that several steroids can induce the promoter activity, thus indicating that they induce the expression of *sip48* and *βhsd*. The highest value of induction was observed when cholesterol was added to the culture medium. On the contrary, no induction of *sip48* promoter was detected when estriol or estradiol was present. Interestingly, all the steroid-inducers could be used as the sole carbon source by *C. testosteroni*, when they were added to M9 minimal medium at a final concentration of 1 mM (data not shown).

4. Discussion

The genetic organization of the genes encoding steroid degradative enzymes in *Comamonas testosteroni* is unknown, although there is evidence that 3-oxo-steroid Δ^1-dehydrogenase and 3-oxo-steroid Δ^4-(5α)-dehydrogenase genes are located in one operon [17]. Recently, two clusters of steroid-regulated genes were reported. One of them corresponds to genes encoding Δ^5-3-ketoisomerase, 3α-hydroxysteroid dehydrogenase and a repressor protein A [21] and the other comprises to *tesB* encoding a steroid-inducible extradiol dioxygenase and three putative ORFs [10]. In this study, we described a novel steroid-inducible gene called s*ip48* located between the previously reported *stdC* and *βhsd* steroid-inducible genes [14,22]. This new gene encodes a protein of 438 amino acids with a predicted molecular mass of 48.5 kDa. The protein encoded by s*ip48* gene shows a high degree of sequence identity with conserved hypothetical proteins of unknown function described in *P. aeruginosa* [23,24], *P. syringae*, *Pseudomonas putida* [25], *B. fungorum*, *S. oneidensis* [26], *P. fluorescens* [27] and *T. aromatica* [28]. Electrophoretic analysis of in vitro labeled proteins demonstrated the presence of 48.5 kDa polypeptide in agreement with the predicted molecular mass.

Northern blot experiments allowed us to identify two distinct *sip48*-specific transcripts of 2400 and 1400 nt that were mapped within the *sip48-βhsd* locus. The 2400 nt transcript is a polycistronic message that includes at least *βhsd* gene, whereas the 1400 nt transcript represents only *sip48* gene. Transcription of the *βhsd* and *sip48* genes as a polycistronic mRNA is supported by the lack of ß*hsd* expression in the *sip48* mutant strain. Moreover, *lacZ* transcriptional fusions integrated into the chromosome of *C. testosteroni* allowed us to locate a steroid-inducible promoter region in the 5′ untranslated region of *sip48*. In addition, no steroid-inducible activity was found in *sip48-βhsd* intergenic region, indicating that ß*hsd* steroid-inducible transcription is regulated by one promoter located 5′ upstream of *sip48* coding sequence. A remarkably high induction of transcription originated by this region was detected when several steroid compounds were added to the culture medium. These data clearly demonstrate that the *sip48* and *βhsd* genes of *C. testosteroni* are organized in a polycistronic operon and that one steroid-inducible promoter located upstream of *sip48* regulates the expression of both genes as a response to the presence of different steroid compounds.

Northern blot experiments shown in Fig. 4 also suggest that the monocistronic *βhsd* and *sip48* mRNAs have different stabilities. Intergenic inverted repeats located behind the *sip48* and *βhsd* genes, respectively (Fig. 1A), could cause partial termination of transcription [38]. Alternatively, these hairpin structures might prevent exonucleotic decay of mRNAs [38]. Specific cleavage by endonuclease can expose new 3′ ends that serve as substrates for processive 3′-5′ exonucleases, rendering the upstream *sip48* mRNA more susceptible to exonuclease degradation [39]. As a result, the polycistronic message is much less abundant than are the monocistronic relatively stable *βhsd* and *sip48* mRNAs. This data could explain the different amounts of transcription–translation products obtained in vitro.

We observed a significant reduction of growth rate of *sip48*::Ω and *βhsd*::Ω strains during the beginning of the exponential phase of growth in minimal media plus testosterone. However, the mutant cells were able to grow in this media after this slow growth period suggesting that the *sip48-βhsd* genes are not essential for testosterone degradation. In the wild type *C. testosteroni*, these genes are induced when testosterone is used as carbon source indicating that the expression of them is under the control of a regulatory system responsive to testosterone or its metabolites. Moreover, it was reported that β-Hsd activity is expressed even when the cells were grown on LB and the activity is higher when testosterone was used as a carbon source [2]. These observations together the present results suggest that the testosterone degradation (β-Hsd activity) measured in *C. testosteroni sip48* and *βhsd* mutants could be attributable to the expression of independent isofunctional testosterone-metabolizing proteins. The redundancy of steroid dehydrogenase activity suggested by the complete testosterone degradation in the mutant bacteria emphasizes the role that these proteins fulfill in the assimilation of steroids in *C. testosteroni*. These results are supported by the complete genome sequence data of *P. aeruginosa*, *P. syringae*, *P. fluorescens* and *P. putida* where several different conserved hypothetical proteins similar to Sip48 are present [24,25]. It has been reported that several genes encoding an extradiol dioxygenase enzyme involved in the *meta*-cleavage pathway of various aromatic compounds and steroids are present in *Rhodococcus rodochrous* [40] and *C. testosteroni* [10]. It is possible that several of the enzymes known to catalyze the degradation of polycyclic aromatic

hydrocarbons could be involved in the steroid catabolic pathways.

In conclusion, we reported a novel steroid-inducible gene that is transcribed as a polycistronic message together with the *βhsd* gene. Although the specific function of Sip48 is unknown, the presence of a typical N-terminal signal sequence suggests that this protein could be located in membrane or periplasmic space supported by the presence of a typical N-terminal signal sequence and could have some role in steroid uptake or metabolism. The observation that *sip48* is induced at the transcriptional level by testosterone is especially intriguing. While the picture of steroid mineralization pathway remains a puzzle, the identification of its pieces will enable, in the first instance an analysis of their role, and secondly, an integrated schema of the structural organization of genes involved in steroid catabolism.

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