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Cloning and Characterization of the Gene Encoding Alpha-Pinene Oxide Lyase Enzyme (Prα-POL) from *Pseudomonas rhodesiae* CIP 107491 and Production of the Recombinant Protein in *Escherichia coli*

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Abstract The alpha-pinene oxide lyase (Prα-POL) from *Pseudomonas rhodesiae* CIP107491 belongs to catabolic alpha-pinene degradation pathway. In this study, the gene encoding Prα-POL has been identified using mapping approach combined to inverse PCR (iPCR) strategy. The *Prα-POL* gene included a 609-bp open reading frame encoding 202 amino acids and giving rise to a 23.7 kDa protein, with a theoretical isoelectric point (pI) of 5.23. The amino acids sequence analysis showed homologies with those of proteins with unknown function from GammaProteobacteria group. Identification of a conserved domain in amino acid in positions 18 to 190 permitted to classify Prα-POL among the nuclear transport factor 2 (NTF2) protein superfamily. Heterologous expression of Prα-POL, both under its native form and with a histidin tag, was successfully performed in *Escherichia coli*, and enzymatic kinetics were analyzed. Bioconversion assay using recombinant *E. coli* strain allowed to reach a rate of isonovalal production per gramme of biomass about 40-fold higher than the rate obtained with *P. rhodesiae*.

Keywords Alpha-pinene oxide lyase · Isonovalal · Bioconversion · *Pseudomonas* · Orphan enzyme

Introduction

The ability of microorganisms to grow on α-pinene as sole carbon source has been reported by several works and involves multiple pathways leading to various products [1–4]. One of these

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catabolic pathways described in *Pseudomonas fluorescens* NCIMB 11671 [5], *Nocardia* sp. P18.3 [3], and *Pseudomonas rhodesiae* CIP107491 [6], leads first to epoxidation of α-pinene substrate by a NADH-dependent monooxygenase before its decyclizing by a α-pinene oxide lyase without cofactors to generate *cis*-dimethyl-5-isopropylhexa-2,5dienal (isonovalal). Finally, isonovalal is probably degraded in isonovalic acid by a NAD^+-dependent dehydrogenase [7].

Since isonovalal is considered as a potential interest in aroma industry [5], several studies have focused on the production of isonavalal using bioconversion process. Notably, development and optimization of isonovalal production from α-pinene oxide were performed using permeabilized cells of *Pseudomonas rhodesiae* CIP 107491 [6, 8]. Bioconversion in a water-organic solvent biphasic medium permitted to recover up to 400 g l^{-1} of isonovalal from 25 g l^{-1} of biomass in 2.5 h. These studies also highlighted that isonovalal or side-products generated during bioconversion process interact with catalytic site and inactivate the enzyme in an irreversible way [9]. The enzyme involved in the cleavage of both rings of the epoxide, α-pinene oxide, to form *cis*-dimethyl-5-isopropylhexa-2,5dienal (isonovalal) was identified as α-pinene oxide lyase. Purification of the enzyme from *Nocardia* sp. 18.3 and *Pseudomonas* species allowed estimating molecular weights about 40–50 kDa (*Nocardia*) [10], 43 kDa (*P. putida*) [4], and 44 kDa (*P. rhodesiae*) [11]. The multimeric structure of α-pinene oxide lyase was also revealed with the identification of dimeric enzyme, composed of two identical subunits of 22 and 21 kDa, respectively, for *P. putida* and *P. rhodesiae*, while two different subunits of 22 and 17 kDa were found for *Nocardia* sp. 18.3, with a possible dimeric (α-β) or trimeric (α-β-β) structure suggested. Analyses of enzymatic properties have shown that α-pinene oxide lyase was devoid of prosthetic groups, had no cofactors requirements, and exhibited a broad pH activity range [4, 11]. In support of enzymatic kinetics analyses, the K_m constant was estimated to 390 mM, 9 μM, and 210 μM, respectively, obtained for *P .rhodesiae*, *Nocardia* sp. 18.3, and *P.putida*, showing a great variability in substrate affinity for α-pinene oxide lyase between these microorganisms [4, 10, 11].

Although α-pinene degradation pathway leading to isonovalic acid was established in various microorganisms, the genes encoding its enzymes remain unknown. Only some studies have reported the ability of cytochrome P450 monooxygenases mutants to catalyze the oxidation of α-pinene in α-pinene epoxides. Two mutants for the gene encoding cytochrome P450cam (CYP101) from *Pseudomonas putida* promote an increased rate of α-pinene epoxides (up to 22%) compared to wild type strain (4%) although *cis*-verbenol and others α-pinene oxidation products remained predominant [12]. On the other hand, modification of the cytochrome $P450_{BM-3}$ from *Bacillus megaterium* by directed evolution [13] and production in *Escherichia coli* whole-cell biotransformation system allowed to obtain the efficient catalysis of α-pinene to α-pinene oxide, verbenol, and myrtenol in a ratio of 5:2:1 [14, 15]. Concerning the gene encoding α-pinene oxide lyase, it has not yet been identified and no study in literature has focused on its characterization, although the identification of this gene is of particular relevance for improved knowledge of alpha-pinene pathway in microorganisms, and could offer promising perspectives for the valorization of this enzyme.

The present study is the first report describing the identification of the gene encoding α-pinene oxide lyase enzyme. In this study, the identification and cloning of the gene encoding α-pinene oxide lyase enzyme from *Pseudomonas rhodesiae* CIP 107491 was investigated using original strategy based on inverse polymerase chain reaction (iPCR). Analysis of amino acids sequence and protein family has been performed based on the study of conserved domains. Expression of recombinant Prα-POL has been carried out in *Escherichia coli*. Its

activity was measured in biphasic aqueous-organic system and its kinetic parameters were compared to those of the native enzyme.

Materials and Methods

Culture and Strains

P. rhodesiae CIP 107491 strain [6] was grown in *Pseudomonas* basal media (K_2HPO_4 6 mM, KH_2PO_4 9.7 mM, $(NH4)_2SO_4$ 7.2 mM, Nitrilotriacetic acid 1 mM, $MgSO_4$ 2.7 mM, $CaCl_2$ 0.7 mM, $(Na)_2MoO_4$ 1.3 μM, $FeSO_4$ 23 μM, Na_2EDTA 7.5 μM, $ZnSO_4$ 26.3 μM, $MnSO_4$ 10 μM, $CuSO_4$ 1.75 μM, $CoCl_2$ 0.95 μM, $Na_2B_4O_7$ 0.5 μM; pH 7.2) as described in Cohen et al. [16], supplemented with sodium lactate (5 g l^{-1}) as carbon source. Conventional Luria-Broth medium (Tryptone 10 g l^{-1}, Yeast Extract 5 g l^{-1}, NaCl 5 g l^{-1}, pH 7.2) was used for culture of *E. coli* BL21(λDE3) strain.

Identification of N-Terminal and Internal Amino Acid Sequence of α-Pinene Oxide Lyase

Sequence of the N-Ter first amino acids of alpha-pinene oxide lyase was obtained by Edmann sequencing from purified *P. rhodesiae* alpha-POL (Prα-POL). Sequences of internal regions were carried out as following: 200 μg of purified Prα-POL were desalted and concentrated in ammonium bicarbonate buffer (50 mM, pH 8) using VIVASPIN 5000 MWCOPES columns (Sartorius®). After trypsine digestion, generated peptides were analyzed by Maldi TOF, separated, and purified by reverse phase HPLC using UP5WTF-25QF column (Interchim®). At least, three peptides were collected and sequenced by the Edman method.

PCR Amplification of Partial Gene of Prα-POL

Genomic DNA was extracted from *P. rhodesiae* biomass using DNAeasy Blood and Tissue Kit (QIAGEN ™). Based on the amino acids sequencing, degenerated oligonucleotides primers were designed in the N-ter region (from QTENKK sequence) and in the internal region of the protein (from YDGGLAG and EVAVQVG motif) to carry out PCR amplification. Codon usage frequency in different *Pseudomonas* species, from Kazuka Codon Usage Database (http://www.kazusa.or.jp/codon/), was applied to reduce the degeneracy of the primers and increase their specificity for the targeted gene. Therefore, forward qtenkk-primer (5′-CAGA C(G/C)GA(A/G) AACAAGAAGG-3′), reverse ydgglag-primer (5′-GCC(G/C)GCCA(A/-G)GCCGCC(A/G)TC(A/G)TA-3′), and reverse evavqvg-primer (5′-(A/G)CC(C/G)AC(T/-C)TG(C/G)AC(C/G)GC(C/G)AC(T/C)TC-3′) were designed to amplify two regions of the gene. PCR products were cloned into pGEM-Teasy vector (Promega) and sequenced (MWG Operon Co, Germany).

Identification, Mapping and Sequencing of the DNA Locus Containing *Prα-POL* Gene

The 300-bp PCR fragment obtained with qtenkk-forward and ydgglag-reverse primers and containing a partial open reading frame (ORF), potentially identified as a part of alpha-pinene

oxide lyase, were used as a probe to carry out a restriction map of the gene locus in order to isolate the complete α-*POL* gene. Genomic DNA from *P. rhodesiae* was submitted to digestion using different restriction enzymes as BamHI, XhoI, PstI, EcoRV, and KpnI. The digested genomic DNA was then separated by gel electrophoresis (agarose), transferred to a nylon membrane, and hybridized with a 300-pb fragment of α-*POL* (α-pol300 probe), radioactively labeled with alpha ^{32}P-dCTP using NEBlo®t Kit (New England Biolabs®).

To perform the sequencing of the locus obtained by restriction mapping, a strategy based on inverse PCR was developed. Inverse PCR method was used to obtain the sequence of the flanking region of a known sequence. Briefly, in support of restriction map obtained with α-pol300 probe, genomic DNA was digested with BamHI, EcorV, and PstI, and the restriction fragments from each restriction digestion were self-ligated using T4-DNA ligase. A PCR using ibam-fwd (5′-GACACGGCCAAGGACTGG-3′) and ibam-rev (5′-GCCTGTACCCTTGAAGCC-3′) primers was then performed on self-ligated fragments using specific primers localized in the α-pol300 fragment and designed in opposite orientation. Amplification products were cloned in pGEM-T-easy vector (Promega®, Wisconsin, USA) and sequenced.

Expression and Analysis of Recombinant Prα-POL in *E. coli*

Plasmid Construction Three plasmid constructions allowing the expression of the native Prα-POL, and N-terminal (N-Ter) and C-terminal (C-Ter) His-tagged Prα-POL were carried. The full-length coding region of *Prα-POL* gene was PCR-amplified with specific primers containing restriction sites (underlined): Nco/pol-Fwd (5′-GGGCCATGGGCATGAGTCAGACAGAAAACAAG-3′) and Xho/pol-rev1 (5′-GGGCTCGAGTTACTTTGCCAGCCAGAACT-3′) for the native Prα-POL; ERI/pol-fwd (5′-GGGGAATTCATGAGTCAGACAGAAAACAAG-3′) and Xho/pol-rev1 for the N-Ter His-tagged Prα-POL; and Nco/pol-Fwd and Xho/pol-rev2 (5′-GGGCTCGAGCTTTGCCAGCCAGAACT-3′) for the C-Ter His-tagged Prα-POL. After restriction digestion and purification, the PCR product was ligated into the NcoI and XhoI or EcoRI and XhoI sites of the pET-28a plasmid (Novagen®) and transformed in *E .coli* JM109 (Promega®). DNA plasmids pET-28a-Prα-POL were extracted from positive recombinant clones by alkaline lysis (Sambrook, miniprep) and sequenced to ensure the integrity of the construct.

Expression of Prα-POL Recombinant Proteins pET-28a-Prα-POL plasmids were transformed in *E. coli* BL21(λDE3) (Promega®) and positive recombinant bacteria were selected on in Luria-Broth (LB) medium containing kanamycin (30 μg ml^{-1}) (Kanamycin sulfate, SIGMA-Aldrich). For expression in BL21(λDE3), 50 ml of LB medium supplemented with kanamycin (30 μg ml^{-1}) was inoculated with 1 ml of a culture grown overnight and cultivated at 37 °C until OD_{600} reached 0.5–0.6. Expression of recombinant protein was then induced with 1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h.

Analysis of Prα-POL Expression For protein analysis in denaturing conditions, SDS-PAGE (Laemmli) was carried out using 12% polyacrylamide gels that were subsequently stained with Coomassie brilliant blue R250 (SIGMA-Aldrich®).

Analysis of Prα-POL protein under native conditions was carried out for the protein dimerization study. Briefly, after centrifugation of 5 ml of an induced culture, bacterial was

incubated in 1 ml of lysis buffer (Na_2HPO_4 50 mM, pH 8, NaCl 300 mM) supplemented with lysosyme (0.1 mg ml^{-1}) for 30 min. Sonication was applied according to 6 pulses of 10 s with a break of 5 s. between each pulse. After centrifugation at 4 °C at 12,000 rpm for 20 min, 10 μg of proteins, from the supernatant (soluble fraction) or after purification of C-ter and N-ter His-tagged Prα-POL using Ion Metal Chromatography Affinity (IMAC) (Protino® Ni-NTA, Macherey-Nagel®), were analyzed on non-denaturing polyacrylamide gel 12%. For the revelation and visualization of protein or dimeric complex, a zymographic assay was developed. Therefore, polyacrylamide gel was immersed in sodium/phosphate buffer (KH_2PO_4/Na_2HPO_4 20 mM pH 7.1) containing alpha-pinene oxide (0.1% *v*/*v*) and incubated with agitation for 30 min. The presence of enzyme or multimeric structure was identified with the formation of a white precipitate resulting from the enzyme activity generating insoluble isonovalal (*cis*-dimethyl-5-isopropylhexa-2,5dienal).

Bioconversions

The bioconversion experiments were carried out in 100-ml Erlenmeyer flasks with recombinant *E. coli* permeabilized cells as described in Fontanille et al. [8]. Briefly, the reaction medium was a two-phase system in a volume ratio 1:1, comprising an aqueous phase made of the permeabilized concentrated cell suspension in phosphate buffer (K_2HPO_4/KH_2PO_4 20 mM, pH 7.5) and an organic layer consisted of an organic solution of α-pinene oxide (SIGMA-Aldrich®) dissolved in hexadecane (Hexodecane Reagent Plus-99%, SIGMA-Aldrich®). Alpha-pinene oxide concentration was ranged from 400 to 800 g l^{-1} with concentrated biomass at 7 to 0.7 g l^{-1} for recombinant *E. coli*. Bioconversion experiments were realized in 50 ml medium during 7 h at 30 °C.

Quantification of Isonovalal Production by Gas Chromatography

Quantification of substrate consumed and metabolite produced (isonovalal) was done by gas chromatography (GC) and GC-MS as described in Fontanille et al. [8]. Briefly, 1 μl of organic phase was directly injected into the apparatus. The chromatograph (HP 6890, Agilent Technologies Palo Alto, CA, USA) was fitted with an apolar SPB-5 (Supelco Inc., Bellefonte, PA) capillary column (30 m × 0.32 mm i.d., film thickness 0.25 μm) and a flame ionization detector. The carrier gas was nitrogen, and the oven temperature was maintained at 80 °C for 5 min, and then raised to 200 °C at 20 °C min^{-1}. The injector and detector temperatures were 250 °C both; the split ratio for injection was 1:5. Concentrations were calculated by assuming that all compounds had the same response factor as α-pinene oxide. The internal standard was heptadecane (SIGMA-Aldrich®).

Enzymatic Assay

The alpha-pinene oxide lyase activity (μmol min^{-1} mg^{-1}) was determined with enzymatic kinetic analysis at 242 nm (242 nm is the maximal absorption wavelength of isonovalal; molar extinction coefficient ε = 10,538 mol l^{-1} cm^{-1}). Enzymatic kinetic was carried out in 1 ml of reaction buffer (20 mM KH_2PO_4/Na_2HPO_4, pH 7.1) containing alpha-pinene oxide (1 g l^{-1}) and 10 to 50 ng of protein in the soluble fraction from total extracts or purified His-tagged α-POL issued from recombinant *E. coli* strains.

Results

Partial Sequencing of the Purified α-Pinene Oxide Lyase of *P. rhodesiae*

Alpha-pinene oxide lyase from *P. rhodesiae* was purified as described by Laroche et al. [11]. Edmann sequencing of purified protein allowed defining Ser-Gln-Thr-Glu-Asn-Lys-Lys-Gly-Phe-Lys (SQTENKKGFK) as the N-terminal sequence of the purified α-pinene oxide lyase. Peptides obtained after hydrolysis of Prα-POL protein using trypsine were analyzed by HPLC coupled with mass spectrometry to obtain their amino acids sequences. Three internal regions of the protein were identified as Tyr-Asp-Gly-Gly-Leu-Ala-Gly (YDGGLAG), Glu-Val-Ala-Val-Gln-Val-Gly (EVAVQVG), and Trp-Thr-Gln-Glu-Glu (WTQEE). A search of these sequences using BLASTP algorithm in the *Pseudomonas* genome database (http://www.pseudomonas.com/) and in NCBI database was performed, but no matching with proteins was revealed. These results seemed to suggest the specificity of Prα-POL only in few organisms holding enzymatic activity associated to alpha-pinene metabolism.

Mapping and Identification of the DNA Locus Encoding Prα-POL Protein

In support of peptides sequences, degenerated primers designed from QTENKK region and two internal motifs YDGGLAG and EVAVQVG were used to perform PCR amplification targeting the beginning of the gene encoding Prα-POL. Two DNA fragments of about 300 bp (named α-pol300) and 500 bp (named α-pol500) were isolated from amplification using QTENKK/YDGGLAG and QTENKK/EVAVQVG primers, respectively. Sequence analysis showed the perfect homology between the two DNA indicating that fragments were amplified from the same genome locus. Interestingly, the global QTENKKGFK N-Ter motif and internal motif WTQEE, previously determined with Prα-POL partial protein sequencing, were also identified in α-pol300 and α-pol500 (Fig. 1). These results strongly suggest that α-pol300 and α-pol500 DNA correspond to the partial sequence of the alpha-pinene oxide lyase from *P. rhodesiae*.

```
  1 ATG AGT CAG ACA GAA AAC AAG AAA GGC TTC AAG GGT ACA GGC CGG ATG AAT GTC GAC ACG GCC AAG GAC TGG
  1 M  S  Q  T  E  N  K  K  G  F  K  G  T  G  R  M  N  V  D  T  A  K  D  W
 73 ATC CAC ACC TTC TTC TTC GGT GGC GCG GAC GTC ATC ATC GAC TAC TAC GCC GAT GAT TTC GTG TTC GAG GAT
 25 I  H  T  F  F  F  G  G  A  D  V  I  I  D  Y  Y  A  D  D  F  V  F  E  D
145 CTG ACC TAC TTC CAG ACC ATC GAC AAC AAG GAA GAC CTA CAC CGC GCG TTC GTC CAG TTC AGC AAC CCG CAG
 49 L  T  Y  F  Q  T  I  D  N  K  E  D  L  H  R  A  F  V  Q  F  S  N  P  Q
217 GCT GCC GAG GGC ACC AGC GGT ACC CAC GAC TTC GAC ATC ATC CGC TAC GAC GGC GGC CTG GCC GGC GAT CAC
 73 A  A  E  G  T  S  G  T  H  D  F  D  I  I  R  Y  D  G  G  L  A  G  D  H
289 CGT GCG GTG ATG CAG CCC AAG CCG GAT CGC TGG ACT CAA GAG GAG TGG GAT CAG TGG ACC GCC GAG GCG AGC
 97 R  A  V  M  Q  P  K  P  D  R  W  T  Q  E  E  W  D  Q  W  T  A  E  A  S
361 CTG GGC GGC CAC CGT AAC GAC TAC GAC GAA TGG GCC AAC ATC ACC TGG ATC TGG CGC GCT ACC CAT ACC GAC
121 L  G  G  H  R  N  D  Y  D  E  W  A  N  I  T  W  I  W  R  A  T  H  T  D
433 GAC GAG TTC TGC GGC CTC ACC GGG ATG AAA GGC AAG ACC ACC TAT GTG CGT GGG CAG ACC TTC CAC CTA TAC
145 D  E  F  C  G  L  T  G  M  K  G  K  T  T  Y  V  R  G  Q  T  F  H  L  Y
505 AAG AAC CGG CAG ATC GTG CGC GAA TAC ACG CAA TGG AAC TTC CGC GAA GTG GCC GTG CAA GTC GGT
169 K  N  R  Q  I  V  R  E  Y  T  Q  W  N  F  R  E  V  A  V  Q  V  G
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Fig. 1 Sequences of Prα-POl300 and Prα-POl500 DNA fragments containing partial α-pinene oxide lyase gene**.** The nucleotide sequence of Pr α-POL300 and Pr α-POL500 DNA fragments obtained with PCR using degenerated oligonucleotides and deduced amino acids are shown. Three internal regions (YDGGLAG), (EVAVQVG), and (WTQEE), preliminary identified from the sequencing of three peptides of *P. rhodesiae* α-POL, are indicated (box)

In order to isolate the entire gene of Prα-POL, a strategy based on inverse PCR (iPCR) was developed. First, a restriction map of the genomic region containing the Prα-POL gene was established using α-pol300 fragment as probe (Fig. 2a). To isolate upstream and downstream flanking region of the α-pol300 locus, iPCR were performed on self-ligated genomic DNA preliminary digested either with BamHI restriction enzyme to obtain the 5′-region or with PstI and EcoRV for the 3′-flanking region, as shown in Fig. 2b. Cloning and sequencing amplification products from iPCR allowed to determine a 3137 nucleotide sequence (GenBank Accession: MF946559) containing a partial open reading frame (ORF) associated to a glutathione S-transferase family protein (GST), and two major ORFs, a 609-bp ORF encoding 202 amino acid corresponding to Prα-POL protein (Fig. 3a) (GenBank Accession: KT265812) and a 1440-pb ORF encoding for a protein showing homology with coniferyl aldehyde dehydrogenase (CALDH) isolated from different organisms. A putative Shine-Dalgarno sequence AGGA was located at position -8 nt from the initiation translation codon AUG of the *Prα-POL* gene (Fig. 3a). No canonical sequences related to bacterial promoter was identified in upstream region of AUG

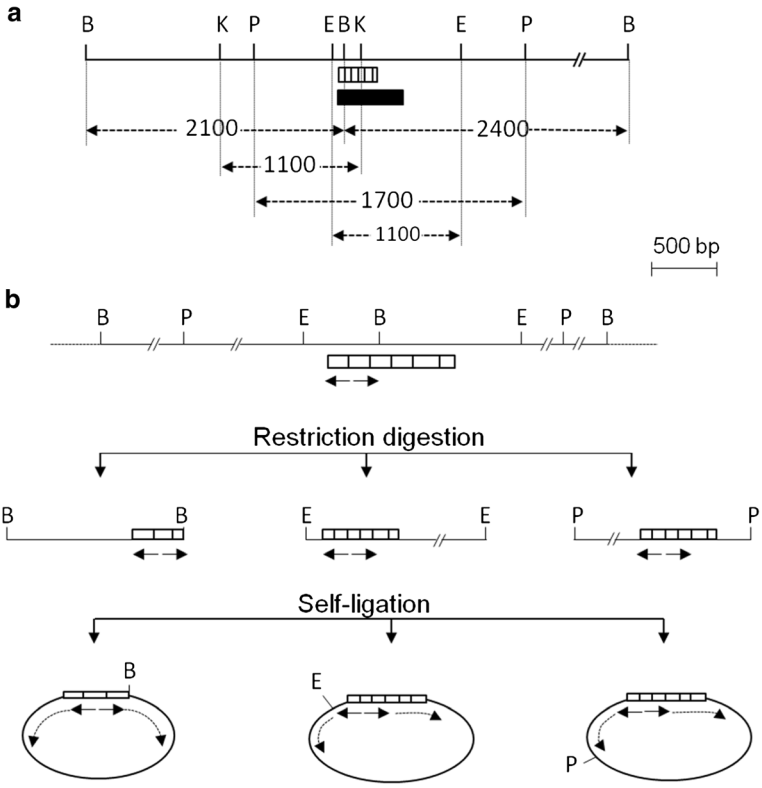

Fig. 2 Restriction map and iPCR strategy for sequencing the alpha-pinene oxide lyase gene locus. **a** Restriction map of the alpha-pinene oxide lyase locus using α-POL300 probe (hatched box) for Bam HI (B), Eco RV (E), Kpn I (K), and Pst I (P) restriction sites. α-POL500 sequence localization is presented in full box. Distance between sites is indicated in kilobase pairs (kbp). **b** iPCR strategy and principle: in a first step, three enzymatic digestions of *Pseudomonas* genomic DNA were carried out independently (BamHI, PstI, and EcoRV). Each digestion product was then self-ligated and a PCR was performed using inverse primers forward qtennkk-primer and reverse ydgglag-primer (arrows, see Mat&Meth)

a

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   1  CGATCGCCCATGGGCGGTTTCCATCCCCAGAGGAGTTATGACA ATG AGT CAG ACA
   1                                               M   S   Q   T
  56  GAA AAC AAG AAA GGC TTC AAG GGT ACA GGC CGG ATG AAT GTC GAC
   5  E   N   K   K   G   F   K   G   T   G   R   M   N   V   D
 101  ACG GCC AAG GAC TGG ATC CAC ACC TTC TTC TTC GGT GGC GCG GAC
  20  T   A   K   D   W   I   H   T   F   F   F   G   G   A   D
 146  GTC ATC ATC GAC TAC TAC GCC GAT GAT TTC GTG TTC GAG GAT CTG
  35  V   I   I   D   Y   Y   A   D   D   F   V   F   E   D   L
 191  ACC TAC TTC CAG ACC ATC GAC AAC AAG GAA GAC CTA CAC CGC GCG
  50  T   Y   F   Q   T   I   D   N   K   E   D   L   H   R   A
 236  TTC GTC CAG TTC AGC AAC CCG CAG GCT GCC GAG GGC ACC AGC GGT
  65  F   V   Q   F   S   N   P   Q   A   A   E   G   T   S   G
 281  ACC CAC GAC TTC GAC ATC ATC CGC TAC GAC GGC GGC CTG GCC GGC
  80  T   H   D   F   D   I   I   R   Y   D   G   G   L   A   G
 326  GAT CAC CGT GCG GTG ATG CAG CCC AAG CCG GAT CGC TGG ACT CAA
  95  D   H   R   A   V   M   Q   P   K   P   D   R   W   T   Q
 371  GAG GAG TGG GAT CAG TGG ACC GCC GAG GCG AGC CTG GGC GGC CAC
 110  E   E   W   D   Q   W   T   A   E   A   S   L   G   G   H
 416  CGT AAC GAC TAC GAC GAA TGG GCC AAC ATC ACC TGG ATC TGG CGC
 125  R   N   D   Y   D   E   W   A   N   I   T   W   I   W   R
 461  GCT ACC CAT ACC GAC GAC GAG TTC TGC GGC CTC ACC GGG ATG AAA
 140  A   T   H   T   D   D   E   F   C   G   L   T   G   M   K
 506  GGC AAG ACC ACC TAT GTG CGT GGG CAG ACC TTC CAC CTA TAC AAG
 155  G   K   T   T   Y   V   R   G   Q   T   F   H   L   Y   K
 551  AAC CGG CAG ATC GTG CGC GAA TAC ACG CAA TGG AAC TTC CGC GAA
 170  N   R   Q   I   V   R   E   Y   T   Q   W   N   F   R   E
 596  GTG GCC GTG CAA GTC GGT GCC TAT CCC ATG CCG GAG AAG TTC TGG
 185  V   A   V   Q   V   G   A   Y   P   M   P   E   K   F   W
 641  CTG GCA AAG TAA
 200  L   A   K   *
```

b

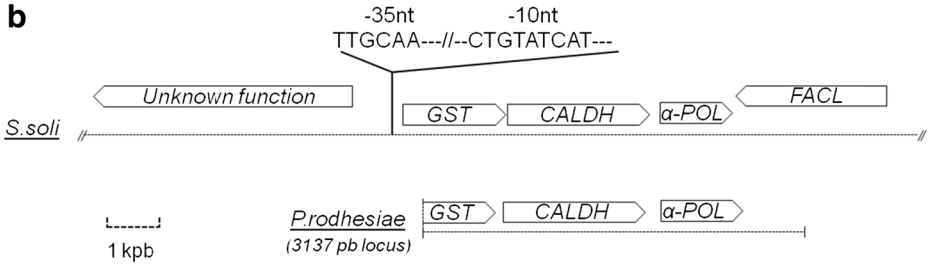

Fig. 3 Sequence of α-pinene oxide lyase gene and structure of associated operon. **a** The nucleotide sequence of *Prα-POL* gene and deduced amino acids are presented with the 45 nt upstream region of ATG START codon. Putative Shine-Dalgarno motif (inset) is identified at position -8 nuncleotide from ATG codon. **b** ORFs organization identified from comparative alignment of the DNA loci from *S. soli* (ID: NZ_AXDW01000010) and *P. rhodesiae* (3137 nt fragment, ID: M946559) with identified ORFs: glutathione S-transferase (GST), coniferyl aldehyde dehydrogenase (CALDH), α-pinene oxide lyase (α-POL), and fatty acyl coA ligase (FACL). The -35 nt and -10 nt indicate a predicted promoter

codon, suggesting the belonging of the *Prα-POL* gene to an operon structure. Search of homology sequence using BLASTn did not permit to identify existing homologous sequences registered in DNA Databank.

Using protparam algorythm (Expasy), the molecular weight of the Prα-POL protein was estimated at 23.7 kDa, in good accordance with the value of 24 kDa found by Fontanille et al. [8] and Laroche et al. [11] for the purified protein from *P. rhodesiae*. A theoretical isoelectric point (pI) of 5.23 was predicted, close to the one (pI = 4) found for *Nocardia* α-pinene oxide lyase [10].

Characterization of the Prα-POL Protein in *P. rhodesiae*

Amino acid sequences deduced from the 609 bp ORF Prα-POL protein were analyzed using BLASTP algorithm in the NCBI protein database. Similar homology was identified in a few organisms belonging to the Proteobacteria and Actinobacteria phyla and was associated to hypothetical proteins with unknown function. Especially, Prα-POL protein showed the highest homology score with GammaProteobacteria group proteins, such as *Solimonas soli* (64% protein identity, ID: WP_028080077), *Nevskia ramosa* (54% protein homology, ID: WP_0022975199), and Gammabacterium BDW918 (53% protein homology, ID: WP_008251196). Weak homology (32%) was observed with a hypothetical protein (ID WP_008357921) from bacteria belonging to Nocardioidacae family whose sequence origin has not been clearly determined (for reference, http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=408672). Although a α-pinene oxide lyase activity was shown in *Nocardia* sp. (Strain 18.1) [3], no homologous sequence with protein Prα-POL was clearly isolated from *Nocardia* protein database.

In addition to the highest homologous score (64% identity) obtained with *Solimonas soli*, the structure of the 3137 nt DNA sequence of *P. rhodesiae* is similar to the *S. soli* DNA locus in which the protein (WP_028080077) homologous to Prα-POL is an integral part of an operon including glutathione S-transferase and coniferyl aldehyde dehydrogenase encoding genes (ID: NZ_AXDW01000010) (Fig. 3b).

Using CDART (Conserve Domain Architecture Retrieval Tool from NCBI), a NTF2-like (nuclear transport factor 2) conserved domain was identified in amino acid positions 18 to 190 and allowed to classify Prα-POL among the NTF2 protein superfamily. NTF2 domains alignment of Prα-POL and Delta-ketosteroid isomerases consensus sequence (referenced as TIGR02096) showed similarity restricted to few dispersed amino acids residue. In addition to Delta-5-3-ketosteroid isomerases proteins, NTF2 family includes members of scytalone dehydratases and the beta subunit of ring hydroxylating dioxygenases.

Bioconversion Assay of Recombinant Prα-POL Produced in *E. coli*

Native recombinant Prα-POL was cloned into pET28a vector and expressed in *E. coli.* Firstly, the ability of recombinant *E. coli* (rec*E. coli*) strain to produce isonovalal was tested during a small-scale bioconversion in presence of α-pinene oxide substrate (400 g l^{-1}) on permeabilized cells concentrated at 7 g l^{-1}. The rapid consumption of substrate observed in less than 1 h and suggesting a high expression level of Prα-POL protein in rec*E. coli*, did not allow to analyze kinetics. A second bioconversion assay was then performed using rec*E. coli* biomass diluted to 0.7 g l^{-1} and α-pinene oxide concentration increased to 800 g l^{-1}. The shape of the curves obtained from analysis of the kinetic behavior of consumed α-pinene oxide and isonovalal synthesis (Fig. 4) was similar to the one obtained by Fontanille et al. for *P. rhodesiae* [8]. Indeed, a regular increasing production of isonovalal during 2.5–3 h of bioconversion (maximal isonovalal concentration 540 g l^{-1}) followed by a plateau was observed. Alpha-pinene oxide was not totally consumed, suggesting that the substrate concentration was not limiting. In our experiment, the reaction rate (gramme of isonoval per hour) was estimated at 4.81 $g_{isonovalal}\ h^{-1}$, corresponding to a specific production rate for rec*E. coli* close to 260 $g_{isonovalal}\ h^{-1}\ g_{biomass}{}^{-1}$. This rate was 40-fold higher to the value of 6.4 $g_{isonovalal}\ h^{-1}\ g_{biomass}{}^{-1}$ for *P. rhodesiae* obtained by Fontanille et al. [8] in an optimized process. These results demonstrated a high expression level of the fully active Prα-POL in rec*E. coli.*

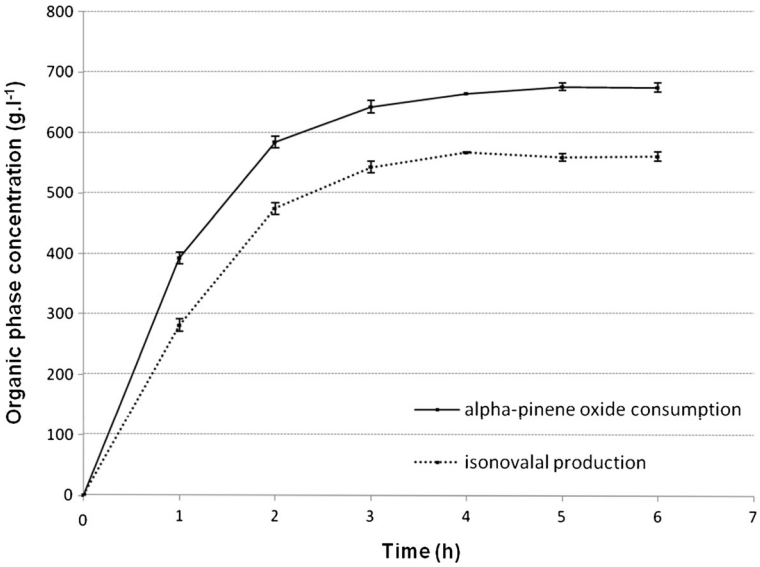

Fig. 4 Time-course of α-pinene oxide consumption and isonovalal production during the bioconversion of α-pinene oxide in a biphasic system hexadecane/phosphate buffer. Analysis of α-pinene oxide utilization and isonovalal production obtained from bioconversion carried out with recombinant *E. coli* permeabilized biomass ($n = 3$)

Expression Analysis and Enzymatic Kinetics of Prα-POL in rec*E. coli* Strain

Expression analysis of native and His-tagged forms of Prα-POL was carried out in all recombinant strains. Total proteins of the soluble fraction, extracted from IPTG induced recombinant *E. coli* strain, and His-tagged Prα-POL proteins purified by nickel chromatography (IMAC) were analyzed both with PAGE under denaturing and non-denaturing conditions, and with a zymogram gel. As shown in Fig. 5, a major band corresponding to Prα-POL was detected in both denaturing and non-denaturing conditions (Fig. 5). In denaturing conditions, the major band was shown in all strains approximately at 25 kDa (Fig 5a), corresponding to the expected size of Prα-POL. In non-denaturing condition (Fig. 5b), this band may be associated to dimeric structure of Prα-POL previously described in *P. rhodesiae* [11]. The patterns of migration were substantially different between strains, notably for N-ter His-tagged Prα-POL compared to the native and Cter-His-tagged Prα-POL (Fig. 5b). This variation may be related to the presence of His-tag which was substantially different in size (N-ter His-tag longer than C-ter His-tag). The native and His-tagged forms of Prα-POL were shown to have functional activity as shown with a zymogram gel (Fig. 5c).

Enzymatic kinetics were carried out to estimate specific enzymatic activity of Prα-POL in all recombinant strains. The enzymatic kinetic was studied by following the production of isonovalal in spectroscopy at 242 nm corresponding to the maximal absorption wavelength of isonovalal. Enzymatic activity was determinate for both total protein extracts and purified His-tagged Prα-POL (Table 1). The specific activity observed for the proteins of the soluble fraction from the recombinant strain expressing non-tagged Prα-POL was 2.8- to 3.8-fold higher to the one obtained for strains expressing the N-ter and C-Ter-tagged proteins. This result could be explained by a weaker expression level of the tagged proteins or by the fact that the His-tag hampered the correct folding of the protein. IMAC-tagged protein purification

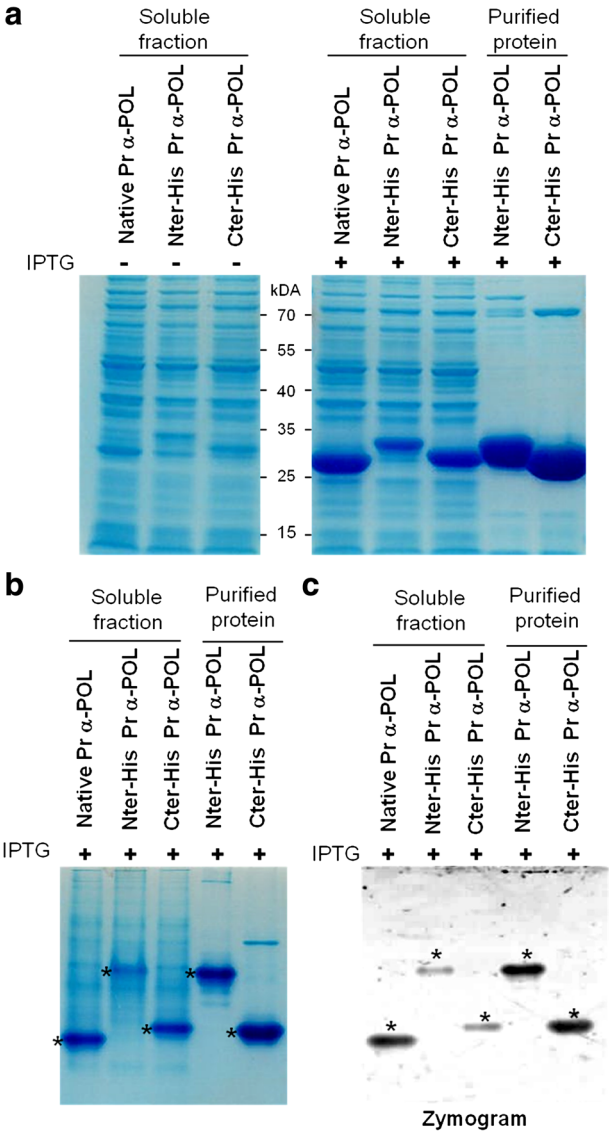

Fig. 5 Polacrylamide gel electrophoresis and zymogram analysis of native and His-tagged recombinant α-POL proteins produced in rec*E. coli.* Total protein of the soluble fraction extracted from rec*E. Coli* and recombinant His-tagged α-POL protein purified with IMAC process were analyzed in denaturing SDS-PAGE conditions (**a**) and non-denaturing conditions (**b**). Size marker indicated weight molecular in kilodalton (KDa). Dimeric forms of the recombinant α-POL protein were indicated by an asterisk (*). **c** A zymogram carried out in non-denaturing conditions allowed to detect the enzymatic activity of Prα-POL shown with the apparition of a precipitate indicated by an asterisk (*)

allowed to increase 3.5- to 5.5-fold the specific activity (Table 1), suggesting that Prα-POL protein was over-expressed at high level, representing at least 25–60% of the total soluble proteins in rec*E. coli*. Specific activity of purified N-ter His-Prα-POL, estimated at 546.37 μmol min^{-1} mg^{-1}, was relatively close to this determinate for *P. rhodesiae* (600 μmol min^{-1} mg^{-1}) [11], while specific activity obtained for purified C-ter-His-Prα-

Table 1 Estimation of α-pinene oxide lyase activity in recombinant *E. coli* strains

IPTG induction	Protein fraction	α-POL	α-POL specific activity (μmol min^{-1} mg^{-1})
NO	SF	Native α-POL	26.57 ± 8.70
		N-ter His-Tag α-POL	
		C-ter His-Tag α-POL	
YES	SF	Native α-POL10	373.30 ± 35.80
		N-ter His-Tag α-POL	98.57 ± 13.59
		C-ter His-Tag α-POL	134.15 ± 11.58
YES	PP	N-ter His-Tag α-POL	546.37 ± 93.73
		C-ter His-Tag α-POL	482.06 ± 77.51

Alpha-pinene oxide lyase activity was determined by spectrophotometry by following the production of isonovalal at 242 nm. Specific activity was performed on soluble fraction of total protein extracts (SF) or purified His-tagged α-POL (PP) from recombinant strains. Three independent experiments were carried out for each strain ($n = 3$)

POL was lower (482.06 μmol min^{-1} mg^{-1}), suggesting a C-ter position effect of the histidin tag on protein functionality.

Discussion

In this work, the gene encoding the alpha-pinene oxide lyase enzyme from *P. rhodesiae* has been isolated for the first time. Although several studies have reported alpha-pinene oxide lyase activity in different organisms [3, 6], the gene encoding the enzyme remains to date unknown. Therefore, alpha-pinene oxide lyase enzyme belongs to the orphan enzymes proteins and is listed as α-pinene oxide decyclase (EC number 5.5.1.1.10) in Database of Orphanzymes Project (http://www.orphanenzymes.org/database/table-s1/). The studies of orphan enzymes are often hampered by the lack of sequence data and hence identification of the gene encoding an orphan enzyme presents a real interest.

Prα-POL is composed of 202 amino acids generating a protein with an estimated molecular weight of 23.7 kDa and a theorical pI of 5.23, values that fit, respectively, to the 24 kDa and pI 5.2 described for the purified protein from *P. rhodesiae* strain [11].

Alpha-pinene oxide lyase activity has been described in only a few organisms, namely *Nocardia* sp. [3] and *P. rhodesiae* [6]. This could explain why amino acid sequence analysis of Prα-POL revealed significant homology (identity > 50%) with only a few proteins with unknown function, suggesting a role of α-POL associated to singular metabolism pathway and limited to a few organisms. High-sequence homology was obtained for two hypothetical proteins identified in *Nevaskia ramosa* and *Solimonas soli* bacteria, originally isolated respectively from air-water interphase and soil [17]. In support of phylogenic properties, *Solimonas soli* was identified and characterized recently by Kim et al. [17]. These authors established a new genus and specie linked to Gammaproteobacteria group, closely related to *Nevskia ramosa* and *P. aeruginosa*. Despite the few data for *Solimonas* et *Nevskia*, our results seem to strongly suggest the existence of alpha-pinene metabolic pathway in these organisms. The involvement of the *Prα-POL* gene, present within an operon structure in association with coniferyl aldehyde dehydrogenase (CALDH) and glutathione S-transferase (GST) encoding genes, remains unclear. Indeed, CALDH and GST proteins were known to operate in different metabolic pathways unrelated to the α-pinene metabolism, such as for example the conversion

of eugenol by CALDH [18] and the degradation of different aromatic compounds by several bacterial GSTs [19].

The sequence homology analysis (BLASTp) revealed other proteins that also belong to the Gammaproteobacteria group, although the homology percentage was much lower (≈ 30%). These results are mainly linked to the presence of a NTF2-like domain identified on Prα-POL. The NTF2 domain does not have a really characteristic sequence motif [20]. Members of the NTF2-like superfamily, among which scytalone dehydratase, Delta-5-3-ketosteroid isomerase, and limonene-1,2-epoxide hydrolase families, are widely distributed among bacteria and eukaryotes. This family is an example of divergent evolution wherein the proteins have many common structural details but diverge greatly in their function, including both non-catalytic and catalytic version. For example, in eucaryotes, the NTF2-like domain, identified first in the NTF2 protein of rat [21], then in various nuclear receptors, is involved in the transport of proteins towards the nucleus [22, 23]. Scytalone dehydratase family is involved in the biosynthetic pathway of dihydroxynaphthalene-derived melanin phytopathogenes fungi [24, 25], whereas the Delta-ketosteroid isomerase (KSI) are involved in the steroid hormone metabolism in animal tissues [26] or in steroid degradation pathway in *P. testosteroni* and *P. putida* [27]. As shown by these examples, all these metabolic pathways are not functionally linked to the α-pinene pathway involving the α-pinene oxide lyase except maybe for the limonene-1,2-epoxide hydrolase. The isolated enzyme from *Rhodococcus erythropolis* [28] allows the growth of the microorganism on a medium containing monoterpene limoneme as sole carbon source. The mechanism of this hydrolase, which catalyzes the addition of water to epoxides to form the corresponding diol, is close to the one of the Prα-POL which cleaves the α-pinene oxide cycle [3, 6]. Activity tests to show the capacity of Prα-POL to use limonene as a substrate gave negative results (data not shown).

The presence of NTF2-like domain in Prα-POL could be required for the 3D structure of the protein. The NTF2-like fold has a cone-like shape that forms a cavity that can be adapted to serve a number of functions among which the interaction with the substrate or protein multimerization as described for calcium/calmodulin dependent kinase II [20, 29]. The NTF2-like domain could thus be involved in the dimerization of Prα-POL.

This work showed that Prα-POL can be expressed in *E. coli* as an active recombinant protein, with enzymatic activities close to those obtained with native Prα-POL of *P. rhodesiae*. Bioconversion for isonovalal production in rec*E. coli* compared to *Pseudomonas* presents a real interest for different reasons: (i) higher level of specific isonovalal production (per unit dry mass of biomass) could be obtained in *E. coli*, (ii) unlike *E. coli*, the enzyme α-pinene oxide lyase synthesis in *Pseudomonas* required a preliminary phase of induction of the metabolic pathway by adding α-pinene substrate, solubilized in hexadecane, in culture medium, (iii) the expression of the recombinant Prα-POL in *E. coli* was constitutive and was not submitted to endogenous pathways regulating the expression/induction of the enzyme as was observed in *P. rhodesiae* [9], and (iv) isonovalal production using purified enzyme could be performed and purification steps of recombinant protein could be facilitated with histidin tag.

In conclusion, the identification of the gene encoding alpha-pinene oxide lyase offered new perspectives. The production of isonovalal, which is considered to have potential interest in the aroma industry, by bioconversion in *E. coli* could facilitate and greatly increase the production yield of isonovalal. The identification of homologous proteins in other organisms also opens new perspectives to study the degradation pathway of α-pinene and should contribute to a better understanding of the metabolism of these organisms and to their valorization. Finally, the Prα-POL affiliation of the NTF2-like superfamily, including scytalone dehydratase, Delta-

5-3-ketosteroid isomerase, and limonene-1,2-epoxide hydrolase families, is of special interest for the potential exploitation and valorization of the enzyme activity. The screening of different substrates, related to NTF-like superfamily, may contribute to explore the activities of enzyme in new metabolic pathways, and could offer new perspectives for the valorization of this enzyme. For example, as ketosteroid isomerase (KSI) enzymes were shown to be involved in catabolism of steroids (e.g., testosterone) in some bacteria as *Pseudomonas testosteroni* and *Pseudomonas putida* [27], it will be relevant to study the ability of Prα-POL to decyclize aromatics steroids in a context of degradation and removal of such compounds from the environment.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

Ethics Statement This article does not contain any studies with human participants or animals performed by any of the authors.

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