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Characterization of 1,8-cineole degradation encoding operon from *Rhodococcus* sp. TMP1

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Recently isolated bacterial strain *Rhodococcus jostii* TMP1 proved to be capable of utilizing 1,8-cineole as its sole source of carbon and energy. Bioinformatic analysis of *R. jostii* TMP1 genome revealed a presence of a novel and more detailed Cin operon – Cin_{TMP1}. It was found that Cin_{TMP1} operon contains genes which are known to be involved in biodegradation of 1,8-cineole by *Citrobacter braakii*. The genes located in this operon – *cin*A1, *cin*B1, *cin*C1, *cin*D1 and *cin*BVMO – were proposed to encode putative cytochrome P450 ($P450_{cin}$), cindoxin reductase, cindoxin, hydroxycineole dehydrogenase and Baeyer-Villiger monooxygenase, respectively. The expression of all recombinant enzymes, except for cindoxin reductase, as well as the coexpression of $P450_{cin}$ system, has been studied and optimized. Recombinant $P450_{cin}$ enzyme of *R. jostii* TMP1 catalyses the initial monooxygenation of 1,8-cineole, yielding 6-hydroxycineole. Furthemore, $P450_{cin}$ performs a subsequent alcohol oxidation to produce 6-ketocineole in *Rhodococcus* cells.

Keywords: 1,8-cineole, *Rhodococcus*, cytochrome P450, cindoxin reductase, cindoxin, hydroxycineole dehydrogenase, Baeyer-Villiger monooxygenase

INTRODUCTION

The rapid growth of chemical industry indicates a clear need for new high throughput technologies. Conventional chemical processes lack specificity, are often too slow and inefficient in physiological environment. It goes without saying that chemical industry is hazardous and therefore all efforts should be concentrated to make it "greener". Biocatalysis is emerging as a more attractive approach than chemical synthesis due to its advantages in terms of economic feasibility and ecology. Moreover, it is considered as one of the "greenest" technologies. Enzymes are versatile biocatalysts and perform chemical transformations maintaining chemo-, regio- and stereospecificity.

However, significant dependence of biocatalytic processes on nature and diversity of reactants has been noticed [1]. Terpenes and terpenoids are a large and diverse class of organic compounds which consists of more than 55 thousands of distinct molecules. Terpenes are the primary constituents of the essential oils, therefore are inexpensive, readily available and renewable. Besides their use as fragrances and flavours, terpenes and their derivatives are known to have antifungal and antimicrobial properties [2]. Since terpenes are major precursors of many active biological compounds such as carotene or steroids, their biotransformations using bacteria, fungi and plants get considerable attention [3].

Biodegradation pathways of various terpenes have been investigated for decades. Extensive research on degradation of one of the most widespread monoterpene camphor has been carried out [4]. The biotransformation pathway of camphor in *Pseudomonas putida* is determined and enzymes as well as genes involved in it are identified [5, 6]. It is established that *P. putida* carries a plasmid reffered to as CAM which encodes enzymes involved in the catabolism of camphor [4]. Along with camphor other monoterpenes such as limonene or α- and β-pinene have been studied [7] [8].

Monoterpenoid 1,8-cineole (1), also known as eucalyptol, is a major constituent of *Eucalyptus* essential oil. It is widely used in flavourings, fragrances and cosmetics [9, 10]. On the other hand, in higher than normal doses it is toxic and therefore 1,8-cineole is used as an insecticide and repellent [11]. Due to its versatile utilization, bioconversions of 1,8-cineole are of special importance, however, enzymology of 1,8-cineole biodegradation is not yet well elucidated.

A metabolic pathway for bioconversion of 1,8-cineole is studied in *Pseudomonas flava* [12], *Rhodococcus* species [13] and *Citrobacter braakii* [14] bacteria (Fig. 1). These bacteria are known to be capable of using 1,8-cineole as its sole source of carbon and energy. Based on 1,8-cineole and camphor

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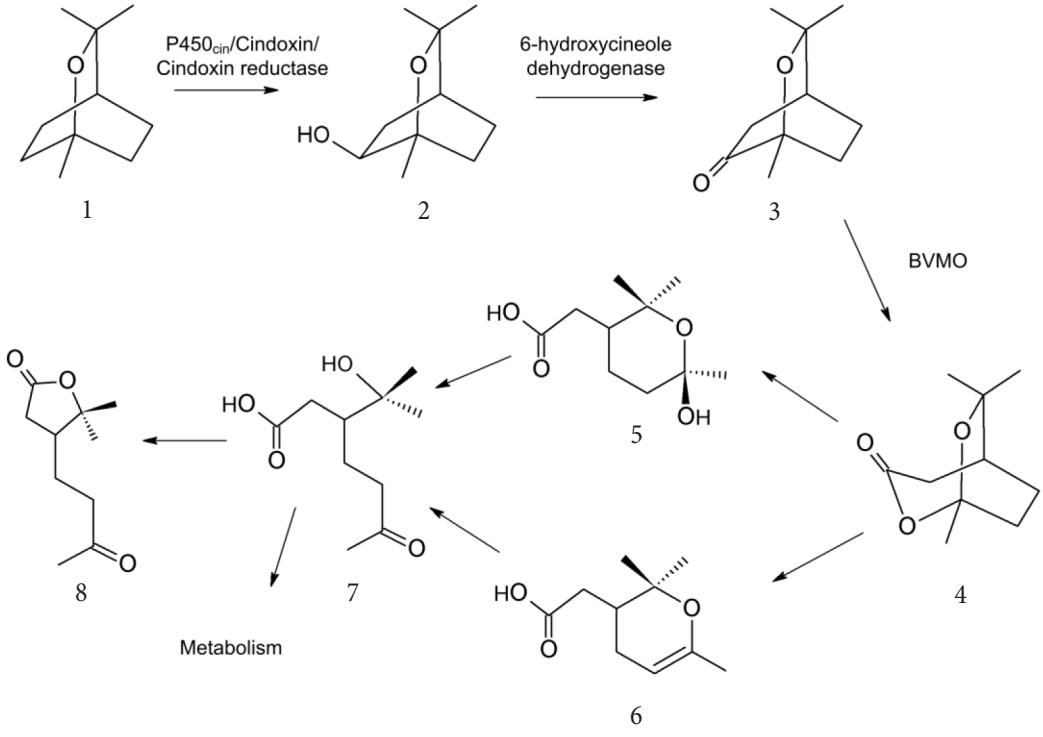

Fig. 1. The proposed pathway of biodegradation of 1,8-cineole. 1,8-cineole (1), (1R)-6β-hydroxycineole (2), 6-ketocineole (3), 1,6,6-trimethyl-2,7-dioxobicyclo[3.2.2]nonan-3-one (4), 2,6,6-trimethyl-5-acetyltetrahydropyran-2-ole (5), 2,6,6-trimethyl-5-acetyldihydropyrane (6), 3-(1-hydroxy-1-methylethyl)-6-oxoheptanoic acid (7), 5,5-dimethyl-4-(3′-oxobutyl)-4,5-dihydrofuran-2(3H)-one (8). Adapted from [13]

degradation analogy, it was proposed that proteins catalyzing the initial decomposition of 1,8-cineole in *C. braakii* are encoded by Cin operon (Fig. 2a) [15]. To date, four genes – *cin*A, *cin*B, *cin*C and *cin*D – have been isolated and cloned from the Cin operon of *C. braakii*. These genes encode cytochrome $P450_{cin}$ [14], cindoxin reductase, cindoxin [16] and (1*R*)-6β-hydroxycineole dehydrogenase [15], respectively.

It is believed that the first step in the metabolism of 1,8-cineole is hydroxylation (Fig. 1). Initial hydroxylation of 1,8-cineole is catalyzed by a three-component cytochrome $P450_{cin}$ system: cytochrome $P450_{cin}$ and its redox partners, cindoxin and cindoxin reductase. When the hydroxylation of 1,8-cineole is completed, the oxidation of 6β-hydroxycineole (2) to furnish the corresponding ketone, 6-ketocineole (3), follows. It has been demonstrated that (1*R*)-6β-hydroxycineole dehydrogenase catalyzes the oxidation reaction of hydroxycineole [15]. According to Williams et al. [13], during the 1,8-cineole transformations in *Rhodococcus* sp., 6-ketocineole is further converted into unstable lactone (4), which then undergoes a spontaneous ring hydrolysis. Although the oxidation

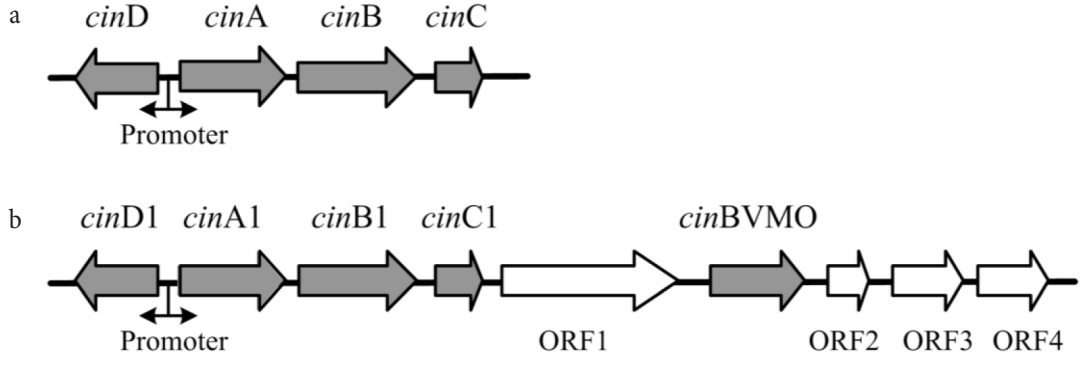

Fig. 2. Maps of the Cin operon of *C. braakii* (a) and Cin_{TMP1} operon of *R. jostii* TMP1 (b). *cin*D(1), 6-hydroxycineole dehydrogenase; *cin*A(1), $P450_{cin}$; *cin*B(1), cindoxin reductase; *cin*C(1), cindoxin; *cin*BVMO, Baeyer-Villiger monooxygenase. ORF is open reading frame. (A) adapted from [15]

products of 6-ketocineole were detected, enzymes performing this catalysis as well as genes encoding them are still undetermined.

To date, only two initial steps and enzymes involved in the conversion of 1,8-cineole in *C. braakii* are known. In addition, researchers know little about biotransformation pathways of 1,8-cineole in other microorganisms. Here we present data on the biodegradation pathway of 1,8-cineole in *Rhodococcus jostii* TMP1 bacteria.

EXPERIMENTAL

Chemicals

Nutrient broth (NB) was obtained from Oxoid (England). 1,8-cineole, streptomycin, SDS and ethyl acetate were purchased from Sigma Aldrich (USA), methanol and thiostreptone from Fluka (Germany). Chloramphenicol was obtained from Serva (Germany), ampicillin and kanamycin from Roth (Germany). All neorganic salts were obtained from Lachema (Czech Republic). PCR primers were purchased from Metabion (Germany). IPTG and all reagents for molecular cloning and DNA manipulation were obtained from Thermo Scientific (USA).

Bacterial strains and plasmids

Escherichia coli DH5α was used for cloning experiments. *E. coli* strains BL-21 (DE3) and Rosetta (DE3) pLysS as well as *Rhodococcus erythropolis* SQ1 were used for protein expression. *R. jostii* TMP1 was previously isolated from a soil sample [17]. The pTZ57R/T vector (Thermo Scientific) was used for cloning. pET21b(+), pET28b(+), pACYC Duet-1 and pCDFDuet-1 vectors (Merck) were used for protein expression and bioconversion of 1,8-cineole in *E. coli*. The pTipQC2 vector was chosen for the same purposes in *R. erythropolis* SQ1 [18].

Bacterial growth medium and conditions

E. coli and *R. erythropolis* strains were cultivated in the NB medium at 30 °C with aeration. *R. jostii* TMP1 was grown in the minimal medium (5 g/L NaCl, 1 g/L $NH_4H_2PO_4$, 1 g/L K_2HPO_4, 0.4 g/L $MgSO_4$, pH 7.2) supplemented with 1,8-cineole (0.1% v/v) in a vapor phase. *E. coli* and *R. erythropolis* strains transformed with recombinant plasmids were cultivated in the NB medium with the required antibiotic/s (50 μg/mL ampicillin, 40 μg/mL kanamycin, 20 μg/mL chloramphenicol, 20 μg/mL streptomycin).

Bioinformatic analysis

The similarity of the sequences of Cin_{TMP1} operon was examined by comparison to the nonredundant nucleotide database at GenBank by using BLAST at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were performed with the Clustal Omega 1.1.0 program (http://www.ebi.ac.uk/Tools/msa/clustalo/) [19]. Phylogenetic trees were constructed using the MEGA 5.1 analysis tool according to the neighbour-joining method with 1000 bootstrap replicates [20, 21].

Cloning into plasmids

Forward primers were designed to *cin*A1, *cin*B1, *cin*C1, *cin*D1, *cin*BVMO containing NdeI, BamHI or NcoI sites at 5′ end. Reverse primers were designed so as to fuse with 6xHis tag. The PCR was carried out with chromosomal DNA of *R. jostii* TMP1 as a template according to the following parameters: 95 °C for 4 min; 30 cycles of 95 °C for 30 s, 58–61 °C for 30 s and 72 °C for 1 min; 72 °C for 10 min. Single PCR products were isolated and cloned into the pTZ57R/T vector. Positive recombinant clones were detected by blue/white colony screening. The resulting plasmids were digested with the restriction endonucleases (*cin*A1 and *cin*C1 with NdeI and XhoI, *cin*D1 and *cin*BVMO with NcoI and HindIII, *cin*B1 with BamHI and HindIII) and inserted into the expression vectors (pET21b(+), pET28b(+), pACYC Duet-1, pCDFDuet-1 or pTipQC2) predigested with the same restriction enzymes. The resulting expression plasmids were sequenced to ensure the lack of mutations.

Expression of recombinant proteins

E. coli (BL-21 (DE3) or Rosetta (DE3)) and *R. erythropolis* SQ1 cells freshly transformed by electroporation with expression plasmids were cultured overnight in the 5 mL NB medium with the respective antibiotic/s. 500 μL was used to inoculate the 20 mL NB medium with the respective antibiotic/s. The flasks were incubated at 30 °C until an A_{600} of 0.7 for *E. coli* cells and 0.3 for *R. erythropolis* was reached. Protein expression was then induced with 0.5 mM IPTG (*E. coli*) or 1 μg/mL thiostreptone (*R. erythropolis*) and incubated at 20 °C (*E. coli* Rosetta (DE3)) or 30 °C (*E. coli* BL-21 (DE3), *R. erythropolis* SQ1) overnight (16–20 h). Cells were harvested by centrifugation (4000 × *g*) for 15 min and then resuspended in 3 mL of lysis buffer (50 mM potassium phosphate, pH 7.2). The cell suspension was sonificated on ice, in addition adding silica beads (0.1 mm diameter) (Roth, Germany) for *R. erythropolis* SQ1. The lysate was centrifuged (4000 × *g*) for 15 min to remove the cellular debris. The expression of recombinant proteins was evaluated by SDS-PAGE (14% gel).

In vivo oxidation of 1,8-cineole

E. coli (BL-21 (DE3)) or *R. erythropolis* SQ1 cells freshly transformed with expression plasmid/s were cultured overnight as described above. 2 mL was used to inoculate the 200 mL NB medium with the respective antibiotic/s. The flasks were incubated at 30 °C until a required A_{600} was attained. The cells were then induced either with IPTG (0.5 mM) or with thiostreptone (1 μg/mL) in addition supplementing with 1,8-cineole (100 μL). The culture was incubated at 30 °C for 7 days. Cells were removed by centrifugation (4000 × *g*) for 15 min, and the supernatant was extracted with ethyl acetate (2 × 50 mL) additionally adding methanol (2–3 mL). The combined organic extracts were dried over sodium sulphate and concentrated *in vacuo*. The residues were analyzed by gas chromatography-mass spectrometry (GC-MS) (RESTEK Rtx-1701 column (30 m × 0.25 mm)) by the following temperature program:

40 °C for 2 min, 20 °C for min^{-1} to 200, 50 °C for min^{-1} to 250 °C. The final temperature was maintained for 2 min. Data was collected and processed using the GCMS Solution software 2.71 (Shimadzu), scanning ions with m/z 30–400 (TIC). Peaks were observed with the mass spectrum consistent with that reported for hydroxycineole and ketocineole [12, 13].

Nucleotide sequence accession number

The nucleotide sequence of Cin_{TMP1} operon of *R. jostii* TMP1 genome determined in this study was deposited in GenBank under accession number KU194228.

RESULTS AND DISCUSSION

Operon analysis

Recently tetramethylpyrazine degrading bacterial strain *R. jostii* TMP1 was isolated from soil [17] [22]. Bioinformatic analysis of the partially sequenced genome of *R. jostii* TMP1 showed several genes which were homologous to *cin* genes from *C. braakii*, known to be encoding biodegradation of 1,8-cineole [15]. To examine if *R. jostii* TMP1 strain is able to use 1,8-cineole as its sole source of carbon and energy, bacteria were cultivated in the minimal media supplemented with 1,8-cineole. *R. jostii* TMP1 proved to be able to grow and develop by degrading 1,8-cineole.

After a more detailed analysis of this identified genetic locus the presence of a novel Cin operon – Cin_{TMP1} – was uncovered (Fig. 2b). Genes located in the Cin_{TMP1} operon of *R. jostii* TMP1 were determined to have a high percentage of identical matches with the corresponding analogues from *C. braakii* (Table). Therefore it was proposed that *cin*A1, *cin*B1, *cin*C1 and *cin*D1 genes encode cytochrome P450, cindoxin reductase, cindoxin and hydroxycineole dehydrogenase, respectively.

Considering the similarity of the initial biotransformation of 1,8-cineole and camphor, it can be presumed that enzymes involved in a subsequent 1,8-cineole degradation steps are analogous. It is determined that after hydroxylation and oxidation of camphor, type II Baeyer-Villiger monooxygenase (BVMO) converts diketocamphane to an unstable lactone which undergoes spontaneous ring hydrolysis. Further camphor degradation includes another oxidation step catalyzed by type I BVMO. In addition to this, Williams and colleagues demonstrated an activity of NADPH-dependent 6-ketocineole oxygenase and linked this activity to the products of oxidation of 6-ketocineole [13]. Moreover, it was observed that type II BVMO involved in the biodegradation pathway of camphor in *P. putida* was able to utilize 6-ketocineole as a substrate yielding the same oxidation products as in the bioconversion of 1,8-cineole [13]. Here, we therefore suggest that the oxidation of 6-ketocineole is catalyzed by a hypothetical monooxygenase, like one of Baeyer-Villiger type. Gene encoding this hypothetical monooxygenase belongs to the Cin_{TMP1} operon and is referred to as *cin*BVMO (Fig. 2).

In order to confirm hypothetical functions of genes located in novel Cin_{TMP1} operon phylogenetic trees were constructed (data not shown). It was shown that *cin*A1, *cin*B1, *cin*C1 and *cin*D1 display a significant homology to *cin*A, *cin*B, *cin*C and *cin*D of *C. braakii* (Table). As for the proposed novel BVMO of *R. jostii* TMP1, the phylogenetic analysis revealed a close homology to luciferases, BVMOs involved in the biodegradation of camphor as well as other monooxygenases of various terpenes (data not shown).

Table. **Sequence homology of genes located in Cin_{TMP1} operon**

Gene	Protein/organism	%I[a]	E-value	Genbank ID	Refs
*cin*A1	$P450_{cin}$/*C. braakii*	93	0.0	Q8VQF6.1	[13]
	Hypothetical protein/*Amycolatopsis thermoflava*	71	0.0	WP_027929004.1	–
*cin*B1	Cindoxin reductase/*C. braakii*	77	0.0	Q8VQF5.1	[15]
	Oxidoreductase/*Pseudomonas thermotolerans*	51	1e-113	WP_027897353.1	–
*cin*C1	Cindoxin/*C. braakii*	83	6e-47	Q8VQF4.1	[15]
	Nitric oxide synthase/*Gordonia* sp.	51	1e-16	ALG85784.1	[23]
*cin*D1	(1*R*)-6β-hydroxycineole dehydrogenase/*C. braakii*	95	5e-137	ACX31575.1	[14]
	3-α-hydroxysteroid degydrogenase/*Salinibacterium* sp.	58	3e-81	WP_010202793.1	[24]
*cin*BVMO	Luciferase/*A. thermoflava*	71	3e-180	WP_027929001.1	–
	Luciferase/*Frankia alni*	59	7e-147	WP_011603674.1	–
ORF1	Hypothetical protein/*A. thermoflava*	51	0.0	WP_051362472.1	–
	Transcriptional regulator/*Intrasporangium chromatireducens*	46	0.0	EWT06391.1	–
ORF2	Hypothetical protein/*A. thermoflava*	58	7e-45	WP_027929040.1	–
	Hypothetical protein/*Saccharopolyspora spinosa*	51	7e-32	WP_010314951.1	–
ORF3	Hypothetical protein/*α-proteobacterium*	62	2e-123	WP_017502621.1	–
ORF4	Short-chain dehydrogenase/*Sphingobium* sp.	59	5e-115	WP_024019859.1	–
	Hypothetical protein/*A. thermoflava*	61	2e-124	WP_027928998.1	–
	Hypothetical protein/*S. spinosa*	62	1e-122	WP_010314958.1	–

[a] Percent identity.

A very close homology of specific genes of gram-positive bacteria *Rhodococcus* sp. and gram-negative *C. braakii* is an unusual phenomenon that can be interpreted as a horizontal gene transfer between distantly related species. Although it is known that gene clusters encoding biodegradative pathways can be successfully transferred, here we cannot predict the direction of the transfer. We can only anticipate that due to toxicity of 1,8-cineole, either of the two bacterial species have evolved to degrade it.

Expression of the recombinant proteins

The genes of *R. jostii* TMP1 (*cin*A1, *cin*B1, *cin*C1, *cin*D1 and *cin*BVMO) were cloned into vectors suitable for expression either in *E. coli* or in *R. erythropolis*. Bacteria of *Rhodococcus* genus were selected as a protein expression host due to uncertain suitability of the *E. coli* system to express genes of *Rhodococcus* origin. Since it is known that the first oxidation step of 1,8-cineole is catalyzed by a three-component cytochrome $P450_{cin}$ system, co-expression strategies using compatible vectors were applied.

Expression of $P450_{cin}$

Plasmids containing *cin*A1 gene (pET21b-*cin*A1, pACYC-Duet-1- *cin*A1 and pTipQC2- *cin*A1) were transformed into *E. coli* BL-21 (DE3), Rosetta (DE3) pLysS or *R. erythropolis* SQ1 cells. Protein expression in *E. coli* was induced with IPTG while thiostrepton was used for gene expression induction in *R. erythropolis*. The best expression of soluble recombinant $P450_{cin}$ protein was obtained either in *E. coli* Rosetta (DE3) pLysS at 20 °C or in *R. erythropolis* SQ1 at 30 °C temperature (Fig. 3).

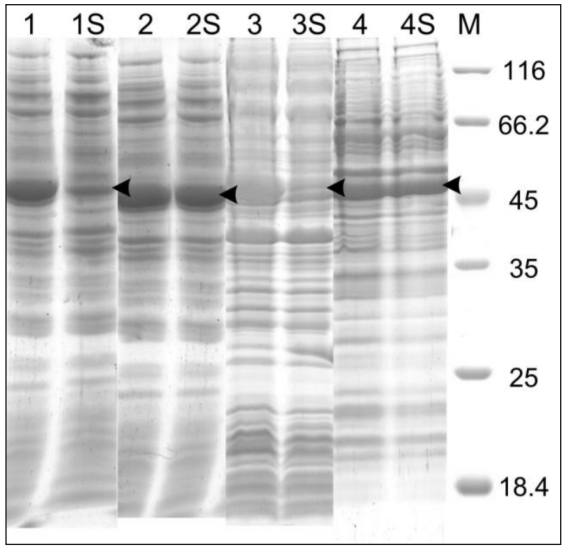

Fig. 3. Expression of recombinant $P450_{cin}$ in *E. coli* BL-21 (DE3) (1, 3), *E. coli* Rosetta (DE3) pLysS (2), *R. erythropolis* SQ1 (4). Plasmids used for induced-protein expression were pET21b-*cin*A1 (1, 2), pACYCDuet-1-*cinA1* (3), pTipQC2-*cin*A1 (4). Arrows indicate the soluble (S) recombinant $P450_{cin}$. M lane, molecular mass marker (kDa)

Expression of cindoxin, cindoxin reductase and hydroxycineole dehydrogenase

Plasmid constructs containing *cin*C1 gene (pET21b-*cin*C1 and pCDFDuet-1-*cin*C1) were transformed into *E. coli* BL-21 (DE3) cells. In both cases, the expression of soluble recombinant cindoxin was obtained after the induction with IPTG at 30 °C temperature (Fig. 4). Moreover, the expression of soluble recombinant 6-hydroxycineole dehydrogenase was observed by applying identical induction conditions (Fig. 4). The expression of the *cin*B1 gene was tested in all above-mentioned bacterial strains including varying the expression temperature. Unfortunately, the expression of recombinant cindoxin reductase has so far proved impossible under the conditions evaluated.

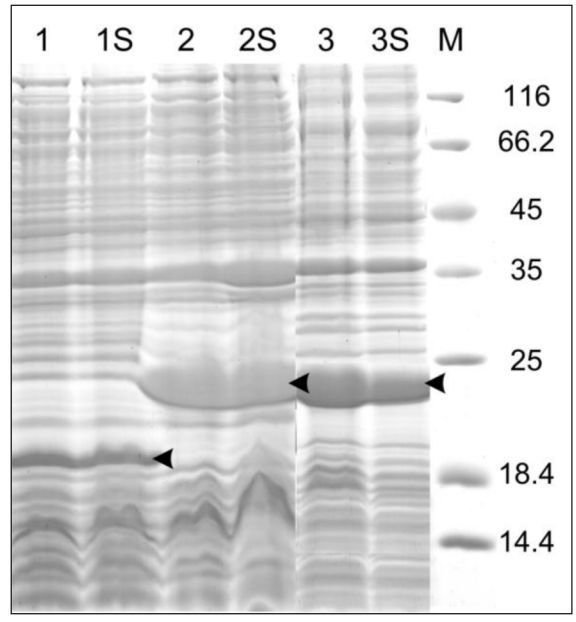

Fig. 4. Expression of recombinant cindoxin and 6-hydroxycineole dehydrogenase in *E. coli* BL-21 (DE3). Plasmids used for induced-protein expression were pET28b-*cin*D1 (1), pET21b-*cin*C1 (2), pCDFDuet-1-*cin*C1 (3). Arrows indicate soluble (S) recombinant proteins. M lane, molecular mass marker (kDa)

Expression of BVMO

In order to examine expression conditions of a hypothetical BVMO, several bacterial strains (*E. coli* BL-21 (DE3), Rosetta (DE3) pLysS, *R. erythropolis* SQ1) as well as different cell incubation temperatures (20–30 °C) were employed. It was revealed that *E. coli* is not a suitable host for the expression of recombinant BVMO (Fig. 5). Since the *cin*BVMO gene has originated from *R. jostii* TMP1, it was proposed that *R. erythropolis* SQ1 may be a more appropriate expression host. This assumption proved to be true and the expression of soluble recombinant BVMO was obtained using the *R. erythropolis* strain (Fig. 5).

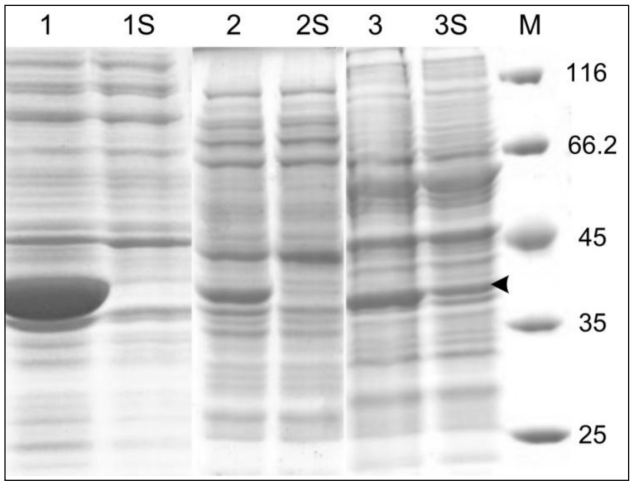

Fig. 5. Expression of recombinant BVMO in *E. coli* BL-21 (DE3) (1), *E. coli* Rosetta (DE3) pLysS (2), *R. erythropolis* SQ1 (3). Plasmids used for induced-protein expression were pET28b-*cin*BVMO (1, 2), pTipQC1-*cin*BVMO (*3*). Arrows indicate soluble (S) recombinant BVMO. M lane, molecular mass marker (kDa)

Co-expression of P450$_{cin}$ complex

Since soluble recombinant cindoxin reductase has yet to be expressed, recombinant *E. coli* flavodoxin reductase was used instead to reassemble the active P450$_{cin}$ system. *cin*A1, *cin*C1 and *fpr* genes were successfully cloned into pACYCDuet-1, pCDFDuet-1 and pET28b vectors, respectively, and either two or three plasmids were cotransformed into *E. coli* BL-21 (DE3) cells. The protein expression in *E. coli* was induced with IPTG at 30 °C temperature. It was observed that the overexpression of flavodoxin reductase lowered cindoxin synthesis in recombinant cells (Fig. 6). In both cases the P450$_{cin}$ concentration was also reduced. These results correspond well to the data already published [15].

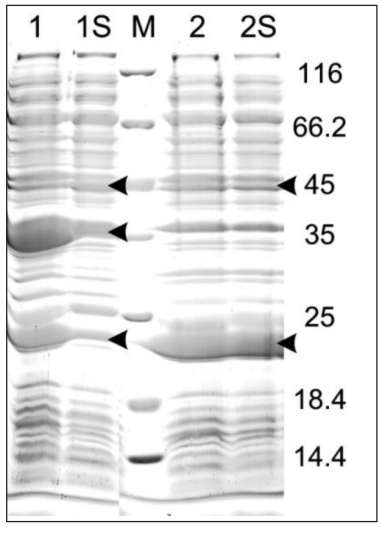

Fig. 6. Coexpression of recombinant P450$_{cin}$, cindoxin and *E. coli* flavodoxin reductase (1) and P450$_{cin}$ with cindoxin (2) in *E. coli* BL-21 (DE3). Arrows indicate soluble (S) recombinant P450$_{cin}$ (45 kDa), cindoxin (23 kDa), flavodoxin reductase (35 kDa). M lane, molecular mass marker (kDa)

Bioconversion of 1,8-cineole by recombinant cells

Next, we set out to determine whether the hypothetical Cin$_{TMP1}$ operon in *R. jostii* TMP1 is responsible for the catabolism of 1,8-cineole. Thus, the initial bioconversion of 1,8-cineole was investigated *in vivo*, both in recombinant *E. coli* and *R. erythropolis* cells.

E. coli BL-21 (DE3) cells transformed with recombinant plasmid or their combinations, each carrying an individual gene (*cin*A1, *cin*C1, *frp*), were cultivated with 1,8-cineole as a co-substrate for a week as described in *Materials and methods*. *R. erythropolis* SQ1 cells were transformed with only one recombinant plasmid encoding P450$_{cin}$. Metabolites were extracted from the bioconversion media and analyzed by GC-MS.

First, the bioconversion of 1,8-cineole was investigated in *R. erythropolis* SQ1 cells carrying a single recombinant P450$_{cin}$ protein. One additional peak was detected and identified as 6-ketocineole (Fig. 7). The mass spectral analysis of 6-ketocineole gave a molecular ion at *m/z* 168(8%), 140(26), 111(10), 97(9), 82(100), 69(21), 67(24), 43(57). This fragmentation pattern was almost identical with that reported earlier [13]. It was determined that a half of 1,8-cineole was converted to 6-ketocineole, while the other half was still present unaffected. Although it is known that the first step in the biodegradation of 1,8-cineole is hydroxylation, it seems that *Rhodococcus* cells have a suitable redox system which is able to assist in further oxidation of 1,8-cineole.

Next, the bioconversion of 1,8-cineole was investigated in *E. coli* cells. When recombinant P450$_{cin}$ alone or along with its redox partner cindoxin was expressed in the *E. coli* system in the presence of 1,8-cineole as a substrate, a peak of 6-hydroxycineole was observed with a mass spectrum consistent with that reported already (molecular ion at *m/z* 170 (14%) with the associated ions at 126(60), 111(34), 108(86), 93(32), 71(61), 69(39), 43(100)) [13, 14] (Fig. 8). The oxidation of 1,8-cineole in the absence of the complete P450$_{cin}$ system can be attributed to the native *E. coli* reductase mimicking the natural redox partners of P450$_{cin}$. These results correlate well with the previously published data of the initial biodegradation of 1,8-cineole in *C. braakii* [16].

When the three-component P450$_{cin}$ system (P450$_{cin}$/cindoxin/flavodoxin reductase) was expressed in *E. coli* in the presence of 1,8-cineole, two products were formed that were identified as 6-hydroxycineole and 6-ketocineole (Fig. 8). Therefore it can be suggested that the three-component P450$_{cin}$ system performs subsequent oxidation of 1,8-cineole to 6-hydroxycineole and 6-ketocineole.

Comparing bioconversion of 1,8-cineole in two distinct systems it is clear that *E. coli* cells generate not only the products of interest but also some secondary compounds (Fig. 8). This can be adressed to a more rapid growth of *E. coli* cells leading to a higher sensitivity and faster metabolism. Furthermore, contrary to the degradation of 1,8-cineole in the recombinant

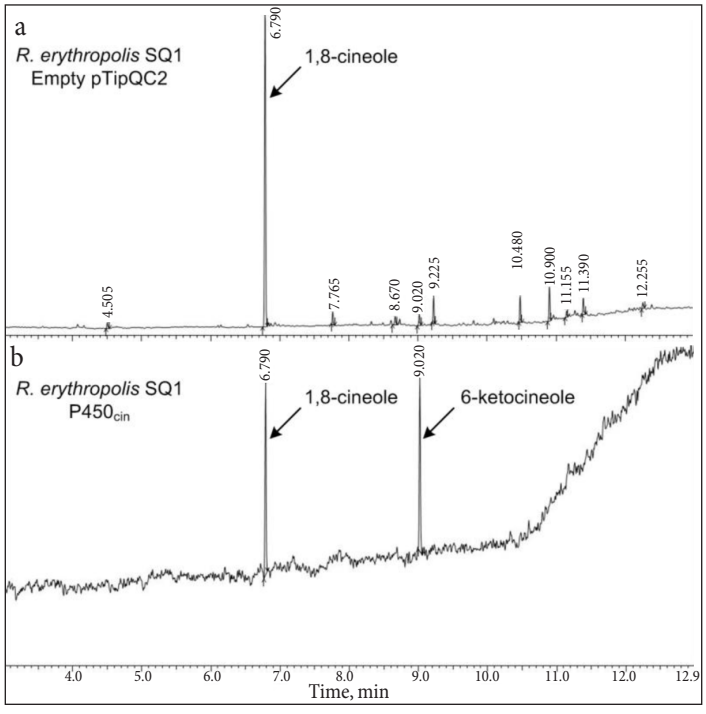

Fig. 7. Gas chromatogram of bioconversion of 1,8-cineole in *R. erythropolis* SQ1 cells. Control *R. erythropolis* SQ1 cells carrying an empty pTipQC2 plasmid (a); *R. erythropolis* SQ1 cells expressing recombinant $P450_{cin}$ (b)

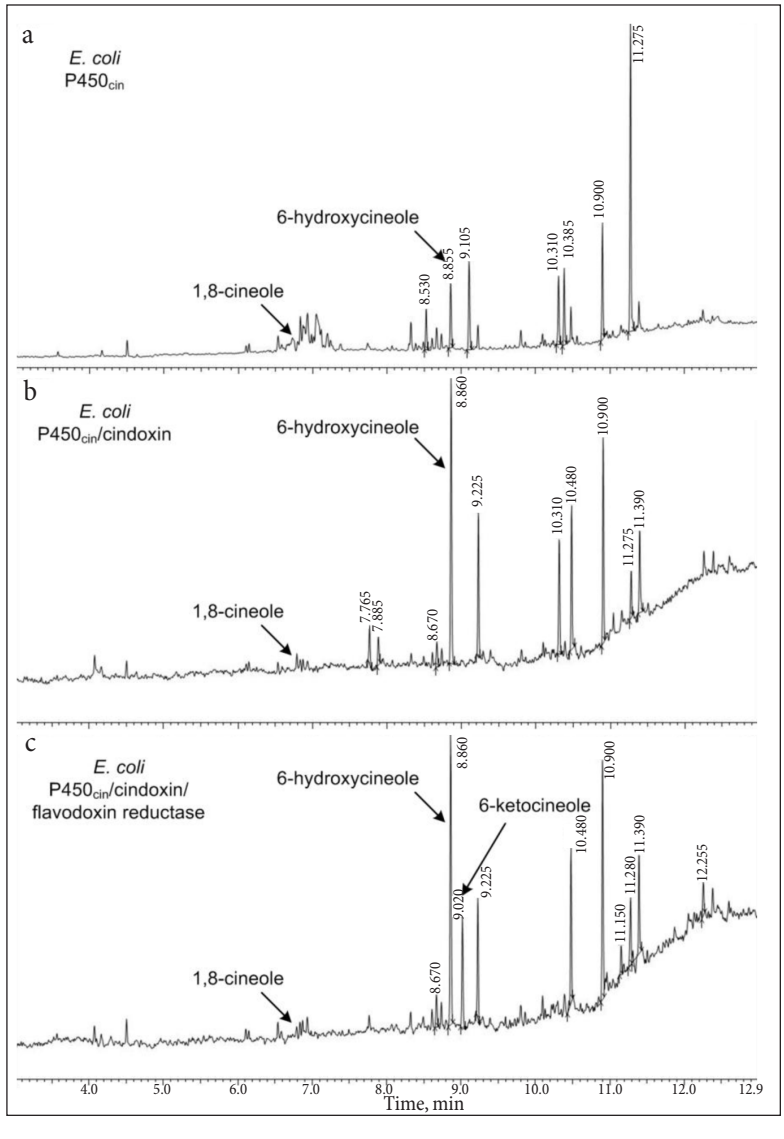

Fig. 8. Gas chromatograms of bioconversion of 1,8-cineole in *E. coli* BL-21 (DE3) cells. *E. coli* cells expressing $P450_{cin}$ alone (a); *E. coli* cells expressing $P450_{cin}$ along with cindoxin (b); *E. coli* cells expressing a three-component $P450_{cin}$ system ($P450_{cin}$/cindoxin/flavodoxin reductase) (c)

E. coli system, 6-hydroxycineole in *R. erythropolis* cells was not detected. We consider that due to the origin of Cin_{TMP1} operon, *Rhodococcus* cells are naturally able to assist the conversion of 1,8-cineole.

CONCLUSIONS

Rhodococcus jostii TMP1 are able to utilize 1,8-cineole as its sole source of carbon and energy. The *cin*A1 gene located in a Cin_{TMP1} operon of *R. jostii* TMP1 encodes cytochrome $P450_{cin}$ that catalyses the initial and subsequent oxidation of 1,8-cineole. The diversity of bioconversion of 1,8-cineole is not fully elucidated, therefore these results promise a greater understanding of the degradation process of this monoterpenoid in various microorganisms. Moreover, a novel variant of cytochrome $P450_{cin}$ opens ways for further development of biocatalytic processes.

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1,8-CINEOLIO DEGRADACIJĄ KODUOJANČIO OPERONO IŠ *Rhodococcus* sp. TMP1 CHARAKTERIZAVIMAS

S a n t r a u k a

Sparčiai vystantis biotechnologijoms, didėja įvairių biokatalizinių procesų poreikis. Vienas svarbiausių biokatalizę ribojančių veiksnių – pradinių medžiagų ir produktų prigimtis bei įvairovė. Terpenai, jų dariniai terpenoidai yra didžiausia gamtinių medžiagų grupė, pasižymi junginių įvairove ir yra biologiškai aktyvių medžiagų pirmtakai. Dėl šių priežasčių terpenų biotransformacijos panaudojant mikroorganizmus sulaukia daug dėmesio. 1,8-cineolas, arba eukaliptolas, yra plačiai paplitęs monoterpenoidas, pasižymintis antimikrobinėmis ir antigrybelinėmis savybėmis. 1,8-cineolas naudojamas farmacijos, žemės ūkio, maisto ir kosmetikos pramonės srityse. Nustatyti tik pirmose dviejose 1,8-cineolo skaidymo reakcijose dalyvaujantys baltymai ir juos koduojantys genai *Citrobacter braakii* bakterijose. Nėra informacijos apie genus, koduojančius tolimesniuose 1,8-cineolo degradacijos etapuose dalyvaujančius fermentus.

Šiame darbe parodyta, kad *Rhodococcus jostii* TMP1 bakterijos gali panaudoti 1,8-cineolą, kaip vienintelį anglies ir energijos šaltinį. Bioinformatinės *R. jostii* TMP1 genomo analizės metu buvo aptiktas pilnesnis CIN operonas – CIN_{TMP1}. Nustatyta, kad CIN_{TMP1} operone lokalizuoti genai pasižymi didele homologija su genais, dalyvaujančiais skaidant 1,8-cineolą *C. braakii* bakterijose. Išsiaiškinta, kad šie genai – *cin*A1, *cin*B1, *cin*C1, *cin*D1 ir *cin*BVMO – koduoja atitinkamai citochromo P450 šeimos fermentą ($P450_{cin}$), cindoksino reduktazę, cindoksiną, hidroksicineolo dehidrogenazę ir Baeyer-Villiger monooksigenazę (BVMO). Ištirtos ir optimizuotos rekombinantinių $P450_{cin}$, cindoksino, hidroksicineolo dehidrogenazės ir BVMO baltymų sintezės sąlygos. Nustatytos *cin*A1, *cin*C1 ir geno, koduojančio *Escherichia coli* feredoksino reduktazę, koekspresijos sąlygos. Parodyta, kad rekombinantinis $P450_{cin}$ katalizuoja 1,8-cineolo virtimą 6-hidroksicineolu. Be to, nustatyta, kad $P450_{cin}$ gali oksiduoti 6-hidroksicineolą iki 6-ketocineolo.